Production and Characterization of Microbial Amylase and Pullulanase for Synthesis of Resistant Starch

by

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By

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Certificate of Approval

This is to certify that the research work presented in this thesis, entitled titled **"Production and Characterization of Microbial Amylase and Pullulanase for Synthesis of Resistant Starch"** was conducted by **Ms. Anum Khan** under the supervision of **Dr. Samiullah Khan.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

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Ms. Anum Khan
Date: 27-01-2021

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List of Abbreviations

AMG	Amyloglucosidase
ANOVA	**Analysis of variance**
CFU	Colony forming unit
Cipro	Ciprofloxacin
DLS	Dynamic light scattering
DNS	3,5 dinitro salicylic acid
EM-RS III	Enzymatically modified Resistant Starch type III
FFAR2	Free fatty acid receptor 2
FFAR3	Free fatty acid receptor 3
FTIR	Fourier transform infrared spectroscopy
FTIR	Fourier transform infrared spectroscopy
GI	Glycemic index
GLP-1	Glucagon like peptide-1
GOPOD	Glucose oxidase peroxidase reagent
HDL	High density lipoprotein
IR	Industrial resistant starch
KOH	potassium hydroxide
LDL	Low density lipoprotein
MPs	Micro particles
MRSA	Man Rogosa sharp agar media
RDS	Rapidly digesting starch
RS	Resistant starch
SCFA	Short chain fatty acids
SCFA	Short chain fatty acid
SDS	Slowly digestible starch
SEM	Scanning electron microscopy
SR-B1	scavenger receptor class B1
T2DM	diabetes mellitus type 2
XRD	X-ray Diffraction

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Abstract

Starch hydrolyzing enzymes, specifically amylases and pullulanase, play vital role in food processing in the manufacturing of resistant starch. Moreover, Resistant starch (RS) is recently documented as a source of fibre and has acquired much attention for both its potential health benefits and functional properties.

In current research work, resistant starch type III from maize flour was prepared using amylase and pullulanase obtained from indigenously isolated *Bacillus licheniformis.* The amylase and pullulanase gene from *Bacillus licheniformis* were amplified and cloned into the*pET*-24(a) and *pGEX-2T* vector respectively, expressed in *E.coli* BL21 (DE3) cells and purified by affinity chromatography. The purified amylase, pullulanase, and combination of both enzymes were used for the formulation of resistant starch which enhanced the yield of resistant starch by 16, 20 and 24-fold respectively in maize flour as compared to native maize flour. Besides, Scanning electron microscopy unveiled that the granular structure of maize flour was disordered into an opaque network with asymmetrical structure, and X-ray diffractograms sustantiated the transformation from an amorphous to a crystalline structure upon enzymatic treatment. In addition, thermogravimetric analysis disclosed increased amylose content while ^{1}H NMR and ^{13}C NMR indicated debranching pattern, of pullulanase treated starch compared to untreated starch. Enzymatically prepared resistant starch was analyzed for antioxidant potential by DPPH radical scavenging assay and reducing power illustrated considerable increase in antioxidant activity of maize flour after enzymatic treatment. Prebiotic effect of EM-RSIII was successfully examined during *in-vitro* experiment and analyzed as fermentation media for promoting growth of probiotic bacteria e.g. *Lactobacillus reuteri.* While, in case of *in-vivo* study EM-RSIII fed to the 12 female Sprague delay rats for a period of 21 days. After 21 days of experiment EM-RSIII fed rats showed significant reduction in body weight gain, fecal pH, glycemic response, serum lipid profile, insulin level and modulation in gut microbiota and their metabolites. Genomic analysis of bacteria from fecal sample of rats revealed that, the numbers of starch-utilizing and butyrate producing bacteria, such as *Lactobacillus, Enterococcus* and *Pediococcus genus*, were increased after the administration of medium and high doses of EM-RSIII, while those of *E.coli* completely suppressed compared to control fed diet rat's from fecal and cecum samples. The microbial metabolites including short chain fatty acids importantly butyrate precisely increased in feces of EM-RS III fed rats. Moreover,

enzymatically treated maize flour caused significant enhancement of the desired properties of maize flour, such as resistant starch content, amylose, milk absorption capacity, iodine and fatty acid complexing ability, and a reduction in swelling power, water binding, oil absorption capacity, and *in-vitro* digestibility compared to untreated maize flour. The low swelling power, water absorption and oil absorption capacity of EM-RSIII was responsible for good handling in processing imparting crispiness and enhanced texture to the end food product like, biscuits, wafers, cookies etc. Significant difference was observed on supplementation of cookies with EM-RSIII for palatability and color parameters when compared to native flour cookies. The sensory analysis was performed by distributing questioners among individual and results were evaluated based on five-point hedonic scale. Result exhibited that consumers portrayed highest acceptability of EM-RSIII augmented cookies specifically for its appearance, sharp aroma, texture, and nutritionally important food item for consumers. EM-RSIII derived drug loaded microspheres were also synthesized by single emulsion evaporation method with average particle size of 964 nm and drug encapsulation efficiency of 86.73 %. Release studies confirmed, stability of drug loaded microspheres at acidic pH while sustained release was observed at pH 7 allowing site specific delivery of drug to colon. For validation of encapsulation efficiency, antimicrobial potential of drug loaded microspheres was analyzed through agar well diffusion method.

This study concluded that amalgamation of amylase and pullulanase works efficiently for the preparation of resistant starch. The prepared resistant starch with unique physicochemical characteristics most importantly its prebiotic nature with enhanced antioxidant potential served as an important ingredient for cookies preparation and coating material for targeted and site-specific delivery of drugs. Therefore, the prepared EM-RS III is an important product to be used in different food applications with fortification of nutritionally important ingredient along with targeted delivery of vitamins, flavonoids, and probiotics.

1. Introduction

'Green chemistry' a term which is used for the designing, development, application of products and processes for elimination or reduction of materials which are hazardous to human health and atmosphere (1, 2). Biocatalysts establish a greener substitute to conventional organic synthesis that offers proper gears for the industrial transformation of synthetic or natural constituents under low energy requirements, mild reaction circumstances, and minimizes the complications of rearrangement and isomerization (3, 4). Also, biocatalysts are biodegradable and can display stereo-selectivity, regio-selectivity, and chemo-selectivity causing declined in by-product formation, eluding the need for functional-group deprotection, protection, or activation (5).

Enzymes are naturally occurring catalytic bodies which are responsible for the catalysis of life. They are produced by living organisms to speed up the biochemical reactions without themselves being used up in the reaction (6, 7). Enzymes lower the activation energy and accelerate the conversion of substrates into their respective products (8). In the absence of enzyme catalysis, many reactions would be too slow to be useful for metabolism. They have specific catalytic ability for a substrate in a chemical reaction which makes them more advantageous for industrial use (9). Enzymes are produced by all living organisms e.g. animals, plants and microorganisms, with different properties relative to their source (10). These sources are used to isolate, purify and characterize many industrially important enzymes. Among them, plant and animal sources have less commercial importance. Microbial enzymes are generally the preferred source because of some economic (bulk production, easy extraction and independence to sessional variation) and technical advantages (single source for a variety of enzymes, stability of enzymes, short generation time, easy genetic manipulation and production on raw substrate)(11). It is estimated that 50% of industrially used enzymes have been extracted from fungi and yeast sources, one third from bacterial sources and remaining from animal and plant sources (6, 12).

Microbial enzymes have been used from the history of ancient Greek and play a crucial role in numerous significant processes including baking, brewing, cheese making and alcohol production(13, 14). With the passage of time and advancement in technologies, role of enzymes in chemical, pharmaceutical, beverage and food

industries are also investigated (5, 7, 15, 16). According to a report, the global market of industrial enzymes was closed to a billion dollars in 1990 and crossed the $2.0 billion mark in 2005 (17). Globally, enzymes market has boosted significantly during last few years. worldwide. The global enzyme market was equivalent to 8.18 billion $ in 2015, and is presumed to breed to 17.50 billion $ by 2024 (18).

Moreover, with growing market demand there is a need to diversify the enzymatic biocatalysts market. About 4000 enzymes are known till date (Li e*t al*.,), but only 200 of them are in commercial use, and only 20 out of them are manufactured on an industrial level (19). Hence,, many developments are still assumed, and will be necessitated in this field in the upcoming years. Baking (8%), animal feed (6%), detergents (37%), starch (11%), and textiles (12%) are the foremost industries operating about 75% of industrially produced enzymes (19, 20). Carbohydrase's have the largest share of about 37% in the global enzyme market with bakery as the largest used application (19).

The current research project has emphasis on investigation of starch hydrolysing enzymes (amylase, pullulanase) from thermophilic microorganism. The isolation of extremophilic microorganisms for exploring the starch hydrolysing enzymes was the main aim of the current study. Since these thermozymes have the ability to withstand harsh processing conditions, they have the potential to be used for the green and economic production of resistant starch (21). However, as expected from previous research work, some enzymes with prominent properties have the problem of low expression which limits the optimum use of such enzymes in particular application. To overcome this problem, the strategy requires overexpressing the genes of selected enzymes into the mesophilic host. To attain maximum level of expression, both for commercial purposes and fundamental studies, mostly remote proteins were expressed in prokaryotic organisms (22). Advanced techniques, novel enzymes with multiple applications, and specifications have been produced, but new eras still need to be explored. These enzymes have a lot of applications in different industries, for example, starch hydrolysing enzymes (23), paper (24), detergent (25) and textile industries (26). Carbohydrase's are utilised in the manufacturing of crystalline glucose, syrups, glucose, prebiotic, maltose syrups and high fructose corn syrups etc. In addition, they are used as additives to eradicate starch-based colours in detergent industry. They are also utilized to reduce viscosity of starch to attain appropriate paper coating. In contrast, amylases are used for sizing textile fibres. The present

research work focused on the recent advancement of starch hydrolysing enzymes for food industries, for preparation of resistant starch as emerging prebiotic (27-29). Resistant starch (RS) belongs to the group of prebiotic as dietary fibres which have the ability to escape digestion from gastric conditions and undergo fermentation by colonial bacteria (30). Resistant starch, in comparison to the traditional fibers, has several dominant functional and physiological characteristics and many pronounced health benefits (32, 33), including the control of risk of diabetes mellitus type II (34, 35) , cardiovascular diseases, colorectal cancer (36), gastrointestinal abnormalities, and assist in metabolism of lipids and glucose (37).Therefore, resistant starch is recommended jointly by WHO/FAO (World Health Organization/Food and Agricultural Organization of United Nations) expert conference as an important nutritional fiber. Besides the health benefits of using resistant starch in starch based products (cookies, pasta, bread, yogurt and budding etc.), it does not affect the food sensory properties while enhancing its physiological properties (38). Additionally, resistant starch also has positive influence on the sensory properties of the food compared to conventional fibers (39). Among its most desirable physicochemical characteristics are their gel formations, water holding capacity, viscosity and swelling ability. These properties makes the resistant starch ideal to be utilized in numerous food formulations (40). Now-a-days the demand of using resistant starch as a functional food ingredient is receiving great attention; therefore, the analysis of resistant starch structure, digestibility, thermal and storage properties is of pronounced importance. In addition to these, understanding the affiliation among the functional, structural, and nutritive properties of RS encourage the food producers in optimizing the conditions for its industrial production. Resistant starch is processed from wheat, maize, rice, potato, oat, barley, tapioca, sago, arrowroot, barley, sorghum, cereal grains, legumes and mung beans (11). However, maize starch is preferred for the production of resistant starch because of its excessive amylose content, large productivity (41) and its use as an important staple food in Pakistan.

Resistant starch based on structural characteristics and conformational changes with substitution or addition to the polymer can be classified into five types, namely RSI-RSV (30). RSI is the physically inaccessible starch for example legumes, pasta, and whole grains. RSII (Native B-Type) with semi crystalline structure is present in uncooked/ partially cooked food for example: green banana and uncooked potato starch. RSIII is the retrograded (recrystallized) starch obtained because of food

processing for example: cooked and cooled rice, potatoes, bread, cornflakes. RSIV is chemically modified starch because of the addition of various functional groups i.e., ethers, esters or phosphate groups rendering it resistant to digestion by hydrolytic enzymes. The spiral shape of RSV which is formed from amylose lipid complexes is usually hard to digest. During cooking process, RSI and RSII can lose their rigidity and resistivity to digestion. In contrast, RSIV is ethically unacceptable to the consumers because of the incorporation of other chemical groups to starch structure. Retrograded starch (RS type III) is preferred over other types owing to several reasons; the most important one is its stability to maintain its resistance during processing conditions.

Due to so many fascinating properties of resistant starch in food industry, its demand in functional food ingredients is receiving great attention. Therefore, many studies have investigated ways to increase the RS content of starch from various botanical sources using physical (30), chemical (42) and enzymatic treatments (43). However, among these (29), enzymatic treatment is preferred due to its environmentally friendly or bio based nature. Enzymatically prepared resistant starch (RSIII) is free of any toxic residues compared to chemically synthesized resistant starch because biocatalysts are highly selective and specific for their substrate and are easily biodegradable (44). For RSIII production, a highly adapted mechanism is the arrangement of linear amylose chains in condensation of double helical structure, after gelatinization process (45). This arrangement form 1-4 glycosidic bonds inaccessible to the digestion by amylase, in small intestine (46). The yield of resistant starch mainly depends on plant origin (47) from different sources with changed amylopectin and amylose ratio, physical form of starch, type of processing (thermal, acidic, or enzymatic), extent of gelatinization and storage conditions (48) . Different methods have been used for increasing the yield of RSIII such as acid hydrolysis and repeated heating-cooling (annealing) process while very less attention has been given to the use of enzymes for increasing the yield of RSIII. Berry (1986) investigated that before freeze-thaw cycle of potato starch, treatment with pullulanase results in the increased yield of RSIII (49). Similarly, research conducted by Zhang & Jin, (2011) also investigated that by hydrolysing the corn starch with pullulanase enzyme successfully increased the RSIII yield (up to 44.7%) (50). In enzymatic processing of RSIII mainly amylases, pullulanase are used. However, many studies have been conducted with

single starch hydrolysing enzyme in order to increase the yield of RS, while less attention has been given for the utilization of both enzymes in single reaction. α-Amylase and pullulanase are starch hydrolysing enzymes which can specifically cleave α-1,4-glycosidic and α-1,6-glycosidic linkages of amylose and amylopectin in glycosidic macromolecules/polysaccharides, resulting in the production of short, linear and branched oligosaccharides of varying length for the formation of resistant starch with more compact crystalline structure (51, 52).Starch is gelatinized at temperatures above 60°C and retrograded at low temperatures below 30°C during the preparation of resistant starch. Therefore, thermostable α-amylase and pullulanase which can withstand temperatures in that range and can easily transform starch into a more organized structure, is required. Additionally, the amylase and pullulanase which have the capability to hydrolyse starch in raw substrate (maize flour) (53) and can withstand the reaction conditions like in the existence of some metal ions present in maize flour e.g. Mg, Ca, Cu, P, K, Na, Zn, Se, Fe etc was preferred over other starch hydrolysing enzymes reported previously in literature. For fulfilments of specified properties as mentioned above, enzymes(α-amylase and pullulanase) isolated from *B.licheniformis* from hot springs in Pakistan, during the current study, were active in presence of metals present in maize flour, and were therefore used in the formulation of resistant starch. In order to make resistant starch, amylose (chain length of 10 to 100 glucose units) is required for RSIII after hydrolysis (54), therefore, it is necessary to optimize the concentration of amylase and pullulanase for enhancing the yield of RSIII. In order to increase the opportunity of alignment and aggregation of amylose chains in less time to form compact crystalline lamellae having ability to resist the gastric digestion, it is more significant to utilize the combination of starch hydrolysing enzymes instead of a single enzyme. As a result, there is an increased opportunity for alignment and aggregation of starch chains favouring the establishment of a perfect compact crystal-like structure. Englyst *et al* suggested that linear amylose is prone to recrystallization and retrogradation, forming tightly packed crystalline structures strengthened by double helices, which would reduce the digestibility of starch (48).

Therefore, the focus of the current study was to enzymatically enhance the resistant starch content of maize flour with the aim to advance its physicochemical characteristics to be used as an important ingredient in foodstuffs (55). Although flour and maize starch are high carbohydrate foods that are substitutable in a few recipes,

however, there are some nutrients in flour which are not present in maize starch. Maize starch is a highly processed carbohydrate without protein, fat, vitamins, minerals, or fiber. It has a nutritional value of approximately 30 calories per 7 grams of carbohydrate or per tablespoon. On the other hand, maize flour, still a processed grain having whole flour, will at least bring some B vitamins and fiber to the food products, instead of maize starch. Therefore, the current study focused on enhancing the physicochemical characterization of the resistant starch content of maize flour instead of maize starch. The resistance of enzymatically produced RSIII to *in-vitro* digestibility is indicative of its ability to bypass small intestine in partially or nearly intact form where it can be utilized by colonic bacteria exhibiting its prebiotic potential by favouring the growth of colonic microflora. Resistant starch, one of the important biopolymers, is a biodegradable and important prebiotic for stimulation of growth of probiotic bacteria in colon (56, 57). It has been studied that native starch is completely broken down by pancreatic enzymes after oral ingestion, with successive absorption from the small intestine into the systemic circulation and has limitation of provoking glycemic index (58). Because of the crystalline structure, the resistant starch is resilient to digestion in the small intestine. To decrease the enzymatic degradation in stomach and to allow acceptable amount of a therapeutic agent to be absorbed, starch in maize flour has been modified with enzymes, α-amylase and pullulanase individually both in combination which resulted in increasing content of resistant starch with compact crystalline.

It has been well studied that the microbiota of colon has significant impact on human health. Whereas participation of human as *in-vivo* model would be considered as gold standard for establishment of prebiotic deep influence on human health, due to ethical, practical limitations and dropout rates of study, participants make the research unreliable and limited.

To overcome this limitation, animal models are considered as a substitute to explore the prebiotic prospect of foods. The second alternative advantage for utilizing animal model is its incessant approach to intestinal matters along with tissues and organs. Generally, most common animal models, utilized for gut microbiota study and to investigate the prebiotic potentials of foods, are rodents like rats, mice and hamsters.

Teixeira *et al* (59) investigated the prebiotic prospective of Amazonian tubers by feeding Wistar rats for 28 days. Prebiotic effect was analyzed by pH and microbiota in feces was gathered from rat's cecum. Samel *et al* (60) investigated the prebiotic

potential of Jerusalem artichoke (*Helianthus tuberosus* L.) with variousconcentration to the diet of rats for 12 weeks and evaluated its beneficial effect on intestinal morphometry, immunity and microbiota of rats. In another study, utilization of 2.5% insoluble fibers from pineapple peel truncatedgastrointestinal transit time, fecal ammonia production and elevated the SCFA production in cecal content with increase in growth of beneficial microbiota like *Lactobacillus* spp. and *Bifidobacterium* spp. in hamsters (61).

However, along with gut microbiota, other factors have deep influence on body metabolism and physiology, most importantly SCFA production, fecal humidity, and pH, which are also directly related to selective prebiotic consumption. In the colon, 95% of produced SCFA were absorbed by colonocytes, whereas remaining 5% expelled in the feces. These microbial metabolites have dual benefit for providing energy source to the host cells as well as regulate the metabolism and energy consumption. The acidification of feces has direct relation with production of organic acids by endogenous beneficial microbiota in the colon (62). Likewise, Hu *et al* (63)and Moura *et al* (64)described the preventive and curative effect of prebiotic on intestinal epithelium in healthy and diseased rats.

Due to such noticeable benefits of prebiotics, its inclusion in industrialized foods has become a healthy and viable substitute for the consumers for maintaining good health (65). Moreover, food industry itself will be benefited by the addition of prebiotic in food products because of functional benefits of prebiotic like improvement of sensory characteristics, nutritional composition and better balance with extended shelf life (66).

Overall, prebiotics are used as ingredient in bakery products, beverages (fruit juices, coffee etc.) breakfast cereals, dairy products and desserts (chocolates, jellies, ice cream etc.) (67). Prebiotics also have emulsifying ability, water retention (e.g. inulin and FOS) and provide spreadable texture, thus providing a pleasant taste and texture to the food along with developing the fast food with low fat content. Among prebiotic categories utilized in the food industry, galactooligosaccarides (GOS) are more stable at high temperature and low pH because of stable beta bonds in their structure as compared to fructooligosaccarides (FOS) and inulin. RS III have potential to increase the dietary fibers of food and can be added to fried battered products for avoiding moisture and absorption of fats (68).

The current research study has objective to explore the ability of enzymatically modified resistant starch (EM-RSIII) from maize flour as a drug carrier for colon targeted drug delivery by exploiting the GIT tract conditions. Using the prebiotic potential of EM-RSIII in vitro, as drug carrier, gives astonishing benefits by stimulating growth of *Lactobacillus reuteri* as probiotic, thus improving colonal health due to production of short chain fatty acids (butyrate, acetate, propionate etc), which is indicated by pH drop in the fermentation medium along with control and site specific drug release. Novel enzymatically modified resistant starch (EM-RSIII) from maize flour, with prominent characteristics including high resistance against enzymatic digestion, low solubility, hardness, birefringence analysis, rheology, pH and thermal stability makes it appropriate for the colon based drug delivery, after testing its digestion against the pancreatic enzymes, which is the main focus of current study. As biobased EM-RSIII has public acceptance, hence its potential use for food and pharmaceutical industry is favoured. Use of EM-RSIII from maize flour as a biopolymer in drug delivery opens up new possibilities for delivery of drugs to particular tissues and cells. It can provide an effective way for drugs to reach the specific sites like local treatment of colon ailments, with reduced administered dose avoiding unnecessary degradation and digestion, for drug administration (69).

In conclusion, the study is based on cloning of the α-amylase and pullulanase gene from indigenously isolated *Bacillus licheniformis* C1, expressed in *E. coli* BL21 (DE3) cells, and purification by affinity chromatography. Moreover, recombinantly expressed purified amylase and pullulanase were effectively studied for its ability to enhance the resistant starch content of maize flour. The impact of treatment with α-amylase and pullulanase individually and in combination of both enzymes on the efficient characteristics of maize flour were analysed, and the *in-vitro* digestion stability with unique physicochemical characterization (water holding, swelling power, oil and milk absorption capacity) of enzymatically prepared maize flour was evaluated compared to that of native maize flour. Therefore, current study was undertaken to introduce new food ingredients as a potential prebiotic prepared through enzymatic hydrolysis as key player for the first time to explore the interlink between pebiotic intake, probiotic stimulation and its effect on blood profile and body physiology. The purpose of recent work was to evaluate the effect of different percentage of enzymatically prepared resistant starch (EM-RSIII) present in the standard control diet on the rat's body weight, fasting and postprandial blood glucose

level, fecal pH, its effects on serum lipid profile, serum insulin level, modulation in gut microbiota and cecum health of control and EM-RS III fed rats to declare it as safe prebiotic. Whereas, aimed to finally utilizing the enzymatically synthesized EMRS-III as a food hydrocolloid by supplementation in cookies ingredients during the current study. Moreover, EM-RS III were attempted to look for the optimum formula of consumer acceptance compared with native flour and mainly assess the suitability and acceptability of addition of EM-RS III as dietary fiber substitute for cookies application by means of sensory evaluation. The study was further based on; validating the prebiotic potential of EM-RSIII for promoting growth of *Lactobacillus reuteri* as *in-vitro* probiotic model (70), the preparation of EM-RSIII coated drug microspheres using ciprofloxacin, a fluoroquinolone antibiotic as a model drug, and finally focused on its characterization with respect to its size, encapsulation efficiency, morphology, chemical and electrostatic interactions and crystallinity of ingredients used in preparation, *in-vitro* release of ciprofloxacin and antimicrobial assays for elucidating encapsulation efficiency.

1.2 Aim and Objectives

Aim of the study:

The aim of this research work was production and characterization of microbial amylase and pullulanase for preparation of prebiotic resistant starch and its applications.

Objectives of the study:

1. Cloning and over expression of amylase and pullulanase gene from *Bacillus licheniformis* into mesophilic host like *E.coli*
2. Purification and characterization of recombinant amylase and pullulanase and their utilization in preparation of RSIII.
3. Structural and physicochemical characterization of enzymatically prepared resistant starch from maize flour
4. Assessing i*n-vitro* and i*n-vivo* Prebiotic potential ofprepared resistant starch
5. Synthesis of food item (cookies) by supplementation of prepared resistant starch
6. Synthesis of drug loaded microspheres with coating of prepared resistant starch in colon targeted drug delivery

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2. Literature Review

Enzymes are naturally occurring catalytic bodies which are responsible for the catalysis of life. They are produced by living organisms to speed up biochemical reactions of the body. Enzymes lower down the activation energy and accelerate the conversion of substrates into their respective products(1). They are highly efficient and can increase the reaction rate up to 10^{12} times. They are neither utilized during the reaction nor appear as a product of reaction. In the absence of enzyme catalysis, many reactions would be too slow to be useful for metabolism (2).

Specific catalytic ability of the enzymes in a reaction makes them more beneficial in industrial procedures. Enzymes have numerous benefits over conventional chemical catalysts. The most important ones are their selectivity and specificity not only for a particular reaction but for their differentiation between similar part of molecule such as regiospecificity and stereospecificity. They have the ability to catalyze the reaction having very narrow ranges of substrates (3). Moreover, during enzyme catalysis, product is produced in uncontaminated state, and further mitigates downstream environmental problems. Enzymatic reactions require very few steps for the generation of desirable end product (4).

From centuries, enzyme existence has been known. However, chemical catalytic action of enzyme was first time studied by the Swedish chemist Jon Jakob Berzelius in 1835. James B. Sumner in 1926, first time isolated, crystallized, and purified the enzyme urease from the jack beans. He got the Nobel Prize in 1947 which is shared with him by John H. Northrop and Wendell M. Stanley due to their contribution in discovering procedure for isolating pepsin. Northrop and Stanley proposed precipitation technique which is still used to crystallize several enzymes (5, 6).

Enzymes are vital units of metabolism and occupy central place for research work all over the world. They are not only the main focus of research in biological community, but also popular in chemical engineering, engineers/designers and also for research purpose in other scientific fields. The use of enzymes in different processes is not only eco-friendly but also the best alternative to the harsh chemicals used in different industries. The output of the processes using enzyme is more as compared to chemicals. Therefore, use of enzyme on industrial level is more beneficial (7, 8).

Enzymes are produced by all living cells but for technical and economic reasons, microbial enzymes are preferred(9). Among microorganisms, fungi and bacteria are main sources of extracellular enzymes production (10, 11). Microbial enzymes are preferred over animal and plant sources (9) because of some economic advantages (bulk production, easily extraction and being independent of sessional variation) and technical advantages (single source for variety of enzymes, stability of enzymes, short generation time, easy genetic manipulation and production on raw substrate) (12-15).Microbial enzymes play a major part in various important processes i.e. brewing, baking, cheese making and alcohol production (16). With the passage of time and advancement in technologies, role of enzyme in chemical, pharmaceutical beverage and food industries are also investigated (17, 18). According to a report published in 2011, the global market of industrial enzymes was close to a billion dollars in 1990 and surpassed the $2.0 billion mark in 2005 (19). In last few years, enzymes market has grown significantly worldwide (20,21). Hence, much has to be explored yet. Animal feed (6%), baking (8%), starch (11%), textiles (12%) and detergents (37%) are the main industries, which use about 75% of industrially produced enzymes (21, 22). Carbohydrases have the largest share of about 37% in the global enzyme market with bakery as the largest used application of enzymes (21).

In modern world, supply of products obtained through enzymatic biocatalysis is increasing continuously, which is linkedwith the rising distresses of consumers and industrialist about the new genera of “green chemistry”(23).In addition to this, with expanding knowledge of biocatalyst structure and characteristics, industrial biocatalyst production inclined towards the production of enzymes from microrganisms. Howevere, towards the expansion of industrialy important biocatalyst production, the credit goes to modern technologies which favoured the expression of selected enzymes in specialized microrgansim (20, 24). Currently, biocatalytic industrial process applications comprising of starch hydrolyzing enzymes find applications in food, pharmaceuticals (25),detergent (26), paper (27) and textile industries (28). Now-a-days the trend of using chemicals for the hydrolysis of starch such as acid is replaced with enzymatic hydrolysis because of numerous advantages of enzymatic hydrolysis over chemical hydrolysis (7, 20). First, due to the specificity of enzyme; sugar syrups are produced with well-defined physicochemical properties. Second, due to the hydrolytic properties of enzymes; fewer undesirable changes or side products are produced. In starch processing industries, those enzymes are mostly

used which have the ability to hydrolyze α-1,4 and α 1-6 glycosidic linkages and have amylolytic ability against complex starch molecules(29).As expected from previous research work, some enzymes with prominent properties have the problem of low expression which limits the optimum use of such enzymes in particular applications. Therefore, there is a need to focus on most advanced and efficient methods for production of enzymes for industrial use (30).

In general, advancement in biotechnology from last decades tailored the performance of microorganisms with enhancing their special features including increased and controlled production of enzymes, metabolites etc. In this perspective, the "omics" technologies including field of genomics, transcriptomics, proteomics and metabolomics emerged. These are the basic tools for the development of methods, protocols and use of instruments for analysis of DNA, RNA and protein structures, leading to the discovery of novel enzymes with increase expression in genetically modified host cells.

The "omics" technologies comprise genome wide sequencing tools, whole genome transcripts, and assessment of protein structure and metabolic profile of microorganisms. Among all "omics" techniques, the most significant and emerging field is "recombinant DNA technology". By utilizing the recombinant DNA technologies, specified gene can be identified, isolated and transformed into well-established industrial microorganisms. In this framework, gene of interest can be cloned and transferred into the host microorganism which is specialized for its expression. The recombinant vectors can be engineered for this purpose by the utilization of restriction enzymes, which allows incorporation of desired gene at specific site, in recombinant vector.

After restriction digestion of both vector and gene of interest, DNA ligase enzyme results in annealing of desired gene into vector. In some cases, multiple copies of specified gene has been inserted into microorganisms in order to potentiate its expression under same promoter (31, 32).

However, development and designing of enzymatic reactors at microscale level are significant advancements in the discipline of proteomics. The active expression of desired enzyme in the new commonly used expression host like *Escherichia coli* and Pichia pastoris is the most highlighted part of biotechnology. The main focus in each sector of omics technologies is associated with cost reduction, which will lead to more significant advancement in bioprocessing and biotechnology in near future (33).

Starch is vital and multifaceted polysaccharides composed of amylose and amylopectin, therefore a combination of enzymes are usually required for its decomposition into smaller low molecular weight compounds. Generally, starch hydrolyzing enzymes can be categorized into following four groups:(34, 35).

1. Endoamylases
2. Exoamylases
3. Debranching enzymes
4. Transferases

Endoamylases are group of important enzymes, like α-amylase, which are responsible for the hydrolysis of internal α-1,4-glycosidic linkage in amylopectin and amylose chain resulting in production of oligosaccharides with different length of glucose ,with α-limit dextrin and with an α-configuration (36).

Exoenzymes are group of important enzymes, like amyloglucosidases or glucoamylases, β-amylases and α-glucosidases, having the ability to attack on the terminal glycosidic bond. β-amylaseis an important member of the group which is able to completely hydrolyze the α-1, 4-glycosidic bond producing β-limit dextrin. In comparison, glucoamylase and α-glucosidase are responsible for the hydrolysis of α-1, 4- and α-1, 6-glycosidic bond resulting in the production of glucose and maltose, as a product. During hydrolysis, α-glucosidase attack on short maltooligosaccharide bonds and result in the release of glucose which is in α-configuration. On the other hand, glucoamylase attack on long-chain polysaccharide. β- amylase and glucoamylase are involved in the conversion of maltose from α to β anomeric configuration (37). Including these, some other important enzymes are the member of exoamylase group, such as cyclodextrin glycosyltransferase and α-amylase, which are responsible for the activity of trans glycosylation and for the production of glucose respectively (36). The third group of starch hydrolyzing enzymes is debranching enzyme, which can hydrolyze the branched point or α-1, 6-glycosidic bond. Member of the group are isoamylase (EC 3.2.1.68) and pullulanase (3.2.1.41) which are responsible for the production of linear polysaccharides by the hydrolysis of α 1-6 glycosidic linkage. The variation amid both of these enzymes is having pullulan hydrolyzing ability. Pullulanase can hydrolyze both pullulan and amylopectin at α-1, 6- glycosidic bond, whereas isoamylase just have the ability to hydrolyze amylopectin at α-1, 6- glycosidic bond. Pullulanase also consists of a class of enzyme known as amylopullulanase or amylase-pullulanase known as pullulanase type II enzyme which

have both characteristics to hydrolyze α-1,4 and α-1,6 glucosidic bond releasing valuable smaller molecular weight end products such as panose, isopanose, maltose, maltotriose etc. (36).Pullulanase known as debranching enzyme belongs to the extracellular carbohydrase family. This enzyme was first time discovered by the Bender and Wallenfels (1961) from mesophilic microorganism which is known as *Aerobacter aerogens* also known as *Klebsiella pneumoniae*. (35, 37-42).

The substrate for pullulanase is pullulan which is a polysaccharide, water soluble and can easily be degraded by microorganism due to hydrolytic enzymes(43). The pullulan itself is produced by the yeast like fungus known as *Auerobasidium pullulan*(44-46). The structural composition of pullulan is repeating units of α-1-4 and 1-6 glycosidic bonds, attached by head to tail like pattern (47). Pullulanaseis particularly responsible for the hydrolysis of α-1,6 bond in pullulan and release maltotriose as an end result (48).

Finally, transferases are the fourth major group of starch hydrolysing enzymes. Transferase have unique mode of action, hydrolyzingα-1, 4-glycosidic bond by donating molecule or functional group (e.g. a methyl or glycosyl group) and transfer it to other glycosidic molecules which act as acceptor and resulted in the formation of new glycosidic bond (37). The most important member of this group is amylomaltase and cyclodextrin glycotransferase, which are involved in the formation of new α-1,4-glycosidic bond. Transferases also include branching enzyme, which is responsible for the formation of α-1,6-glycosidic bond. The difference between cyclodextrin glycosyltransferase and amylomaltase during trans glycosylation reaction is their end product. The end product of cyclodextrin glycosyltransferase is cyclic compound which have 6, 7 or 8 glucose molecules while amylomaltase produce product in the form of linear chain (36). The most common and complex substrate for all the major four groups of carbohydrate hydrolyzing enzymes are complex polysaccharide most importantly “starch”(48).

Starch is majorly occurring type of polysaccharides present on earth which is considered as one of the most valuable asset for life due to its extensive usage as a source of energy, particularly for humans and animal feed all over the world (49). It has numerous uses in almost all the fields of industry, including food, feed, baking, brewing, detergent, textile, oil drilling, ethanol production and bioplastic formation (50).

Starch is abundantly present as a reserve material in various parts of plants including leaves, fruits, flowers, stem, grains, tubers, roots and seeds. Starch exists in the form of semi-crystalline granular structure and acts as a source of carbon and energy as glucose to fuel up the metabolic reactions of plants e.g., sprouting of seeds and to cope up with water shortage(51).

Starch is a polymer comprised of two homo-polysaccharides of D-glucose namely amylose and amylopectin constituting about 98-99% of starch. Starches obtained from most plants consist of about 20-30% amylose and 70-80% amylopectin(52).

The two types of α-glucans are composed of several glucose molecules connected with α-(1,4) and/or α-(1,6) glycosidic bonds(53).Amylose is one of the major components that constitute polymeric starch , which gets scientifically defined as a glucose homo-polymer that commonly occurs in linear configuration combined by α-1-4 glycosidic linkage whereas, in some cases it also exhibits 1-6 branch linkage which repeats itself in a ratio of 1:180-320 units (54). The grade of polymerization of amylose is observed to be 6000 units of glucose whereas, it exhibits an average molar mass of around 105-106 g/mol. Amylose is most commonly found in a right handed spiral or helical structure (28).

Amylopectin has a densely branched polymeric network composed of glucose molecules in which 95% of glucose molecules are linked together with α I,4 glycosidic bond whereas the remaining 5% units are combined by α I,6 glycosidic bond. Amylopectin is the other major constituent of polymeric starch densely branched joined together by 1-6 glycosidic bonds. This homopolymer is rich in glucose units arranged in linear configuration containing α 1-4 glycosidic linkages. The branches linked together with 1-6 bonds constitute only 4-5% of the overall bonds exhibited by amylopectin (55). Amylopectin is one of the massive polymers as a result of which its molar mass is observed to be huge, whereas its degree of polymerization is observed to be 2×10^6(56). Figure 2.1 shows a cluster model of amylopectin`s structure. Amylopectin molecules are constituted of a core chain commonly known as C chain combined with various branches containing a reducing end group, which then constitute A chain and B chain. The A chain is rather small and short with approx. 12-16 DP and while constituting a double helix, it exhibits a cluster type appearance. This cluster constitutes around 80-90% of the overall chain and is connected to the B chain which is rather longer and comprises the remaining 10-20%

of the overall chain. Mostly, the branches extend to form 2(40 DP) or 3(70 DP) clusters, whereas rarely some branches combine to form a 110 DP cluster (57, 58).

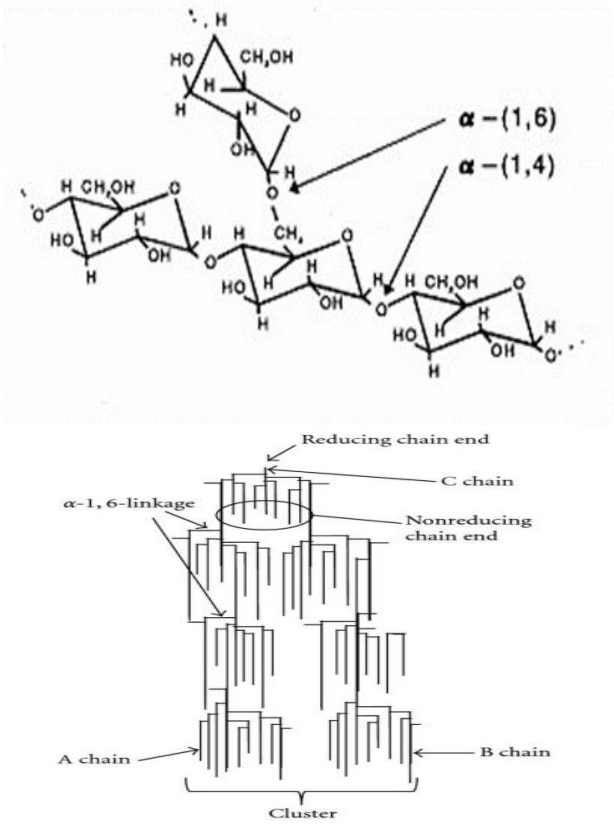

Figure 2.1: Amylopectin cluster model indicating A, B and C chains of glucose (**59**).

The consumption of α-amylase and pullulanase in starch-based industries have been well recoqnized for hundreds of years and various microrganisms found for the extraordinary production of these biocatalyst, even though few selected strains of bacteria and fungi meet the criteria for extracellular production of theses biocatalyst on industrial scale. *Bacillus strains* are recognized to be efficient producers of α-amylase and pullulanase and these have been extensively utilized for commercialization of the enzyme for different industrial applications. The biocatalyst from the *Bacillus* species are of key interest and focus of current study for large-scale biotechnological processes due to their significant activity against raw substrates and because efficient expression systems are available for their enzymes to be used in food industry. During the current study, given the effect of pH shift, effect of different temperature ranges and metal ion concentrations for valuable utilization of amylase and pullulanase on raw substrate (maize flour) modification, were analyzed from *Bacillus licheniformis*. To be an excellent candidate for modification of raw substrates the amylase and pullulanase should be stable and capable of hydrolysis in broad range

of pH, temperature and in the presence of metal ions (as raw substrates (maize flour) naturally enriched with valuables metals ions). During current study maximum amylase activity was observed at acidic pH 6 and it was 1.82U/mg. *Bacillus amyloliquefaciens* has been reported for production of amylase stable at pH 5 to pH 7. It showed optimum amylase activity at pH 5 and it retained 60%-80% of its activity at pH 6 and pH 7 (60, 61). Ali *et al*. have also reported amylase production at acidic with optimal activity at pH 5 (62). Extracellular thermophilic amylase of *Bacillus amyloliquefaciens* P-001 has also been reported for showing optimum activity at pH 6 (63). While in case of exploitation of temperature effect our finding is in parallel to the findings of Poli*et al*. who reported a thermophilic amylase producing specie of *Anoxybacillus amylolyticus* and reported the optimum amylase activity at 50°C (64). Parakash*et al*. reported that amylase purified from *Chromohalobacter sp*. TVSP 101 showed optimum amylase activity at 55°C (65). Carvalho and Côrrea reported thermophilic amylase from *Bacillus sp*. (66). Whereas, during the current study, stimulation of amylase activity by calcium is in accordance of Asgher*et al*. who reported thermostable calcium dependent alpha amylase from *Bacillus subtilis* (67). In case of purified pullulanase optimum temperature was 50°C with maximum specific activity of 191.4 U/mg. Our findings were in close similarity with other reported researches as Roy *et al*. (2003) reported that optimum activity of pullulanase at 50°C which was produced by thermophilic, *Bacillus sp*. US149 (68). Devi and Yogeeswaran (1999) observed that the mesophilic strain of *Micrococcus halobius* OR-1 produced pullulanase which have the optimum activity at 60°C (69).Whereas according to Stefanova *et al*. (1999) the optimum activity of pullulanase from *Bacillus acidopullulyticus*was observed at 50°C (70). In case of pullulanase enzyme production optimum pH ranges varied between pH 4.0-7.0. In current research work, maximum pullulanase activity was obtained using *Bacillus licheniformis* at pH 6. According to the previous literature most of the pullulanase from the microorganism having maximum activity at acidic or neutral condition and optimum pH for the enzyme activity mostly ranges between pH 5.0 to 7.0. Such as research conducted by Gomes *et al*., (2003) reported that for pullulanase from *Rhodothermus marinus* the optimum pH range was 6.5 -7.0 (71). Roy *et al.,* (2003) also reported the production of pullulanase type II by *Bacillus* having maximum activity in acidic pH range (68). *Thermotoga maritima* produced acidic pullulanase with optimum pH of 5.9. Beside this, Nisha & Satyanarayana (2016) and Meng *et al.* (2020) also investigated the

production of acidic pullulanase enzyme from *Clostridium thermo sulfurogenes* SV2 *and Bacillus sp* (72;73). DSM 405, respectively with optimum activity at pH 6.0.In addition to this, during present study Ca^{+2} and EDTA has no noticeable effect on pullulanase activity. These results suggesting that Ca^{+2} is not requiring for activity of pullulanase and no effect of chelating agent suggested that it work efficiently in the presence of different metals. The given results are parallel to Chengyao *et al.* reported that, pullulanase activity was independent of Ca^{+} ions in the reaction mixture (74).Therefore, during current study the amylase and pullulanase were investigated for their significant characteristics and recommended as an excellent catalysts for the modification of raw starch substrate (maize flour).

2.1 Classification of Starch based on Digestion:

Several enzymes act upon the starch converting it into its monomers (glucose) starting with the action of α-amylase present in the oral cavity followed by digestion carried out by pancreatic α-amylase, sucrose isomaltase and glucoamylase, in the small intestine. The glucose released is absorbed by the membrane of the small intestine.

Starch plays a vital role in human metabolism. Recently, the scientific community has understood the importance of starch in human health and is taking keen interest in research work based on different aspects of starch structure, digestion and health benefits (75,76).

The classification of starch is principally done for nutritional purposes and is mainly based on the extent of starch digestibility(77).

Based on the digestibility pattern, Englyst and its Colleagues classified starch into following three categories:

2.1.1 Rapidly Digestible Starch:

Rapidly digestible starch **(**RDS) is that component of starch which upon ingestion gets rapidly hydrolyzed by the enzyme, only in 20 minutes. On ingestion, it gets broken down into smaller molecules by enzyme of the small intestine. This induces a fast release of glucose in bloodstream, increasing insulin levels and hence can be a cause of diabetes and cardiovascular diseases (78).

2.1.2 Slowly Digestible Starch:

Slowly digestible starch **(**SDS) is that fraction of starch which hydrolyzes into glucose monomers by the action of intestinal α-amylase after first 20 mins and the complete hydrolysis is achieved within 120 mins, as proposed by *in-vitro* digestion models of SDS. This takes more time to be hydrolyzed as compared to RDS primarily

because of its amorphous structure being physically less susceptible to enzymatic attack, leading to a slow but prolonged glucose release in the blood (79).

2.1.3 Resistant Starch:

Resistant starch (RS) is that portion of starch which resists digestion by gastric enzymes and undergoes partially or fully fermented in the large intestine, by colonic bacteria. RS is known as an essential component of total dietary fibre (TDF) and resists hydrolysis by enzymes of the small intestine (80). It is a linear molecule consisting of α 1, 4 D-glucan with 10 to 100 glucose units tightly packed in double helical structure and is primarily a derivative of retrograded amylose. A positive correlation exists between the RS and amylose contents of native maize, as proposed by Evan and Thompson (81).

Fermentation of resistant starch by colonic microflora results in the reduction of intestinal pH, production of ammonia, phenols and production of short chain fatty acids (SCFAs), particularly butyrate with anti-carcinogenic and anti-inflammatory properties, promoting the colonic health (82).

2.2 Classification of Resistant Starch:

RS on the basis of structural and conformational changes (83)is categorized into five types, as follow.

2.2.1 Resistant Starch Type I:

RS1 is that type of starch which is rather difficult to digest because of the hard-external coating set-up surrounding the grain, which delays its digestion. It is also referred to as physically inaccessible starch due to its entrapment inside whole or partially milled seeds and grains. In most cases, the outer protective matrix is constituted of protein-based substances or it might be able to acquire protection from cell wall, as it mostly occurs in legumes, pasta and whole grains. These also contain granules present in cereal grains that can be found in the protein`s hard matrix. The swelling of these granules while cooking is delayed owing to the presence of a protein matrix present just outside the starch that behaves like a fence and stops the action of amylase. The matrix protecting the starch eventually gets broken down when it is milled and meshed causing the starch on the interior of this matrix open for the amylase to act (84).

In some foods e.g. pasta, RS1 type is present inside the compact protective matrix composed of proteins. This proteinaceous hard shell acts as an obstacle by not allowing water to diffuse easily (85). Therefore upon heating, the pasta granules do

not undergo complete gelatinization rendering it less susceptible to attack by hydrolytic enzymes (86). Upon attack by proteases, the outer protective layer of proteins is removed, resulting in increased accessibility of the substrate to the hydrolytic enzymes and hence enhances starch hydrolysis. Cooking leads to an enhanced rate of starch hydrolysis by gelatinization, rendering it more accessible for enzymatic attack (87).

2.2.2 Resistant Starch Type II:

RSII is that type of resistant starch which resists the enzymatic digestion owing to the organized structure and configuration of the raw native granules. Because of the tightly compact and dense structural configuration of the RSII, it can resist enzymatic attack by various types of amylases. It is also known as Native B-Type with semi crystalline structure and is present in both B and C polymorphic types. In its native resistant form, RSII mainly occurs in green banana and uncooked potato starch (80, 88). The resistance to enzymatic attack is particularly ascribed to the occurrence of B-type crystalline structure of the starch pellets. On contrary, upon heating, RSII undergoes a conversion from B-crystalline structure to a very digestible amorphous structure, making it more vulnerable to enzymatic hydrolysis (89). The resistant starch levels correlate with the level of amylose present in the native starch structure. Upon heating, the highly resistant granular conformation of the RS type II becomes open by conversion of crystalline structure into amorphous form, which is easily accessible to digestive enzymes (88). RSI and RSII are considered native starches because of their susceptibility to chewing, heating, milling, moisture, and boiling. Therefore, during cooking process, RSI and RSII can lose their rigidity and resistivity to digestion (90).

2.2.3 Resistant Starch Type III:

RS-III is the only form of resistant starch which is most commonly used in many food formulations. It is mainly comprised of retrograded amylose because of its high affinity to re-associate. It can be synthesized by thermal, acidic, or enzymatic treatment (91). Among these, enzymatic synthesis is preferred due to its environmentally friendly or bio-based nature making resistant starch free of any toxic residues as compared to chemically synthesized resistant starch. Importantly, biocatalysts are highly selective and specific for their substrate and after reaction, they can easily breakdown into simple nontoxic compounds and become inactive (92).

Therefore, the most preferred way to further increase the quantity of RS-III is through enzymatic debranching, which yields in all linear glucans that have strong tendency to re-associate. Debranching enzymes like pullulanase and amylase have drawn increased attention in starch conversion processes (93). These specifically hydrolyze α-1, 6-glucosidic bonds and produce linear polymer associated by α-1, 4-glucosidic bonds from amylopectin molecule into condensed double helical structure, which facilitate starch retrogradation. This structural modification renders α-1,4-glucosidic bonds inaccessible to amylase hydrolysis in gastrointestinal environment (94). However, synthesis of resistant starch totally rely on the ratio of substrate, amylose and amylopectin (95), type and arrangement of crystalline structure of starch (96), degree of polymerization of amylose chain, autoclaving (gelatinization), retrogradation temperature and most critically, percentage of protein content (28). Amylose and long chain amylopectin, upon hydrolysis, aid to increase the apparent amylose concentration of starch. Whereas in some cases the chains of amylopectin do break down, but in such a scenario it can form small double helices which directs the formation of a strong dense crystalline structure (97).

2.2.4 Resistant Starch Type IV:

This type of starch is also called as chemically modified starch because it is revised by the supplementation of various functional groups including ethers, esters or phosphate groups, rendering it resistant to digestion by hydrolytic enzymes (98).Esterification of starch with the addition of various functional groups such as acetic anhydride, propylene oxide and octenyl succinic anhydride leads to an increased resistance to hydrolysis by amylolytic enzymes (99). Esterification of starch is usually achieved by usage of the functional groups containing hydrophobic regions; they contribute to enhanced resistance to amylolytic enzymes. This is mainly because hydrophobic moieties prevent the solubilization of starch molecules, thereby delaying the accessibility of the hydrolytic enzymes. The resistant starch content can be enhanced by the addition of different cross linkers to the starch molecule. The commonly used cross linkers include phosphate oxychloride and sodium trimetaphosphate (100). The cross linker molecules attach to the starch, decreasing its flexibility, and when subjected to enzymatic attack, cross linker can be bonded to the active sites of the enzymes as inhibitor (101). Additionally, crosslinking of starch molecule leads to a compact structure, restricting the swelling ability of the starch molecules, thus ultimately inhibiting their expansion when subjected to cooking.

However, RSIV is ethically unacceptable to the consumers because of the incorporation of other chemical groups to starch structure. Chemical modification can increase the RS content almost up to 80%. Accompanying downsides are linked to consumer acceptability and environmental concerns. There are strict regulations of FDA to use a modifier in food starch, within given limits (102).

2.2.5 Resistant Starch Type V:

RSV is also a type of resistant starch that synthesizes the complex by utilizing linear amylose and lipids. It gets synthesized from high amylose starch that rapidly gelatinizes at high temperatures and is quite prone to retrogradation (103).

2.3 Methods for Preparation of Resistant Starch:

Many procedures are being practiced to make/or increase the yield of resistant starch. The effectiveness of these methodologies largely depends on the botanical origin of the starch. Autoclaving cooling cycle is successful in increasing the RS content in almost all types of starches, but it requires more input in terms of manpower and time. Therefore, more research work is needed for rapid extraction of RS through autoclaving cooling cycles. Following are different strategies/methodologies described to elevate the production of resistant starch content.

2.3.1 Physical Modifications:

Physical amendment of starch can be achieved by subjecting starch to thermal treatment and high-pressure gelatinization, ultimately storing it at low temperature(104). Moreover, physical modification of starch includes freezing, annealing, hydrothermal and pressure treatments. Retrogradation of RSIII in crystalline structures is accomplished by consistent autoclaving and refrigeration of the starch during RS production. A commonly used method of physical modification is hydrothermal treatment during which starch is treated when it has 30% of its moisture content and then it is subjected to subsequent heating at 100°C for about half an hour. For the formation of retrograded starch, the starch obtained from the above hydrothermal treatment is refrigerated at low temperature which facilitates annealing of the linear molecules into a more crystalline resistant structure which is characteristic of RSIII (105). Dual autoclaving-retrogradation treatment led to a substantial increase in the RS content of rice cultivars, treated rice starch samples showed greater RSIII contents (30.31-38.65%) in comparison to their native starch (4.42-10.94%) as reported by Ashwar *et al.,* (106).

Autoclaving-cooling treatment resulted in the increased RS content of corn-based rice analogues. The results of current study indicated that cooling at 40°C for 60 hours led to upsurge in RS content from 6.27% to 15.38%. Longer cooling is associated with greater percentage of retrograded starch, ultimately showing higher RS content (106). Physical modification methods are frequently used because of its safety, simplicity and cost-effectiveness but it is one of the most laborious method requiring extended gelatinization and retrogradation times(107).

2.3.2 Chemical Modifications:

Chemical modification is another method which is used to render enhanced resistance to digestion by the formation of starch chemical/functional group phosphodiester bonds which may lead to production of 40-80% of resistant starch among the total dietary fiber (108). This encompasses the introduction of different functional groups within the starch polymer without disturbing size distribution and morphology of starch granules. This results in important changes in various starch properties including starch gelatinization, retrogradation, swelling, pasting and textural capacities (109). Food and non-food industries lead to the improvement of inherent starch properties through chemical modifications.

Chemical modifications result in increased amylose content which contributes to greater retrogradation capability of the RSIV. Treatment with acids and alcohols are included in chemical modifications which cause hydrolysis of starch amorphous regions particularly the amylopectin branches producing linear residues which then retrograde at low temperature forming crystalline structure of RSIV (110). Similarly, RS levels of rice starches significantly increased from 0.9% (before treatment) (111), to about 38.3% after cross-linking treatment using12% STMP/STPP sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) taken in the ratio of 99:1.This method is very useful, feasible and extensively used in the industries to produce RS IV but it is costly and has safety and ethical issues, which makes its usage limited in some cases (84).

2.3.3 Enzymatic Debranching:

Enzymatic treatments modify the starch making it more resistant to digestion. The increase in RSIII is primarily because debranching enzymes exclusively slice the α-(1,6)-glycosidic bonds present in the amylopectin molecules and α-(1,4)-glycosidic bonds of amylose resulting in an increased amylose content with shorter chain length. About 10 to 100 DP of glucose units are optimum for forming double helix's

crystalline lamella. This causes an increase in its retrogradation which results in amylose units forming tightly packed, organized crystals contributing ultimately to enhanced crystallinity (112). Pullulanase, Isoamylases, and α-amylases are the most commonly used starch hydrolyzing enzymes preferably used for preparation of resistant starch. The amylose units can form double helices which are maintained by formation of hydrogen bonds leading to a further increase in RSIII (113).

2.3.3.1 Reaction Pattern of Pullulanase:

For the formation of RSIII, enzymatic method is the most commonly used method primarily because it is cost-effective, less time consuming, safe for human health and there is no need to lower down the temperature for enzyme activity (thermostability), making it more feasible with respect to industrial processes (114). Starch is constituted of amylopectin which is a densely branched structure linked together with α-(1,6)-glycosidic bonds. The enzymes which exclusively act upon these sites are called debranching enzymes e.g., pullulanase and isoamylases. Once provided with optimum conditions, these enzymes are capable of hydrolyzing pullulan, amylopectin and all other saccharides containing α-(1,6)-glycosidic linkages (115). Zhao and Lin, proposed that debranching of retrograded starch with pullulanase results in increasing its RS contents(116). Heating of starch slurry results in the formation of gel which when attacked by pullulanases, the α-(1,6)-glycosidic linkages of the starch gel get broken down by the enzyme producing short linear chains of amylose. These short amylose chains are refrigerated and they re-associate into a more highly packed, organized crystalline structure which is thermostable and has increased resistance. Pullulanase is more preferably used as compared to debranching enzymes (e.g.: isoamylases) because of its broad substrate specificity (117).

2.3.3.2 Reaction Pattern of α-Amylase:

Amylolytic enzymes are the class of enzymes which can cleave the glycosidic bonds present in the starch molecule. These enzymes are produced by all living organisms (118). Amylolytic enzymes can be split into two classes centered on their mode of action i.e., endo-acting and exo-acting enzymes. As the name indicates, endo-acting enzymes hydrolyze the starch molecule internally by cleaving the glycosidic bonds resulting in a sudden decrease in the starch viscosity and hence are also known as liquefying enzymes. On the other hand, exo-acting enzymes are those which act on the ends of the starch molecule liberating oligosaccharides only from the non-reducing end of the starch polymer. But the viscosity of the starch molecule remains

significantly unaffected by exo-acting enzyme (118). Hydrolysis of starch molecule by α-amylases results in cleavage of the α-1,4 glycosidic linkages liberating oligosachharides with α configuration. The α-amylases which can attack multiple chains of starch molecule are usually preferred (34).

2.4 Quantification of Resistant Starch:

Various other approaches are extensively used for the quantification of RS content in various food items. Some of the most commonly used methods include Berry method (119), which directly quantifies RS in food residues after exclusion of digestible starch whereas indirect methods are also used for determination of RS considered as the portion obtained after subtracting solubilized starch from the total starch (120). Other methods include usage of enzymes i.e., amylases, proteolytic enzymes, amyloglucosidases, etc. Resistant starches are also quantified as part of the total dietary fibre (TDF) content (121). Megazyme assay is nowadays most extensively used method in which firstly the digestible portion of starch is digested with prepared solution of enzymes containing a combination of amyloglucosidase (AMG) (3 U/mL) and pancreatic α-amylase (10 mg/mL).This assay most preferably utilize the *in-vitro* quantification of resistant starch in the sample(122).

2.5 Factor Effecting Resistant Starch Production:

Various factors affect the synthesis and yield of resistant starch by enzymatic hydrolysis, described as follow.

1. **Interaction with Proteins:**

Proteins have been negatively associating with the formation of RS and starch-protein interaction results in reduction of RS contents synthesis (91) as observed in case of results in which mixtures of potato starch and albumin, when subjected to high-pressure autoclaving-cooling, decreased the RS contents (123). Addition of 5% albumin reduced RS level by 50%. Proteins limits the accessibility of the starch to enzymatic hydrolysis due to the physical barrier by encapsulating the starch granular structure and thereby results in decreased synthesis of RS contents (124). Unless this proteinaceous barrier is removed by proteases or other treatments, digestion by α-amylase and amyloglucosidase is significantly retarded, resulting in reduced RS contents in starch. Previous reports (125) stated that an extensive proteinaceous network strengthened by disulphide bonds was formed around starch granule of corn and sorghum. Cooking caused the network to be collapsed and intertwined over the granules. The longer the cooking time, greater the conformational changes occurring

in protein from α-helix to β-sheet (extensive cross-linking), thereby resulting in exposure of starch for digestibility (126).

2. Enzyme Inhibitors:

Various substances act as inhibitors and inhibiting α-amylase activity including proteins, glycoproteins existing in legumes, anti-nutrients such as tannins, polyphenols, phytic acid, lectins, some hydrolysis products specifically maltose and maltotriose (80) therefore have negative effects on RS preparation.

1. Ions:

Escarpa*et al.*, suggested that presence of some micronutrients in starch gels such as calcium and potassium resulted in decreased RS contents primarily because of their intervention in the hydrogen bonding formed between chains of amylose and amylopectin.

2. Lipids and Emulsifiers:

Björck*et al*., and Crowe *et al.*, proposed that complexes of amylose with lipids showed resistance to *in-vitro* digestibility when compared with free amylose. The extent of the decrease in digestion basically depends on the ratio of amylose to amylopectin along with the type of lipid forming complexes with monoglycerides forming complexes which are greatly resistant to hydrolysis (127).

2.6 Physicochemical Properties of Resistant Starch:

2.6.1 Amylose Content:

Amylose matter of starch is considered as one of the essential properties rendering resistance to enzymatic digestion. It is usually determined by the method based on iodine binding capacity of starch. The amylose content influences several other properties of starch including swelling power, gelatinization, retrogradation capacity and susceptibility to enzymatic hydrolysis (128). Amylose content of starches depends on the botanical source and usually range from 20-30% in majority of plants (129).

The relative amounts of amylose and amylopectin constituents of starch are important because amylose shows more resistant to gelatinization and when subjected to cooling results into retrograded starch at a much faster rate. The amylose can also form complexes with lipids thereby reducing the extent of digestibility further as compared to the carbohydrate alone. Therefore, RS V containing starch shows greater resistant to enzymatic digestibility and might take about several hours to digest. The reason is that the complexes of amylose-lipid in RS V limits swelling of granular starch while

cooking, rendering it resistant to enzymatic hydrolysis because of physical inaccessibility of the starch granule, due to lipid layer (130).

The amylose levels are directly proportional to the level of resistivity of starch, subsequently increasing RS content. The amylose level can be increased by hydrolysis with pullulanase which debranched amylopectin chains of the starch molecule to produce amylose chains, ultimately enhancing the RS content significantly. This is because hydrolysis of α-1,6-glycosidic bonds of amylopectin produces short linear amylose chains which have stronger retrogradation tendency and recrystallize into compact structure, contributing to the increased RS content. Hence, debranching of starch with pullulanase enhances amylose and RS content (131).

2.6.2 Water Absorption Capacity:

Water absorption capability (WAC) is the extent of water holding ability of starch. It is an essential parameter for processing of starch that has implication for viscosity. The WAC of the starch plays a crucial part in the development of functional and sensory features of starch during food preparation. The presence of hydrophilic components in both proteins and polysaccharides help in interaction with water in foods. The differences in WAC of starch can also be due to differences in protein structures, concentration, and interaction with water (132). Additionally, WAC denotes the water mixing ability of a product under limited water conditions (133). The increased WAC of starch has been directly related to increased leaching and solubility of amylose and loss of starch crystalline structure. Due to high concentration of amylose, compact crystalline structure of resistant starch renders to have low WAC. WAC of starch is useful for its application in sausages, doughs, processed cheese and bakery industry (134).

2.6.3 Swelling Power and Water Solubility Index:

Swelling power and water solubility index (WSI) of prepared resistant starch helps in processing and better handling, leading to crispiness, crunchiness, expansion and enhanced texture to the formed product. Moreover, these properties of resistant starch make it suitable candidate for the baking industry (cookies, biscuits, cereals, etc.). The swelling power of starch is usually evaluated over different temperatures and is dependent on temperature, amylose/amylopectin ratio and structural and chemical composition of starches. Granules which have greater crystalline areas and stronger bonds will lead to less swelling in both cold and heated waters, resulting into weaker gels with greater retrogradation tendency. Heating starch granules in excess amounts

of water causes disruption of the crystalline structure. This disruption led by hydrogen bond between water molecules and hydroxyl group of amylose and amylopectin chains resulted in dissolution and swelling of starch. Once gelatinization has occurred, it results in swelling of starch which enhanced with increasing temperature. Swelling pattern can also be influenced by the number of other components including amylose, protein, phosphorous, lipids and amylopectin (135).

Water solubility which is the amount of extent of the solutes/hydrolysis of bonds which get leached out from starch granules when subjected to swelling. The WSI of starches is significantly dependent upon source, amylose/amylopectin content, temperature, thermal stability, as well as type of starches. WSI is considered as an indicator of destruction of starch components. Generally, below the gelatinization temperature, all starches remain less soluble. Some studies showed low solubility of starches even at high temperatures because of the semi-crystalline granular structure of starch (136).

In comparison, the undefined, unorderly structure of some native starches gets easily destroyed and results in hydrogen bonding of water with hydroxyl group of amylose (137).

2.6.4 Water Binding Capacity:

According to Franks *et al,* water structure greatly modifies and influenced by number of interacting biologicaly important molecules. Like particularly in case of bonding pattern with oxygen and hydrogen molecules interacting differently at extrem conditions like during ice formation (138). The difference in behavior of WBC of starch is associated with the granular morphology and internal granular structure. The bound water includes both the water absorbed by the starch granules as well as adsorbed by their surfaces.

Some researchers reported an increase in WBC of starch pastes after pre-gelatinization, primarily because heat-moisture treatment causes an increased hydrophilic tendency of starch (139). Some modifications result in the weakening of internal granular structure of starch, making it more susceptible to bind water molecules. Contrarily, modified starch can also show decreased WBC which might have been due to the enhanced crystallinity caused by the debranching and recrystallization process, done during its preparation. This gave molecular chains a greater opportunity to undergo aggregation, resulting into a compact and rigid crystal (140).

2.6.5 Oil and Milk Absorption Capacity:

Food formulations are related with water, milk, and oil absorption capacities of starch. Different means have been used to govern oil holding capacity of starches (141). Several factors are responsible for increased oil uptake of starch including alterations in the amylose/amylopectin ratio, chain length distribution, larger surface area and porosity of the particles and presence of greater hydrophobic proteins which assist oil absorption (142). The amount of carbohydrates in mixtures also result in the elevated OAC. Oil absorption capacity of starch might include the physical entrapment of oil inside the porous structure of starch, due to capillary forces and by the development of complexes of amylose with lipids within the helical starch structures (106). The OAC of flours is an essential factor for the improvement of the flavor, mouth feel, and texture of food items. The increased oil uptake of native starch might be because of the varietal differences, greater surface area and porosity of the particles. Compared to native, RS had a compact structure due to debranching and ordered conformation of granules could have resulted in low porosity that restricted oil uptake (143).

Milk absorption capacity of starch samples is related to the interaction between amylose and milk proteins which form a complex, and hence greater the amylose contents of starch greater will be the ability of milk absorption (144).

2.6.6 Iodine Staining Index:

The iodine staining index (ISI) is considered as one of the essential properties of any starch sample primarily because of the insertion of iodine residues inside the helical structure of starch. It is a basic tool to indicate the efficacy of the complexing ability of the starch chains with other organic compounds. This is a very important property of RS for its application in various processes such as cooking, baking, drug carrier and as encapsulating agents because these processes require interaction of starch with other compounds. The procedure extensively used for determining iodine staining index of starches is a modified procedure used for the determination of amylose by Knutson, (145). The iodine binding ability of the starches differs in various aspects such as starch chain length, level of polymerization, concentration, and conformation of starch molecules. The absorbance values of starch-iodine complexes at a wavelength of 460nm are indicative of presence of short chains with degree of polymerization of glucose units (DP),less than 30,whereas absorbance values at 570 nm are representative of longer chains having DP of around 35-300 (146).

The ISI of debranched starch samples such as the RSIII was observed to be higher as compared to their corresponding native starches indicating higher complexing ability possessed by the resistant starch (147).This is because of native starch which showed lower absorption values at both 460 and 570 nm. The primary reason can be the inner branched chains of amylopectin molecules embedded in the form of highly organized crystals making it harder for native to form iodine complexes. Similar results were observed in findings of Precha-Atsawanan *et al*., (148) in which the inclusion complexes of iodine with debranched starches (RS) displayed greater absorption values as associated to native starches. It was also stated that increasing hydrolysis of starch with debranching enzymes (pullulanase) increased the absorbance values of partially debranched RS-iodine complexes at both wavelengths, specifically at 570 nm than the 460nm indicating presence of longer chains. Klaochanpong *et al*.,(147) also suggested the involvement of longer chains in formation of efficient iodine complexes. Hydrolysis of the branch points of amylopectin lead to release of smaller hydrolyzed products consisting of various sizes of branched and linear dextrin's which upon complete hydrolysis linearize into straight chains. This makes the inner helical cavities of the starch accessible for iodine molecules to penetrate and form starch-iodine complexes, hence greater absorbance values are obtained (147).

2.6.7 Fatty Acid Binding Capability:

Fatty acid-binding capability of starch samples is calculated by the method described by Klaochanpong (147) with slight changes and is based on evaluating the iodine staining index before and after the addition of fatty acids. The fatty acid binding capability of the starches was determined by difference of ISI values of iodine complexing with starch–FA complexes and of free starch. The fatty acid complex forming ability is elucidated by the measurement of the free debranched starch sample residual in the mixture, detected by iodine staining because of the quick, efficient, and feasible procedure. This method also reveals lengths of chains of the resistant starch (DBSs) that participate in forming inclusion complexes with fatty acids by following the reduction in absorbance values at different wavelengths (147).

Palmitic acid (PA, 16) and butyric acid (BA, 4) are classified as long chain and short-chain fatty acids, respectively. The complexes formed between starch-FA are assumed to be more stable as compared to those formed between iodine and free starch. All the related studies showed a substantial increase in complexation with iodine as well as fatty acids when debranching was done by enzymes. Therefore, RS can be employed

as an alternative source of coating material for delivery of active compounds including flavonoids, vitamins, drugs and antioxidants (149).

2.6.8 Light Transmittance:

One of the most common use of starch is to impart viscosity to food. The effect of refrigerated storage is usually used to predict the behavior of starch containing material when placed at low temperature. Substantial reduction in the light transmittance of both native and modified starches when subjected to refrigerated storage was observed over the period of 5 days (120hrs). This decrease in transmittance is mainly related to changes in different properties of starch gels including granule swelling, amylose and amylopectin contents and their leaching during storage, all of this contributing to the development of turbidity and retrogradation (140).

This reduction in the light transmittance of starch samples with the rise of refrigerated storage time can be attributed to the retrogradation ability of starch during which the initial broken bonds re-associate into a compact, organized structure (140).

Light transmittance of modified, treated or RS was greater as compared to their native counterparts after equal storage periods. Yadav*et al*.,2013 reported similar results of light transmittance on arrowroot starch gel (151). One of the reasons of reduced %transmittance of native starch is the phenomenon of Raleigh light scattering which takes place when suspended starch molecules are so small that they are comparable to the wavelengths of incident light and its extent varies with degree and dispersion of starch molecules. Enzymatically produced RS results in greater debranching of amylopectin chains, greater amylose and RS content, greater compact and organized structure which influences its retrogradation tendency and hence decreased transmittance of starch samples (152).

2.6.9 Antioxidant Potential of Resistant Starch:

2.6.9.1 DPPH Radical Scavenging Activity and Reducing Power:

The commonly used methods to assess the antioxidant potential of the starch samples include the DPPH radical scavenging activity and reducing power assays of starches. DPPH radical scavenging assay depends on the quenching of purple colored DPPH free radicals by antioxidant molecules. During the assay donation of electrons or hydrogen atoms possibly following a free radical scavenging mechanism on the DPPH molecule. This leads to the production of a colorless product which is spectrophotometrically detected. Contrarily, the yellow colored solution turns to

various shades of green in reducing power assay. The change in color from yellow to green depends on the reducing power possessed by the sample. Ferricyanide complex (Fe^{+3}) undergoes reduction into its ferrous form (Fe^{+2}) due to the existence of reducers.

Polysaccharides possess one or more free hydroxyl group which are directly related to the extent of antioxidant potential of starch molecules (138). Similarly, hydroxyl groups of starch structure contribute to its free radical scavenging ability. The increased antioxidant activity of modified starches by different methods including autoclaving, irradiation or enzyme debranching could be associated to the abundance of greater hydroxyl groups because of modification treatments, thereby showing increased radical scavenging ability by both DPPH scavenging and reducing power methods (106).

2.7 Digestion of Starch:

Starch digestion starts in mouth by mechanical degradation of starch by teeth during chewing and mastication. It is also accompanied with the secretion of salivary α-amylase which hydrolyses glycosidic bonds of the starch molecule. The α-amylase leads to the random cutting of α-1,4glycosidic bonds resulting in the formation of products such as maltose, maltotriose and dextrin. The digestion process continues in the stomach but mainly this results in the activation of pepsin, by HCl. Pepsin assists starch hydrolysis by degradation of proteins which acts as a protective barrier of starch, interfering with the activity of amylolytic enzymes(154). Most of the digestion is basically carried out in the duodenum by pancreatic enzymes(155). Pancreatic amylase acts in similar manner as the salivary α-amylase which results in the formation of oligosaccharides. Oligosaccharides are hydrolyzed to form monosaccharides by the activity of enzymes such as sucrose, glucose, maltose and dextrin,once entered the mucosa. Maltase breaks down maltose and maltotriosethat result in the release of glucose units. Sucrase when hydrolyzes sucrose, produces glucose as well as fructose. Lactase also produces glucose and galactose by the breakdown of lactose. Glucose then gets transported to the epithelial cells in the small intestine through the mucosal lining as a result of active transport.

Remaining undigested portion of the starch such as the RS bypasses the small intestine entering the large intestine where it gets microbially fermented by probiotic bacteria, resulting in production of short chain fatty acids. The colonocytes are provided with nutrition in the form of SCFAs by the colonic microflora (156).

2.7.1 *In-vitro* Digestibility of Resistant Starch:

Previously, there has been an cumulative interest in the structural design of food-based delivery systems to encapsulate, protect, and release bioactive modules alleged to be beneficial for human health(157). These delivery schemes may be premeditated to liberate the bioactive components at a specific location in the human gastrointestinal (GI) tract, repeatedly in retort to an environmental trigger, such as pH, ionic strength, or enzyme activity. The delivery system may also be intended to control the degree of bioactive release, i.e., burst release or prolonged release. Testing the efficacy of newly established delivery systems varies on the availability of digestion models that precisely simulate the complex physicochemical and physiological events that occur in the human GI tract. In vivo feeding procedures, using animals or humans, typically deliver the most accurate results, but they are time consuming and costly, that is why much effort has been dedicated to the development of in vitro procedures (158). Several approaches are used for the *in-vitro* elucidation of starch digestion in laboratory settings as described in figure 2.2. The enzymes which are commonly used in these *in-vitro* enzymatic digestion include pancreatic α amylase, amyloglucosidase, pepsin and lipases etc. (159).

One of the methods used for enzymatic hydrolysis involves the gelatinization of starch with water and then incubated with α-amylase in shaker water bath at 37°C for 30minutes and the digestion products are quantified using dinitro salicylic acid assay(DNS) as proposed by Goyal *et al*., (160).

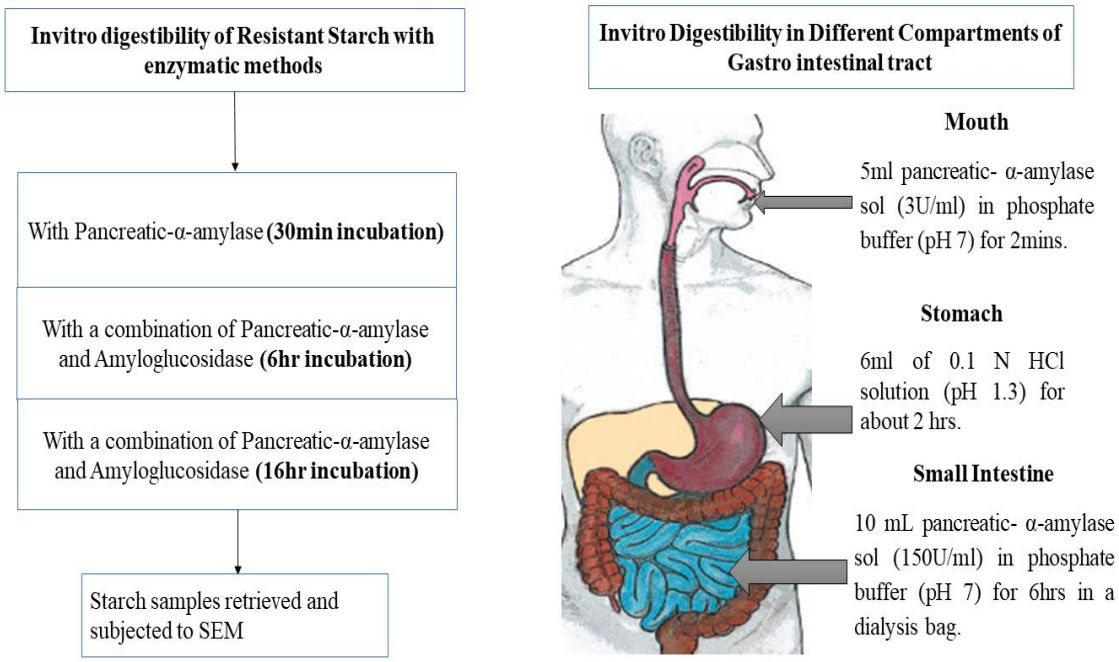

Figure 2.2: *In vitro* enzymatic digestions mimicking gastrointestinal tract of human

In vitro enzymatic digestions are also performed by digesting starch samples sequentially with salivary α-amylase, pepsin and pancreatin (Sigma Chemicals Ltd.) mimicking the situations of mouth, stomach and small intestine correspondingly (161). The pH of the solution is first maintained at 6.9 (mouth), then changed to a pH 2 (stomach) by using Hcl and later again maintained at pH 6.9(small intestine) with the addition of NaOH solution. All steps of *in-vitro* digestion is performed at 37°C to mimic human body temperature. To mimic the intestinal absorption of the digested products, the mixture at the end of incubation is transferred to a dialysis bag and dialyzed overnight at 37°C against distilled water to predict the absorption and release of digested products (162).

In some cases, the simulated gastric juice is artificially prepared by the addition of different salts as well as pepsin to predict the digestion of RS by gastric juice, as proposed by Wichienchot *et al*.,(163). *In-vitro* enzymatic digestion can also be done and determined by Megazyme kit in which the starch sample is incubated with a combination of pancreatic α-amylase and amyloglucosidase (Sigma Ltd.) for a period of about 16hrs in a 37°C shaker water bath.

In all the above described methods, starch digestibility varied depending upon several factors including starch retrogradation rate, amylose and RS contents, and presence of cross linkers. Increased resistance by chemical modifications e.g., addition of cross linker also renders the RS less vulnerable to enzymatic hydrolysis (164).

It was demonstrated that the diet containing the highest RS content showed lowest degree of digestibility as compared to the highly digestible native flour with lower RS content. In this study, successive autoclaving and cooling treatment causes a significant increase in the RS contents and resistance to enzymatic digestion(165).

The increased resistance of RSIII to enzymatic hydrolysis is primarily because of realignment of starch linear amylose chains in more tight, compact crystallites, making the double helices of the crystallites less susceptible to attack by α-amylases, thus decreases hydrolysis and enhances resistance. It was also proposed that for a starch to serve as a prebiotic, it does not need to be completely indigestible because its SDS and RDS portions disintegrate quickly, once accessed by enzymes, but as a prebiotic, RS reaches the colon and undergoes fermentation by the colonic bacteria. RS is used as a carbon source resulting in the release of gases, SCFAs, smaller amounts of organic acids and alcohols and stimulates the growth of beneficial probiotic bacteria, particularly *Bifidobacteria* sp. and *Lactobacilli*(166). Among SCFAs, butyrate is of important due to its role as an energy source for colonocytes. Other health benefits of RS include decreasing the incidence of obesity, diabetes mellitus, *inflammatory bowel disease* (*IBD*), insulin and cholesterol preventing detrimental metabolic disorders and its role in colonic cancer prevention (167).

2.8 Maintenance of Body Weight:

The epidemic obesity reminded the scientists to find effective protection tools for weight loss. Successive maintenance and weight loss are reliant on energy balance; the overall difference is between the energy intake and utilization. Lower intake than utilization results in weight loss while larger intake than utilization results in weight gain. There are several features of resistant starch which could indorse weight loss and the maintenance includes reduced postprandial insulin level in blood and augmented release of gut satiety peptides, lower fat storage in adipose tissues, increased fat oxidation and preservation of lean body mass. During weight loss, the maintenance of lean body mass would decrease the basal metabolic rate thus decrease total energy expenditure during weight loss (168).

2.9 Hypoglycemic and Insulinemic Effects:

The ratio of amylose and amylopectin of starchy foods alter the glycemic index (GI) of food. It is also affected by other factors like gelatinization of starch, native environment of the starch, baking temperature, and water content of the processed foods. Therefore, the GI value of RS is defined by the production/yield/content of RS. The food incorporated with RS reduces the digestion rate so by reducing digestion, RS has a property of controlled glucose release (80). It can be expected that there will be greater use of stored fats and insulin response will be lowered (169. RS can help in treating diabetes by changing the dietary plan of diabetic patients (170).

The incretin hormone, Glucagon like peptide 1 (GLP-1) perform a major part in glucose homeostasis by improving insulin homeostasis, lowering plasma glucose concentration and preventing beta cell function of pancreas. The intestinal L-cells are the endocrine cells of intestinal epithelium which secret GLP-1 in response to various types of nutrients. In both animal and human models, it has been observed that SCFAs production in the intestine increases the GLP-1 secretion. Furthermore, Freeland and Wolever reported that the diet rich in SCFAs is always needed for hyper insulinemic subjects to increase concentration of GLP-1. The ability of SCFAs to induce GLP-1 secretion has been widely reported. SCFAs production occurs in the colon while the L-cells which secrete GLP-1 hormone are mainly located in distal ileum and colon. In addition to this, the expression of free fatty acid receptors (FFAR2 and FFAR3) was also found in the colon and specifically related to strong expression of GLP-1 producing L-cells. More than 15 years ago, Dumoulin and coworkers showed the evidence that SCFAs provoke the secretion of GLP-1 in the plasma but the mechanism which links the production of SCFAs with the GLP-1 hormone is not fully understood. Tolhurst and Colleagues recently presented that the primary colonic cultures expressing mRNAs for both FFAR2 and FFAR3 and the propionate and acetate also stimulate the secretion of GLP-1 by activating receptors (FFAR2). Moreover, it has been found that mice with deficient FFAR2 have significantly reduce GLP-1 protein content which shows that may be FFAR2 is involved in function of L-cells(171).The slow digestion of RS reduces postprandial blood glucose level and insulinemia which in turn increases the period of satiety (172, 173). Using a commercial RS III ingredient (CrystaLean®) from a human study, even the maximum blood glucose level was noticed to be lower significantly as compared to other carbohydrates i.e., oligosaccharides, simple sugars and common starch. Food

containing RS III declined postprandial blood glucose level and play an important role in delivering enhanced metabolic control in type II diabetes (80). In order to confer benefits to glycemic and insulinemic response, the contribution of RS is at least 14% of total starch taken (175-177). It has also been shown by a recent study that resistant starch reduces glucose dependent insulinotropic polypeptide mRNA level in the ileum and jejunum in both diabetic and normal rats (178). The other types of RS i.e. SIV are chemically changed starches and have also been found to produce different glucose responses. The two different diet effects were compared, one having 1-2% acetylated potato starch and other have 2-3% beta cyclodextrin enrich potato starch. The study was conducted on humans. Only the beta cyclodextrin enrich potato starch is found to be effective and lowers the blood glucose level. The reason can be that the distal absorption of beta cyclodextrin occurs more in the intestine resulting in delayed gastric emptying (80). It is clear from different studies that RS has low glycemic response.RS is becoming a more attractive ingredient for many food manufacturers specially to the breads and cakes or other backing products, which results in low GI value after consumption (169).

2.10 Hypocholesterolemic Effects:

It has been reported in many studies on rats that RS affects lipid metabolism (169). Effective hypocholesterolemic effect of RS has been widely demonstrated . In a study conducted on rats, RS diet with 25% raw potatoes raised the cecum size and cecum pool of SCFAs, and also elevated the absorption of SCFAs, lowering the plasma triglyceride and cholesterol level (80). The trials on feeding RS containing food like Adzuki bean starch(AS) and Tebou bean starch(TS) to rats show that the food have more effect on lowering serum cholesterol level due to increase in hepatic SR-B1(scavenger receptor class B1) and cholesterol 7α hydroxylase mRNA (179). The total cholesterol, VLDL, IDL, and LDL cholesterol are lowered by utilizing bean starches and increase the SCFAs concentration in cecum particularly butyric acid and also neutral sterol excretion in feces. Another study conducted on hamsters fed with cassava starch extruded with 9.9% oat fiber and cassava starch extruded with 9.7% RS results showed lowered serum cholesterol level and improved cardiovascular health (90). The risk of coronary heart diseases can be decreased by the intake of adequate level of dietary fibers. With the decrease in total lipid profile and cholesterol absorption by utilization of RS containing diet, re-absorption of bile acid and alteration in hepatic metabolism and plasma clearance of lipoproteins occur (180).

2.11 Microbial Modulation of Gut by Taking Resistant Starch Containing Diet:

It is reported that colonic fermentation of RS is involved in lowering blood glucose level with modulation of beneficial gut microbiota which ferments RS and produces SCFAs for maintaining good health (181). The three major phyla i.e. *Firmicutes*, *Actinobacterium* and *Bacteroidetes* were claimed to be involved in RS fermentation (182).

Lactate and acetate are the major fermentation products produced by *Bifidobacteria* specie. Acetate and propionate are the major products produced by *Bacteroides* specie. *Fusobacterium* and *Butyrivibrio* produce butyrate as a major product after RS fermentation from human fecal samples (183). Moreover, it is also stated that *Lactobacilli*, *Bifidobacterium* and *Clostridium butyricum* are the most efficient microbes involved in RS fermentation as compared to other strains of bacteria (169). Taking RS containing food increase the proportion of microbes like *Lactobacillus, Bifidobacteria, Ruminococcaceae, Lachnospiraceae, Clostridium* and *Akkermansia* in rats (170-172).The dominant genera in the stomach, small intestine, and colon are listed, based on 16S rRNA gene sequence studies as described in figure 2.3(188).

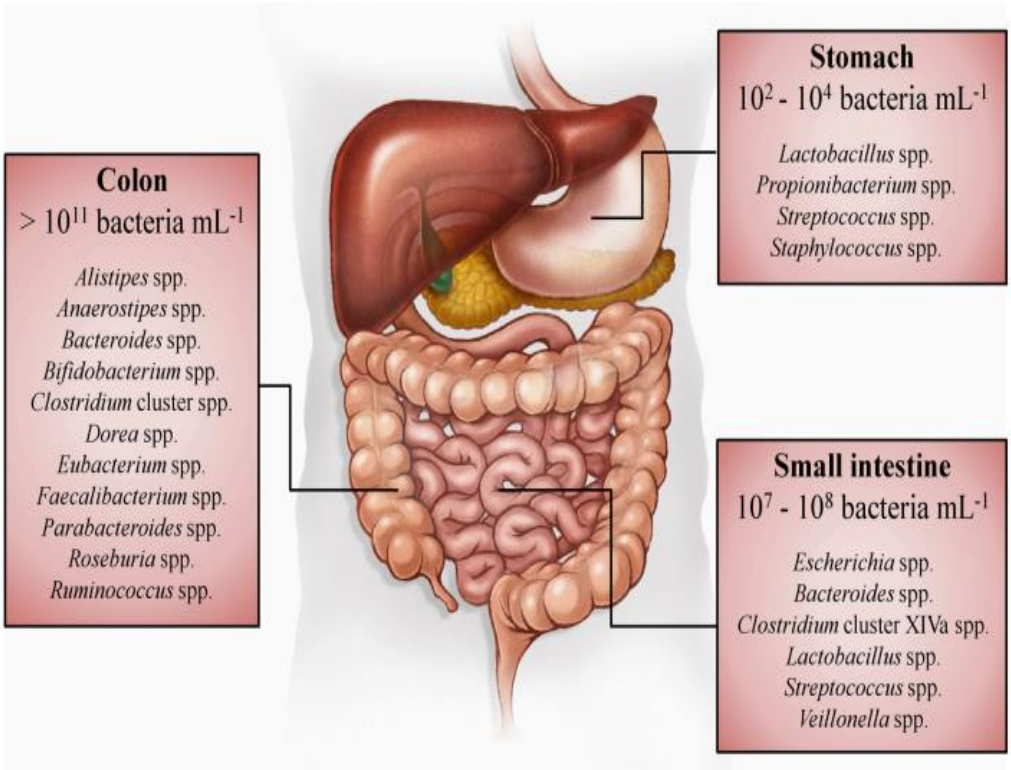

Figure 2.3: Spatial distribution and concentration of bacteria along the gastrointestinal tract of human (189)

2.12 Short Chain Fatty Acids Production by Resistant Starch Fermentation:

Gas products like methane, hydrogen and carbon dioxide are produced by RS fermentation but the most important are the SCFAs i.e. acetate, propionate and butyrate production by different probiotic bacteria in the colon by fermentation of RS (182, 190, 191). Iso-butyrate and iso-valerate are the lesser amount of SCFAs produced. Other organic acids like lactate, succinate, formate and alcohols like ethanol and methanol are also produced as a result of fermentation (192). In another study it has been reported that feeding lotus seed RS enhanced the components of formic, butyric, acetic, so-butyric, propionic and lactic acids. Moreover the intestinal microbiota has been reported to convert acetic and lactic acid to butyrate using acetyl CoA pathway (185). In another study after fermenting four novel RS containing diets using *in-vitro* fermentation system in the presence of human fecal slurries, a significant amount of beneficial SCFAs like propionate, acetate and butyrate were detected (192). Another research study showed the production of SCFAs using *in-vitro* RS fermentation by human fecal slurries (193). The fermentation of RS and production of SCFAs were detected in different research studies specially acetate, propionate and butyrate (194-197).

2.13 Applications of Resistant Starch:

RS has catching immence attention from nutritionalists, food processors and consumers due to its overwhelming benefits upons body physiology and food sensory properties. The reason is consumer consciousness and knowledge about the healthy food utilization with pronounced health merits. Along with this, researchers, food producers and nutritionalists marketing/advertisments towards healthy food consumptions hunts the consumer attention. In order to fulfil the need of fibers in daily intake of food, the RS supplemented cookies occupied the extraordinary attention from consumers. First commercially launched product of RS was described in 1990s. Currently, RS supplemented powders are manufactured by a many companies engaging technologies developed at Kansas State University from maize hybrid they manufactured amylose and resistant starch rich (198). RS products are of enriched benefits and cannot be swapped by traditional insoluble fibers. RS is supplemented in the production of products free of moisture. Howere, the products aimed to have pulpy texture, low Ph with high temperature storage mostly supplemented with chemical stractures obtained from potato, topiaca and maize cross bonded with other functional groups (199). With advancement in sensory, texture and

organoleptic characteristics bakery, pasta and beverages were fortified with RS supplementations. Large quantity of products enriched with insoluable fibers is available in the market, for example, high-fiber bread, and breakfast cereals. But others, like white bread, biscuits, cookies, and cakes are not yet fortified with fiber. The most eye-catching applications of RS with current need of time are described below.

2.13.1 Preparation of Resistant Starch Supplemented Cookies:

Cookies formulation is majorly associated with water, milk, and oil absorption capacities of starch. Presence of hydrophobic proteins, differences in the ratio of amylose/amylopectin and the chain lengths of starch perform a key role in oil absorption and in the development of a product with desirable properties (200, 201). Compared to native, RS III had a compact structure due to debranching and ordered conformation of granules which could have resulted in low porosity that restricted oil uptake. Milk absorption capacity of starch samples is related to the interaction between amylose and milk proteins which form a complex and hence greater the amylose content of starch, greater will be the ability of milk absorption. The supplementation of resistant starch (RS) ingredients in cookies formulas is gaining great attention, dominatly with the current positive health outcomes attributed to supplementation of RS and the demand of novel RS ingredients in the market. Nevertheless, the focus of current study is on the use of RS III prepared by enzymatic hydrolysis (EM-RS III) from maize flour in cookies making (126).

2.13.2 Application of Resistant Starch in Drug Delivery to Colon:

Polymer based microparticles for site-specific delivery of a drug to colon is becoming a hot spot of applied research. Utilizing specialized polysaccharide as a colon specific drug carrier employ tremendous benefits including its biocompatibility, inexpensiveness, non-toxicity, resistivity to digestion in upper GIT tract for site specific delivery to colon and biodegradation with bundles of benefits including stimulation of probiotic growth in colon with minimal loss of loaded compounds during the early stage of digestion (202). In this respect, unique or specialized polysaccharides harboring such characteristics including resistant starch as target drug carrier are the sustainable and novel approach (203).

Resistant Starch, a natural or modified polysaccharide, is a renewable and biodegradable resource with the advantages of being indigestible in upper GIT tract and fermented by colonic bacteria (204). Native starches are not appropriate for direct

use due to limitation of self-assembly and gastric acid sensitivity and more susceptibility to enzymatic hydrolysis (205). Novel enzymatically modified resistant starch (EM-RSIII) from maize flour, with prominent characteristics including high resistance against enzymatic digestion, its low solubility, hardness, birefringence analysis, pH and thermal stability makes it appropriate for the colon based drug delivery after testing its digestion against the pancreatic enzymes, which is the main focus of current study. As biobased EM-RSIII has public acceptance, hence its potential use for food and pharmaceutical industry is favored (75). Use of EM-RSIII from maize flour as a biopolymer in drug delivery opens up new possibilities for delivery of drugs to particular tissues and cells. It can provide an effective way for drugs to reach the specific sites like local treatment of colon ailments, reduced administered dose avoiding unnecessary degradation and digestion, improved bioavailability of drugs and most importantly in remediating the patient compliance for drug administration (203).

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Chapter 3: Preparation of Resistant Starch using α-Amylase

Paper 1:

Title: Preparation and characterization of resistant starch type III from enzymatically hydrolyzed maize flour

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3.1. Abstract

Background: Polysaccharides including resistant starch are categorized as dietary fiber and are used as an important prebiotic. Similar to soluble fibers, resistant starch also has a number of physiological effects that have been shown to be beneficial for health. Starch hydrolyzing enzymes, most importantly amylases, play essential roles in the production of resistant starch. This study aimed to develop amylase-treated maize flour with slow digestibility and unique physicochemical characteristics compared to native maize flour.

Methods and Results: In the current study, resistant starch type III from maize flour was prepared using amylase obtained from indigenously isolated *Bacillus licheniformis.* The amylase gene from *Bacillus licheniformis* was amplified and cloned into the pET-24(a) vector, expressed in *E.coli* BL21 (DE3) cells and purified by metal ion affinity chromatography. The purified enzyme enhanced the yield of resistant starch 16-foldin maize flour. Scanning electron microscopy revealed that the granular structure of maize flour was disrupted into a dense network with irregular structure, and X-ray diffractograms confirmed the transformation from an amorphous to a crystalline structure upon amylase treatment. Thermogravimetric analysis revealed increased amylose content of amylase-treated maize flour. Moreover, amylase-treated maize flour resulted in a significant enhancement of the desired properties of maize flour, such as resistant starch content, amylose, milk absorption capacity, and iodine and fatty acid complexing ability, and a reduction in swelling power, water binding, oil absorption capacity, and in vitro digestibility compared to untreated maize flour.

Conclusion: Resistant starch type III showed low digestibility and increased complexing ability with iodine and fatty acid and therefore could be a safe and beneficial alternative as a coating material for the delivery of active, sensitive ingredients to the colon.

Keywords: α-Amylase, *Bacillus licheniformis*, Resistant starch, Slow digestibility, Resistant starch with unique characteristics

Highlights:

- Resistant starch type III with unique properties was prepared from maize flour by amylase
- The resistant starch content of maize flour was increased 16-fold by treatment with amylase
- Resistant starch was prepared with desired properties such as site-specific digestion
- The milk absorption capacity of native and enzymatically treated maize flour was reported for the first time.
- Enzymatically prepared resistant starch with desired properties is a good alternative for targeted drug delivery to the colon.

3.2. Introduction

Starch is an important biomass on earth and is used as an energy source from microorganisms to humans. Starch is a complex macromolecule composed of two homopolysaccharide units: amylose, a linear and flexible polymer, and amylopectin, a branched polymer of amylose chains [1]. Starch is categorized into three groups based on its digestive properties: rapidly digestible starch (RDS),slowly digestible starch (SDS) and resistant starch (RS) [2]. Resistant starch on the basis of physical characteristics is classified into four types i-e RS-I, RS-II, RS-III, and RS-IV. According to Englyst *et al*., 1992 : RS-1; physically inaccessible starch for example whole grains: RS-II; native granular starch for example green banana, sweet potatoes: RS-III; retrograded or recrystallized starch for example bread, corn flakes: RS-IV; chemically modified starch for example addition of ether or ester groups by cross linking, conversion or substitution [1]. Currently, RS has attracted much attention in human societies because of its functional and physiological properties [3]. RS has the potential to mend human health by preventing the risk of chronic diseases such as diabetes mellitus type II [4, 5],colorectal cancer [6], obesity [7], and cardiovascular diseases[8]. Furthermore, RS has possible coating potential for active sensitive ingredients or drugs, which escape digestion in the stomach and small intestine and reach the colon undigested[9], where RS is fermented by colonic bacteria and acts as a prebiotic similar to dietary fiber. Additionally, RS also has a positive effect on the palatability of food compared to traditional dietary fibers [10]. Among its most desirable physicochemical characteristics are its abilities to decrease oil absorption [11] and swelling power [12], to increase complexing ability [13] and to lower digestibility with similar GIT conditions during in vitro examination [14, 15].

In the case of the human body, to comprehend the physiological effectiveness of resistant starch, it is recommended that daily carbohydrate intake must contain 10 to 20% resistant starch, whereas most of the marketable foodstuffs have less than 5%, and most of the conventional food products lose resistant starch content during processing. Therefore, due to the related health effects of resistant starch, it is essential to design a process for improving the resistant starch content of food with consumer safety and acceptability of food products in mind. Recently, many studies have investigated ways to increase the RS content of starch from various botanical

sources using physical [16], chemical [17, 18] and enzymatic treatments [19]. However, among these, enzymatic treatment is preferred due to its environmentally friendly or biobased nature [20]. Enzymatically prepared resistant starch is free of any toxic residues compared to chemically prepared resistant starch because biocatalysts are highly selective and specific for their substrate and are easily biodegradable [21].

For this purpose, α-amylase, one of the starch hydrolyzing enzymes, was used that can specifically cleave α-1,4-glycosidic linkages of amylose and amylopectin in glycosidic macromolecules/polysaccharides, resulting in the production of short, linear and branched oligosaccharides of varying length [22].Amylose and amylopectin molecules are linked with glycosidic bonds, which are specifically hydrolyzed by α-amylase. For the required chain length of amylose (10 to 100 glucose units) after hydrolysis, it is necessary to optimize the concentration of amylase. As a result, there is an increased opportunity for alignment and aggregation of starch chains favoring the formation of a perfect compact crystalline structure. It has also been reported that released chains possess molecular similarities with amylose with a degree of polymerization ranging from 10 to 100 units of glucose [23]. Englyst *et al*.[24] suggested that linear amylose is prone to recrystallization and retrogradation, forming tightly packed crystalline structures strengthened by double helices, which would reduce the digestibility of starch.

Therefore, the focus of the current exploration is to enzymatically enhance the resistant starch content of maize flour with the aim to advance its physicochemical characteristics to be used as an important ingredient in foodstuffs [25]. Although maize starch and flour are both high-carbohydrate foods that are interchangeable in some recipes, flour contains some nutrients that are not present in maize starch. Maize starch is a highly processed carbohydrate without protein, fat, vitamins, minerals, or fiber. It has a nutritional value of approximately 30 calories per7grams of carbohydrate or per tablespoon. On the other hand, maize flour, still a processed grain but where whole flour is added instead of maize starch, will at least bring some B vitamins and fiber to the food products. Therefore, the current study focused on enhancing the physicochemical characterization of the resistant starch content of maize flour instead of maize starch.

In conclusion, the study is based on cloning of the α-amylase gene from indigenously isolated *Bacillus licheniformis*C1,expressed in *E.coli* BL21 (DE3) cells, and purification by metal ion affinity chromatography. Moreover, recombinantly

expressed purified amylase was effectively studied for its ability to enhance the resistant starch content of maize flour. The impact of treatment with α-amylase on the efficient characteristics of maize flour was analyzed, and the in vitro digestion stability of enzymatically prepared maize flour was evaluated compared to that of native maize flour.

3.3. Materials and Methods

3.3.1 Materials

Maize flour was purchased from the local market of Mansehra (KPK Pakistan). Protamex® **(**endoprotease with a broad specificity from *Bacillus* Sp.P0029) was purchased from Sigma-Aldrich, USA. Other solvents and chemicals used in this work were obtained from Merck, Germany, and were of analytical grade. The kit for the estimation of resistant starch concentration was purchased from Megazyme International Ireland Ltd.

3.3.2 Cloning of the α-amylase Gene from *Bacillus licheniformis* C-1

The gene encoding α-amylase was PCR amplified from genomic DNA of indigenously isolated *Bacillus licheniformis*C1. Primers (F 5' ATGCAACACAAACGGCTTTATGC-3' and the downstream primer R 5' GAACATAGATCGAAACCG-3') were used to amplify the coding sequence of α-amylase from the strain submitted to the NCBI server (accession number: AF438149). The optimized PCR conditions for complete amplification of amylase were 30 cycles at 94°C for 45 s, 51°C for 1 min and 72°C for 2 min. The PCR products were analyzed on an agarose gel, purified, and sequenced. The amplified gene was inserted into the vector pET-24a (+) by cloning between Nde1 and BamH1 and placing it under the control of the T7/lac promoter including the C-terminal hexa-histidine tag. The resulting recombinant plasmid was transformed into *E. coli*BL21 (DE3) by the heat shock method. After transformation, the positive clones from LB-agar plates were further cultured into LB broth with antibiotic supplementation (50 µg/µL kanamycin) and placed at 37°C for 16 hours.

3.3.3 Expression and Purification of α-amylase

The α-amylase was produced in a 1 L batch reactor at 37°C, pH 7, using a defined medium with 50 µg/mL kanamycin and a dissolved oxygen tension above 40%. Expression for theα-amylase gene was induced at OD_{600nm} =0.6 by supplementation with 0.5 mM IPTG(isopropyl beta-D-thiogalactopyranoside); the temperature was

reduced to 20°C after induction, and induction was continued overnight at 180 rpm. After that, the reactor was terminated, and the culture was centrifuged for 10 min at 4°C at 6000 rpm.

The biomass was collected, and the cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.75 mM NaCl, 5% Triton X-100) and disrupted by sonication for 3 x 30 sec with a 3mm titanium probe using a sound intensity of 60% (UP400S: Dr. Hielscher, (Sartorius, Göttingen, Germany). Thereafter, the mixture was further centrifuged at 15000 rpm for 50 min to separate soluble proteins from cell debris and insoluble proteins. The supernatant was filtered through a Minisart high-flow filter (0.45 µm), degassed and purified through an AKTA prime system(GE Healthcare, Fairfield, USA) using immobilized metal ion affinity chromatography with nickel as a ligand.

For that purpose, the column was first equilibrated with binding buffer (50 mM Tris-HCl, pH 7.5, with 700 mM NaCl, and 20 mM imidazole), protein was loaded, and the column was washed with binding buffer to remove the unbound protein. The protein was finally eluted with elution buffer (50 mM Tris-HCl, pH 7.5, with 700 mM NaCl and 250 mM imidazole). The fractions containing the purified protein (α-amylase) were pooled together and further dialyzed against 50 mM citrate buffer, pH 5, using a Spectra/Pro dialysis membrane with a 35 kDa molecular weight cut-off(Spectrum laboratories, Rancho Dominguez, CA, USA) overnight. Enzyme homogeneity and the molecular weights of purified amylase were estimated on a 12% SDS-PAGE gel.

3.3.4 Protein Analysis

The total protein concentration of the pooled fraction was measured using the Bradford method [26] (Sigma, Steinheim, Germany) using bovine serum albumin (Sigma-Aldrich) as the standard.

3.3.5 Application of α-amylase for the Synthesis of Resistant Starch

Five grams of maize flour in 0.5M citrate buffer (pH 5) was dispersed, and the mixture was pregelatinized by autoclaving (121°C for 20 min). The mixture was cooled, protease (200 U/mL) was added, and the reaction was run for 30 min at 37°C.The sample was placed in a water bath at 99°C to inactivate the protease. The gelatinized slurry was cooled again to 50°C, and purified α-amylase (200U/mL) was added and incubated at 50°Cwith shaking for 16 hours. The starch paste was heated to 99°C to inactivate α-amylase. Then, a second cycle of autoclaving (121°C, 15 psi) for 1 hour was carried out, and the paste was cooled at -4°Cfor 24 hours. Finally, the

treated starch sample was crushed into powder. A negative control without any enzymatic treatment was run in parallel.

3.3.5.1 Quantification of Resistant Starch, Non-Resistant Starch and Total Starch

The maize flour after enzymatic digestion contained different types of starch in different ratios, which were calculated as follows.

Resistant Starch

Resistant starch was quantified by using the following formula:

Resistant starch (g/100 g sample) = ΔE x F/W x 90

where:

ΔE = absorbance of reagent blank = measured absorbance for 100 µg of D-glucose using glucose oxidase/ peroxidase; (GOPOD) reagent into microgram

F = conversion from absorbance to micrograms (the absorbance obtained for 100 µg of D-glucose in the GOPOD reaction is determined and F = 100 (µg of D-glucose) divided by the GOPOD absorbance for this 100 µg of D-glucose.

W = dry weight of the analyzed sample

The resistant starch content of native maize flour and α-amylase-treated maize flour was further determined by a Megazyme Resistant Starch Assay kit, and the sample codes were used for native maize flour (NMF) and α-amylase-treated maize flour (ATMF) during characterization.

Total starch was quantified by using the following formula:

TS = RS +NRS

where:

TS = Total starch

RS= Resistant starch and NRS = Non-resistant starch

3.3.5.2 Optimization of Reaction Conditions for Hydrolysis of Maize Flour by α-Amylase

To determine the optimum conditions for the synthesis of resistant starch, the enzymatic reactions were carried out at various pH values (4.5, 5.0, 5.5, 6.0, 6.5, and 7.0), times(4, 8, 12, 16, 20, and 24 hours), amounts of α-amylase (0, 50, 100, 150, 200, 250, and 300 U/mL)and protease concentrations used for pretreatment (0, 50, 100, 150, 200,250 and U/mL).The synthesis of resistant starch produced under these conditions was quantified by using a Megazyme Resistant Starch Assay kit.

3.3.6 Morphological Characterization of Maize Flour

3.3.6.1 Light Microscopy

Light microscopy was performed to investigate the morphological and structural characteristics of the native and enzymatically treated maize flour. Samples were prepared for microscopic study by suspending the native and enzymatically treated maize flour samples NMF and ATMF in a drop of 1:1 glycerol/water (v/v); after 30 min, samples were examined at 40 X resolution, and images were captured.

3.3.6.2 Scanning Electron Microscopy

Structural characterization of native and enzymatically treated maize flour was determined by scanning electron microscopy (VEGA-3 by TESCAN Czech Republic with oxford EDS detector, Oxford Instruments). Samples were dried to a fine powder and fixed on an aluminum stub coated with a film of gold via double-sided stick tape. Finally, samples were observed at an increasing voltage of 20 kV.

3.3.7 Structural Characterization of Native and α-Amylase-Treated Maize Flour

Structural characterization was carried out using Fourier transform infrared spectroscopy (FTIR). Samples were prepared by taking 2 mg powder of α-amylase-treated maize flour and native maize flour and mixing with potassium bromide (KBr) pressed under a sheet. The samples were scanned to generate the spectra using a Nicolet Avatar 360 FRIR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

The spectra were recorded at room temperature at a resolution of 4 cm^{-1} in transmission mode from 400 to 4000 cm^{-1}.

3.3.8 Thermal Analysis

Thermogravimetric analysis (TGA) was performed at a heating rate of 10°C/min to 300°C(Perkin Elmer 8000 TGA apparatus). The sample amount was2-3 mg in each case, and the experiments were performed in the presence of nitrogen as the inert carrier gas.

3.3.9 X-ray Diffraction

X-ray diffraction (XRD) was determined through X-ray diffractograms (D/Max-2200 X-ray diffractometer, Rigaku Denki Co., Tokyo, Japan). The powder samples were placed in a closed desiccator at room temperature to equilibrate the moisture content before analysis. The samples were then subjected to strong radiation of Cu Kalpha at a speed of 2 °C/min. The radiations were generated from an X-ray diffractometer with an angle of 2 theta (2θ) and a temperature range of 5°C to 55°C at 40 kV and 30 mA.

3.3.10 Physicochemical Characterization of Native and α-amylase-Treated Maize Flour

3.3.10.1 Amylose Content

The amylose content of enzymatically treated and native maize flour was quantified following the method previously described by [27]. Initially, 0.1 g of the starch sample was dissolved in 1 ml of absolute ethanol followed by the addition of 10ml of 1 N NaOH; the remaining volume was filled to 100 ml with distilled water , and the sample was incubated overnight at 25°C.Approximately 2.5 ml of the sample was mixed with 20ml of distilled water containing 3 drops of phenolphthalein as an indicator and titrated against 0.1N HCl until the disappearance of pink color was observed. A total of 1 ml of iodine reagent was added to this mixture, the final volume was adjusted to 50ml, and the absorbance was measured at 590nm. The amount of amylose present in the starch samples was calculated from the amylose standard curve. A total of 1ml of iodine reagent in 50ml distilled water was considered as the blank.

3.3.10.2 Water Absorption Capacity

The water absorption capacity was estimated by mixing 0.25g of α-amylase-treated maize flour and native maize flour in 12.5 ml of distilled water, and the mixture was heated at 30, 60, and 90°Cin a water bath for approximately 30 min with shaking every 5 min. The samples were then chilled at room temperature and centrifuged for 20 min at 3000×g. The pellet was dried in an oven for 4-5 hours at 110°C and weighed afterwards. The water absorption capacity was determined by the following formula:

$$\text{WAC (\%)} = \frac{\text{(sediment) pellet wt}}{\text{sample wt}} \times 100$$

3.3.10.3 Swelling Power

Swelling power was estimated by mixing and heating the native and enzymatically treated maize flour similar to the case of water absorption capacity except that the sample weight was 0.5 g in 25 ml, and swelling power was calculated at different temperatures (30°C, 60°C, and 90°C) as follows:

$$\text{SP (\%)} = \frac{\text{wt of sediment}}{\text{wt of dry starch} - \text{wt of dissolved starch}} \times 100$$

3.3.10.4 Water Binding Capacity

Samples were prepared for water binding capacity similar to the case of water absorption capacity except that 0.25 g of sample was dissolved in 5 ml distilled water, and the binding capacity was calculated as follows:

$$\text{WBC (\%)} = \frac{(M2 - M1 - Mo)}{Mo} \times 100$$

Where Mo = weight of initial sample taken

M1 = empty centrifuge tube

M2 = weight of centrifuge containing the dried pellet.

3.3.10.5 Oil Absorption Capacity

A total of 1 g of each sample (native and α-amylase-treated maize flour)was mixed with 6 ml of olive oil in a falcon tube. The sample was vortexed for 5 min and left for 30 min to allow oil absorption. Then, the samples were centrifuged for 20 min at 3000 rpm, unabsorbed oil was removed, and the OAC was determined by the gain in weight as follows:

$$\text{OAC (\%)} = \frac{\textit{weight of starch with oil} - \textit{original weight of starch}}{\textit{original weight of starch}} \times 100$$

where the weight of the starch with oil absorption = Wt. of the tube with pellet- Wt. of the empty tube.

3.3.10.6 Milk Absorption Capacity

Milk absorption capacity was determined in the same way as the determination of oil absorption capacity except that the sample was 0.1 g in 2 ml of raw milk, and it was calculated by the following formula:

$$\text{MAC (\%)} = \frac{\textit{weight of starch with milk} - \textit{original weight of starch}}{\textit{original weight of starch}} \times 100$$

where the weight of the starch after milk absorption = wt of the tube with pellet- wt of the empty tube.

3.3.10.7 Iodine Staining Index and Fatty Acid Binding Properties

The iodine staining index (ISI) is used for the quantification of iodine binding and the fatty acid complexing ability of native and enzymatically modified maize flour samples. For these, two replicates of both samples were prepared by dissolving 0.25 g of α-amylase-treated sample in 2.5ml of 90% DMSO, while the untreated sample was dissolved in 25ml of deionized water. Both samples were heated in a water bath (boiling temperature) for half an hour, and the volume of α-amylase-treated sample was adjusted to 25ml with deionized water. Now, one replicate of

samples was processed for ISI measurement. For this purpose,50 µl of the sample solution was transferred into a new test tube, which was wrapped with aluminum foil for light protection, followed by the addition of 5 ml of 0.6 mM iodine solution in 10% DMSO for 30 min. A total of 0.25 ml of butyric acid was added to the second replicate, followed by incubating the solutions for 15 min in a boiling water bath with intermittent mixing. The mixture was then cooled and kept at room temperature for approximately 30 min to increase the opportunity for complex formation. Finally, 50 µl of this solution was processed in the same way as was done for the ISI measurement with fatty acid complexation. The iodine staining index of the iodine complex was determined at an absorbance of 460 nm, while butyric acid complexing ability was determined at 570 nm.

3.3.10.8 Light Transmittance Properties

A total of 1% of native and enzymatically treated maize flour was processed at 90°C in a water bath for 30 min at 75 rpm. The resulting suspension was then cooled for 1 hour and refrigerated for 5 days. The light transmittance was spectrophotometrically determined every 24hours at 640nm.

3.3.10.9Antioxidant Potential

The oxidative properties of both samples were determined by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.

DPPH radical scavenging activity was determined according to the method of Brand *et al.*[28] with slight modifications. A DPPH solution of 316 µM was prepared in methanol, and its absorbance at 517nm was measured. Each sample (1 mg/ml) was mixed with DPPH solution (1:1) and incubated at 37°C for 1 hour. Pure methanol and DPPH solution (1:1) was used as a negative control, and ascorbic acid with DPPH solution was used as a positive control.

Percentage radical scavenging was measured by the following formula:

$$\text{DPPH radical scavenging activity \%} = \frac{1 - absorbance\ of\ sample}{absorbance\ of\ control} \times 100$$

3.3.10.10 Reducing Power

The reducing ability of the samples was measured as suggested by Oyaizu.M [29].Each sample (5mg/ml) was mixed with 2.5 ml of 1% potassium ferricyanide, followed by the addition of 2.5 ml of sodium phosphate buffer (0.2 M) at 50°C for 20 min after the addition of 2.5 ml of trichloroacetic acid (10%) and finally centrifuged for 10 min at 750×g. The supernatant was taken and mixed with 1ml of ferric chloride

(1%) and 5 ml of distilled water. The optical density was measured at 700 nm, and distilled water treated in the same way was used as a blank.

3.3.10.11 *In-vitro* Digestibility of Enzymatically Treated Maize Flour in Different Compartments of the Gastrointestinal Tract

In vitro digestibility of α-amylase-treated and native maize flour was performed as described by Dona *et al.* [30] with slight changes. A total of 1g of sample from each gelatinized sample was added to 100ml of water. From this sample,2ml was taken and treated with α-amylase (3 U/ml) in 5 ml of potassium phosphate buffer (5ml, pH 7), followed by incubation at 37°C for approximately 2 min in a shaking water bath. Samples were withdrawn every 30sec up to 120 sec and centrifuged, and the supernatant was collected to mimic salivary amylase. To mimic the stomach conditions, 6ml of each sample was mixed with 0.1 N HCl solution, and the pH was adjusted to 1.3 to mimic the gastric pH; the samples were incubated in a shaker incubator at 37°Cfor approximately 2 hours. Samples were withdrawn every 40 min up to 120min and centrifuged, and the supernatant was collected. To mimic small intestine conditions, the sample (10 mL) was mixed with pancreatic α-amylase (300 U/mL) in a dialysis bag with a molecular weight cutoff of 14kDa. The dialysis bag was placed into a beaker containing phosphate buffer (350 mL at 37°C) with gentle agitation (75 rpm). Dialysate (2 mL) was collected after 1, 2, 3, 4, 5, and 6 hours of incubation. The enzyme tube was inactivated in a water bath (99.9°C).The digestion rate of native and α-amylase-treated maize flour from each compartment of the gastrointestinal track was measured by the dinitro salicylic acid method.

3.3.10.12 Microscopy of the Pellet Obtained after *In-vitro* Enzymatic Digestion of Samples

Light microscopy and scanning electron microscopy of α-amylase-treated maize flour and native maize flour obtained after in vitro digestibility was performed. The samples were suspended in a drop of 50% glycerol solution on a glass slide and examined under a microscope at 100 X resolution. For scanning electron microscopy, dried samples were prepared and examined as described earlier.

3.4 Results and Discussion

α-Amylases are the major group of industrially important enzymes that can hydrolyze starch molecules at 1,4-α-glycosidic linkages. They have broad commercial applications in starch processing, textile, brewing, baking and other related industries

[31].In this project, the aim was to use α-amylase from indigenously isolated *Bacillus licheniformis*C1strainsfor the green synthesis of resistant starch from maize flour. Starch is gelatinized at temperatures above 60°C and retrograded at low temperatures below 30°C during the synthesis of resistant starch. Therefore, thermostable α-amylase, which can withstand temperatures in that range and can easily transform starch into a more organized structure, is required. α-Amylase from indigenously isolated *Bacillus licheniformis*C1 is a unique enzyme with properties such as those listed above, and its gene was cloned and expressed in *E. coli* BL21(DE3).

3.4.1 Cloning and Expression of α-amylase from *Bacillus licheniformis* in *E.coli* BL21 (DE3)

In the present study, the raw starch-hydrolyzing α-amylase gene from an indigenously isolated *B. licheniformis*C1 strain was successfully cloned and overexpressed in *E. coli*BL21 (DE3),and the enzyme was successfully used in the green synthesis of resistant starch from maize flour. The α-amylase gene from *B. licheniformis*C1is a 1.5 kb DNA fragment with 1542 bp, and the molecular weight of the expressed enzyme was 58 kDa. Most of the expressed recombinant protein was in the soluble fraction when checked by SDS-PAGE (Fig. 1). The overexpression of the α-amylase gene in *E. coli*BL21 (DE3) gave rise to 21500 U/mL of enzyme activity with 187.4 U/mg specific activity (Table 1).

Table 3.1: Summary of the purification step of recombinant α-amylase expressed in *E.coli* BL21 (DE3)

Purification Steps	Total Activity (AU) [a]	Total Protein (mg)[b]	Specific Activity (U/mg)	Recovery (%)	Purification (fold)
Cell-free supernatant	9900	1800	5.5	100	1
IMAC*	4218	22.5	187.5	42.6	34

[a]: Enzyme activity was measured in 0.1 M citrate buffer pH 5 at 50°C using 5 µg/µL (w/v) starch as the substrate.

b: Protein concentration was measured by the Bradford method using BSA as the standard.

IMAC*: Immobilized metal affinity chromatography used for purification of α-amylase

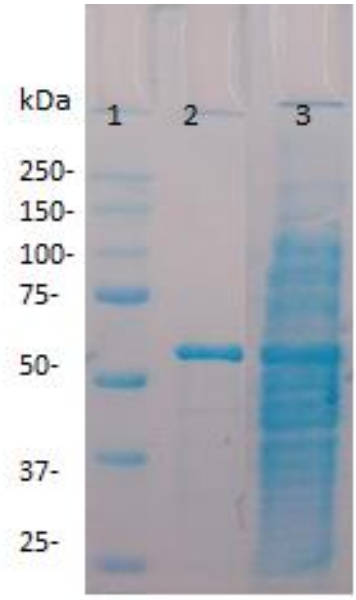


Fig.3.1: SDS-PAGE of α-amylase Lane 1: Protein ladder, Lane 2: Purified enzyme and Lane 3: Crude enzyme

3.4.2 Green Synthesis of Resistant Starch with α-amylase

For the green synthesis of resistant starch, the reaction conditions were optimized as described below.

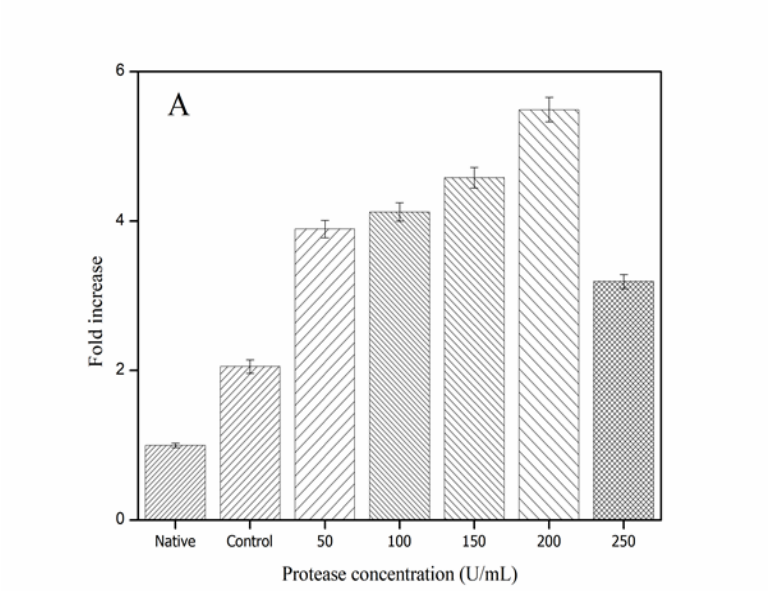
3.4.2.1 Optimization of Reaction Parameters for the Green Synthesis of Resistant Starch

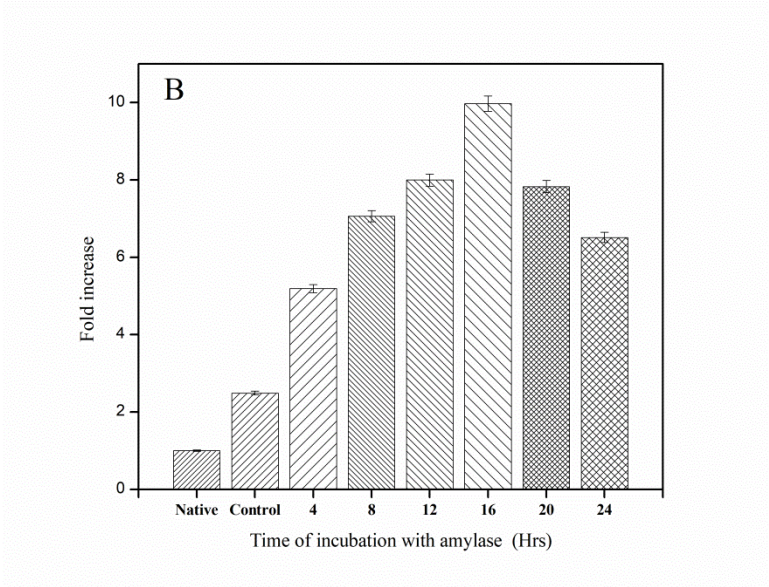
Maize flour is generally composed of carbohydrates, proteins, fats, vitamins, and ash [32].Proteins and starch interact mainly by hydrogen bonding, which greatly influences the physicochemical characteristics and nutritional benefits of starch protein-rich food products. Protein-starch interactions result in the formation of a physical barrier by encapsulating or surrounding starch granules, thus interfering with the in vitro preparation of resistant starch. Therefore, for the enzymatic hydrolysis of maize starch to increase the resistant starch content of maize flour, the protein content should be removed to expose starch to the starch hydrolyzing enzyme [33]. Therefore, maize flour was pretreated with protease to remove the hard cover of proteins for α-amylase activity.

Protease pretreatment was performed at 37°C, pH 7, and the optimum concentration of protease (200U/mL) (Fig. 2 A).

After protease treatment, starch chains were accessible to α-amylase, which formed a crystalline structure by molecular contact in maize flour. A major portion of maize flour is carbohydrates, which are important glycosidic macromolecules composed of amylose and amylopectin. In the current study, α-amylase remarkably reduced the chain length of amylose, which majorly contributed to the higher yield of resistant starch. The reaction time for α-amylase hydrolysis was analyzed from 0 to 24 hours, and a maximum of 9.9-fold resistant starch content was observed over the control after 16 hours (Fig. 2 B). However, an increase in the reaction time to longer than 16 hours caused an obvious decrease in resistant starch yield. A marked reduction from 9.9-fold to 6.5-fold was observed when the reaction time was increased from 16 to 24 hours. This indicated that the majority of the amylose chains in the starch molecule were hydrolyzed into glucosidic micro molecules (small glucose chains: monomers),which were unable to form double helices. The optimum chain length for a double helix is10 to 100 glucose units, which can align together to form compact crystalline structures upon retrogradation and become resistant to further hydrolysis by α-amylase.

α-Amylase activity was determined at different pH values (3.5 to 6.5) (Fig. 2 C)to determine the optimum pH for maximum production of resistant starch. The maximum resistant starch yield increased 11.2-fold over the control at pH 5. The amount of resistant starch prepared was reduced as the pH increased to higher than pH 5, demonstrating a reduction in α-amylase activity at higher pH because the activity depends on the optimum stability of α-amylase with substrate in a particular pH range, which results in the maximum yield of resistant starch. α-Amylase used in the synthesis of resistant starch from native/raw maize flour resulted in a reduction of the degree of polymerization of starch and enhanced the fluidity of amylose chains, which are responsible for the recrystallization and retrogradation of starch macromolecules. As shown in Fig. 2 D, the maximum resistant starch yield, 16.7-fold over the control, was obtained using 200 U/ml α-amylase, but a reduction in resistant starch content was observed with a gradual increase in the amount of α-amylase used. This is because the increased concentration of α-amylase resulted in rapid and unorganized hydrolysis of the maize starch producing small glucosidic micro-molecules, which will decrease the apparent amylose, thus preventing the crystallization of amylose upon retrogradation and ultimately causing a negative effect on increasing the yield of resistant starch [34].


A
Fold increase
0
2
4
6
Native
Control
50
100
150
200
250
Protease concentration (U/mL)


B
Fold increase
0
2
4
6
8
10
Native
Control
4
8
12
16
20
24
Time of incubation with amylase (Hrs)

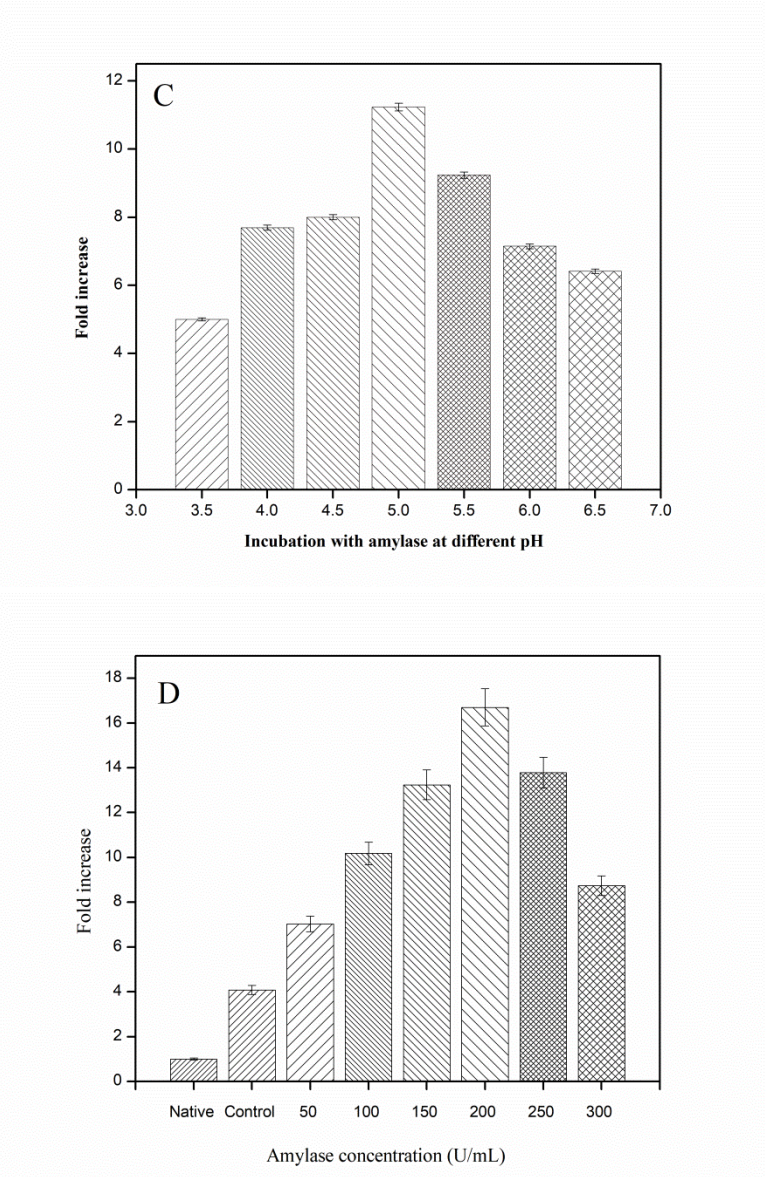


Fig.3.2: Optimization of different parameters for the enzymatic synthesis of resistant starch type III (RSIII). A:Effect of protease concentration on RSIII yield, B:Effect of reaction time on RSIII yield C:Effect of different pH on RSIII yield, D:Effect of α-amylase concentration on RSIII yield

3.4.3 Morphological Characterization of Native and α-Amylase-Treated Maize Flour

The shape, structural appearance and physical changes during the processing of native and α-amylase-treated maize flour are shown in Fig. 3 (A-F). Light microscopy and scanning electron microscopy demonstrated that native starch appears round, bell-like oval or pear in shape, and no structural deformity was present at the outer surface of native granules, while α-amylase-treated maize flour looked flat and looked roughly bread-or block-like in terms of the outer shape. The resistant starch that was formed

with the retrogradation and enzyme hydrolysis looked smooth, and layered strips were also seen on the surface. Fig. 3 E and F designates the clear physical changes during the processing of heat-treated and α-amylase-treated gelatinized maize flour. α-Amylase-treated maize flour appeared to be porous, sticky and deformed compared to heat-treated gelatinized maize flour, indicating the hydrolytic activity of the α-amylase. The sponge-like structure is from the leaching of amylose due to enzymatic hydrolysis of the starch granules.

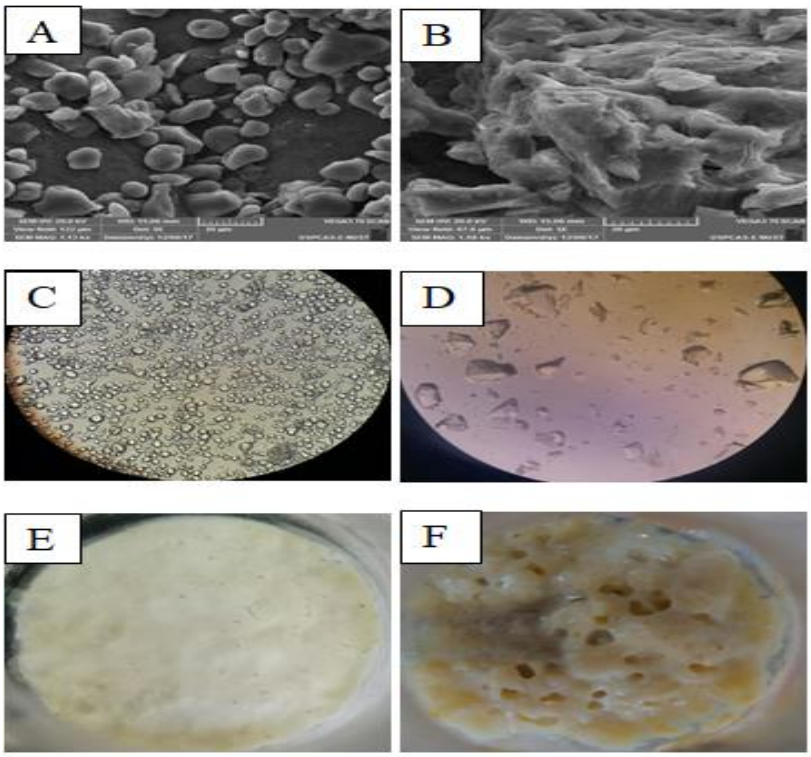


Fig. 3.3: Morphological characterization of native and α-amylase-treated maize flour (A): Scanning electron micrograph of native (B): Scanning electron micrograph of α-amylase-treated maize flour, (C): Light micrograph of native and (D): Light micrograph of α-amylase-treated maize flour, (E) Gelatinized native maize flour (F) Gelatinized α-amylase-treated maize flour.

3.4.4 Change in Crystallinity of Starch Samples by X-ray diffraction

The X-ray diffractograms of native and α-amylase-treated maize flour are presented in Fig. 4A**.** The starch molecules consisted of amylose and amylopectin arranged in a complex structure within the granules comprising crystalline and amorphous regions. Commonly, amylopectin short chains were arranged into double helices, which may contribute to the semi crystalline form of starch molecules.

The double helices and crystallites ordered into the semi crystalline part of the starch granule were not associated with the disordered (or amorphous) part, which comprised amylose and longer chains of amylopectin.

Katz and Itallie classified the crystal structure of starch into three major categories: shorter chains as A-type, longer chains as B-type and mixture of A and B-types as C-type.

In the current study, the native or raw maize starch powder showed strong diffraction peaks at 10.25°, 22.17°, 18.07°, and 22.91°, indicating that maize starch has properties of A-type crystal structure. However, the presence of very strong and high peaks at 2θ= 14.58°, 20.12°, 25.15° and 22.5° are representative of the formation of a new crystalline structure of the α-amylase-treated sample. The change in crystalline pattern is consistent with a previous report on the preparation of resistant starch from maize starch, in which the appearance of a peak at 20.12° 2θ indicates the disappearance of an amorphous region based on amylopectin compared to the control, caused by the enzymatic hydrolysis and amylose chain rearrangement upon retrogradation at a low temperature [35, 36]. The difference in the degree of crystallinity among samples might be ascribed to the number and size of the crystalline region, which is mostly related to the amylopectin content and its chain length. The crystallinity of starch was also affected by the interaction and orientation of the double helix within the crystalline domains. As suggested by Miao et al., the X-ray diffraction pattern revealed that RS content partially contained crystalline and amorphous parts because of the arrangement of double helical structures composed of partially ordered structures[37].

3.4.5 Structural Analysis by Fourier Transform Spectroscopy

The FTIR spectra of native and α-amylase-treated starch found in maize flour are shown in Fig. 4B. Both types of native and α-amylase-treated maize flour samples had almost the same spectral results, which shows that there is no chemical modification in either molecule, but the small variation in the intensity of the spectra of the treated starch indicates that the molecule underwent some physical modification. The peak at approximately 3420 cm^{-1} represents OH-group stretching vibrations in the α-amylase-treated sample.

3.4.6 Thermal Decomposition Measurement by Thermogravimetric Analysis

The thermal decomposition of starch molecules based on molecular structure was investigated by TGA (Fig. 4C). Both samples showed a single step major weight loss

in addition to the slight weight loss occurring below 150°C. The weight loss below 150°C is due to the loss of water molecules. Native maize flour showed up to 7% weight loss due to water evaporation, while amylase-treated maize flour showed up to 6% weight loss below 150°C. The major weight loss started at 280°C in the case of native maize flour, while this major thermal event occurred at a slightly lower temperature (273°C) in the case of α-amylase-treated maize flour. However, when the temperature reached 600°C, native maize flour lost up to 88% weight, while α-amylase-treated maize flour lost up to 76%. Under oxidative atmospheres, thermal treatment of starches normally leads to its depolymerization when the applied temperature exceeds 300°C [38].The starch macromolecule under goes a series of irreversible changes, and the structural changes initially result in the formation of pyrodextrins; at higher temperatures, the starch molecule degrades, resulting in the formation of levoglucosan, furfural and low molecular weight volatile products, and ash/carbon products remain [39].

Starch is composed of two major molecules, amylose, a linear structure containing α-1-4- glycosidic bonds, and amylopectin, a highly branched polymer of short glucose units linked by α-1-6- glycosidic bonds. The amylose to amylopectin ratio in starch molecules influences various properties, including gelatinization, viscosity, retrogradation, water interaction, and physical and chemical behaviors. The amylose to amylopectin ratio has a major contribution to the thermal decomposition and activation energy of starch. An increase in the amylose to amylopectin ratio decreases the thermal decomposition temperature and sequence of activation energy primarily because higher molecular weight amylopectin molecules require more energy to breakdown.

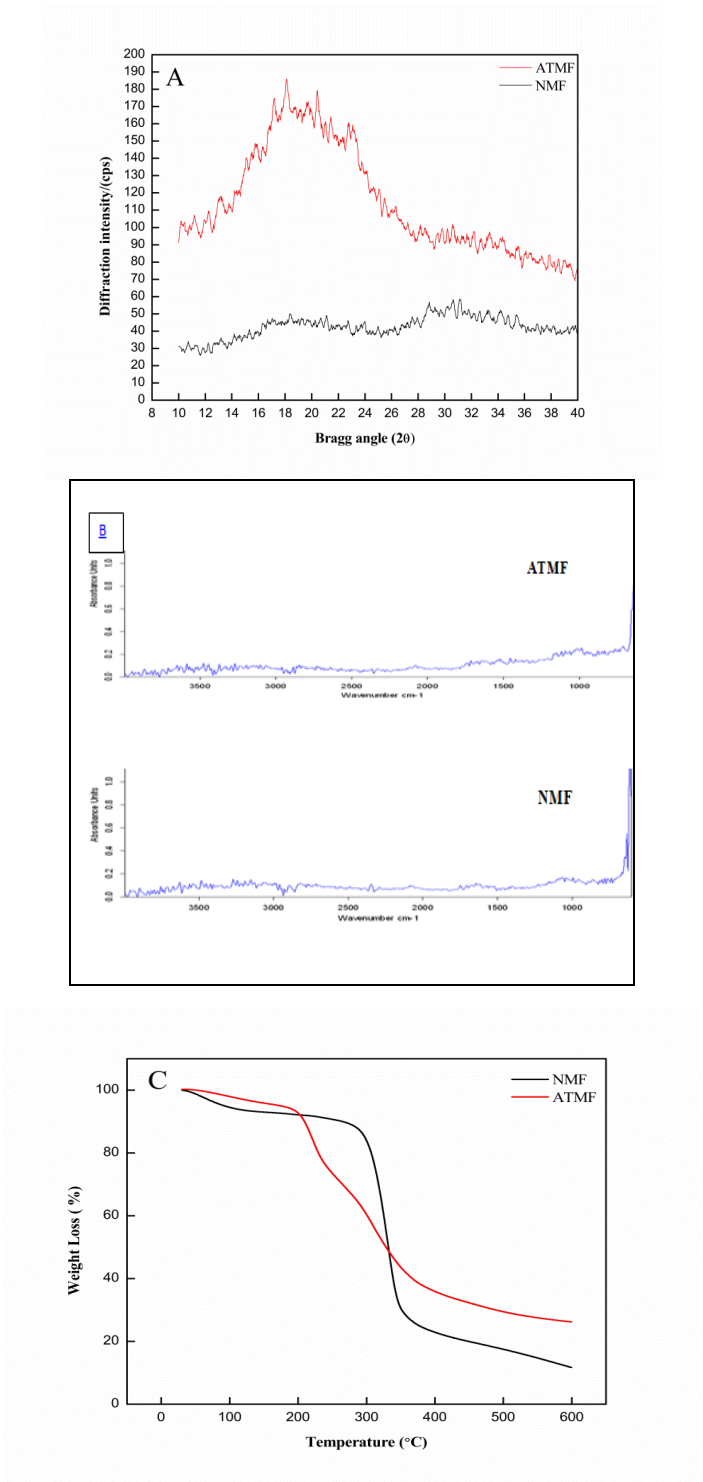


Fig.3. 4: Structural characterization of native and α-amylase-treated maize flour A**:** X-ray diffraction patterns of native control (NMF) and α-amylase-treated samples (ATMF), B: FTIR spectra of native sample (NMF) and α-amylase treated sample (ATMF) and C**:** TGA thermogram of native control (NMF) and α-amylase treated samples (ATMF)

3.4.7 Amylose Content of Native and Amylase-Treated Maize Flour

An increase in amylose content was observed for enzymatically produced maize flour (30%) in comparison to native maize flour (19.87%) (Table2). The amylose content is directly related to the resistivity of the starch sample [40],resulting in a higher resistant starch content compared to native maize flour. This is because native maize flour is unprocessed with intact amylopectin chains and lower amylose content, while exposure to α-amylase leads to the release of linear amylose, resulting in greater amylose content, which upon retrogradation organizes into a crystalline compact-resistant structure, i.e., higher resistant starch content. The result was in accordance with Rosida*et al*., 2016, wherein the water yam (*Dioscoreaalata* L.) flour with the highest resistant starch content (9.0%) depicted high amylose content due to increased formation of amylose as a result of the depolymerization process, leading to the formation of crystals contributing resistance [41].

3.4.8 Swelling Power, Water Absorption Capacity and Water Binding Capacity

The swelling power, water absorption capacity, and water binding capacity of native and enzymatically produced maize flour were analyzed at 30°C, 60°C and 90°C(supplementary data Table S1). A significant variation in starch samples was observed at the three different temperatures. These parameters were significantly dependent upon amylose/amylopectin content, temperature, thermal stability, and type of starch. Native maize flour and heat-treated maize flour was run as a control with each analysis to analyze the possible effect of ingredients like protein or smaller peptides etc in maize flour as compared to starch.

Native maize flour showed higher swelling power, water absorption capacity and water binding capacity at all three temperatures compared to the lower values exhibited by α-amylase-treated maize flour. This might be because swelling of granules is attributed to amylopectin content and is inhibited by amylose content. Therefore, native maize flour has higher amylopectin content than α-amylase-treated maize flour, which results in increased swelling power. The water absorption capacity and water binding capacity of native starch was greater than those of the enzymatically produced resistant starch because it is an unprocessed powder form, and when dispersed in excess water, its loose structure allows water to come in contact easily, thereby increasing the solubility of starch in water. The increase in the water absorption capacity of native maize flour has always been associated with an increase in amylose leaching and solubility. Moreover, this might also indicate that

enzymatic treatment of maize flour has resulted in increased amylose and resistant starch content, hence a rigid compact crystalline structure leading to reduced water absorption capacity, water binding capacity and water swelling power compared to unprocessed, raw native maize. The reduced swelling power was due to increased amylose content, as starches with higher amylose content are more rigid and reinforced, resulting in less swelling when heated as proposed by [42]. The results were in agreement with those reported by Osundahhunsi *et al*., where increased resistant starch content decreased water swelling power, water absorption capacity and water binding capacity [43]. However, increasing the temperature from 30 to 90°C resulted in an increase in the swelling power, water absorption capacity and water binding capacity of the native maize as well as α-amylase-treated maize flour, but native starch still had higher values observed at all temperatures. Due to the low water absorption ability, swelling power and water binding capacity, enzymatically prepared resistant starch from maize flour helps in processing and better handling, leading to crispiness, crunchiness, expansion and enhanced texture to the formed product. Moreover, enzymatically prepared resistant starch is suitable for the baking industry (cookies, biscuits, cereals).

3.4.9 Oil and Milk Absorption Capacity of Native and Amylase-Treated Maize Flour

The oil absorption capacity of enzymatically produced and native maize flour might include the physical entrapment of oil inside the porous structure of starch due to capillary forces and by the development of complexes of amylose with lipids within the helical starch structures [44].

Therefore, native maize flour showed an increased oil absorption capacity of approximately 90.91% because of its loose structure compared with the enzymatically treated maize flour (Table 2). The increased oil uptake of native starch might be because of the varietal differences in the ratio of amylopectin and amylose, degree of polymerization and chain length distribution [45], larger surface area and porosity of the particles and presence of greater hydrophobic proteins [46], which assist in oil absorption. In comparison to the native maize, enzymatically produced resistant starch samples showed lower oil absorption capacity. This is primarily because hydrolysis with α-amylase leads to greater realignment of starch linear amylose chains in more tight, compact crystallites, making double helices in the crystallites result in low porosity that restricts oil uptake [47]. Therefore, the enzymatically treated maize flour

sample had a more compact structure due to increased resistant starch content and a lower oil absorption capacity of approximately 75.76% compared to native maize flour. Moreover, protease pretreatment during enzymatic synthesis leads to the removal of hydrophobic proteins, which might contribute to low oil absorption in α-amylase-treated samples compared to native untreated samples. Resistant starch has a lower oil absorption capacity, which is useful in the synthesis of reduced caloric/fat-containing formulations and enhances crunchiness and crispiness (texture) of food.

3.4.10 Milk absorption capacity

Milk absorption capacity was reported for the first time in the current study for comparing the effect of enzymatically treated and native maize flour characteristics. Milk absorption capacity is related to the interaction between amylose and milk proteins, which form a complex, and hence, the greater the amylose content of starch is, the greater its milk absorption ability. Native maize showed a lower milk absorption capacity than enzymatically treated maize flour, which demonstrated greater milk absorption capacity. A possible explanation for this increased milk absorption capacity of resistant starch samples is that the use of enzymes leads to hydrolysis of starch by α-amylase, leading to increased amylose content, which results in a greater amount of amylose available to form complexes with milk proteins, ultimately leading to more milk bound to starch samples and hence greater milk absorption capacity. The greater milk absorption capacity of the resistant starch sample renders it a suitable candidate for use in the manufacture of cereals (such as cornflakes) and in confectionary (dairy products).

3.4.11 Iodine Staining Index and Fatty Acid Complexing Abilities

The iodine staining indices and fatty acid complexing abilities of native maize and enzymatically produced resistant starch from maize flour measured at 460 and 570 nm are given (Supplementary data Table S2). The native maize flour depicted significantly lower values of light absorption at both 460 and 570 nm, even at a high starch concentration of approximately 5%. In contrast, the ISI and fatty acid complexing ability of enzymatically produced resistant starch (α-amylase treated) at different concentrations exhibited a higher absorbance, indicating higher complexing ability.

Native maize flour showed lower absorption values primarily because the inner branched chains of intact amylopectin molecules are embedded in the form of highly organized crystals, making it difficult to form iodine and fatty acid complexes [48].

This is because hydrolysis by α-amylase releases amylose molecules and other smaller hydrolyzed products consisting of various sizes of branched and linear dextrin, which upon complete hydrolysis linearize into straight chains, leading to high amylose and ultimately increasing resistant starch content. Therefore, releasing chains from the helical structure makes accessible for complexing iodine, as shown by the greater values of the absorption of α-amylase-treated maize flour RS-iodine and fatty acid complexes compared to control native maize flour. The absorbance values of the treated sample at 460 nm were greater than the ISI values at 570 nm for all tested starch concentrations, indicating a greater amount of shorter chains due to enzymatic hydrolysis. Comparable results were reported by [49], in which the inclusion complexes of iodine with debranched starch (resistant starch) for all the starch concentrations displayed greater absorption values compared to native starch at 460 nm. The results were in accordance with the reports of [48],indicating ISI values of the FA-complex solutions observed for both native and RS samples. The ISI values were lower than those of the solutions without the addition of fatty acids, demonstrating that starch chains, whether in intact or hydrolyzed forms, can have complexing ability with fatty acids (Supplementary data Table S3).

The greater complexing abilities of α-amylase-treated maize flour with both iodine and butyric acid compared to untreated native maize flour indicate that resistant starch can be employed as an alternative source of coating or encapsulation for delivery of active compounds, including flavonoids, vitamins, and drugs, for targeted delivery as microparticles to the colon. The encapsulation of drugs with prebiotics is a wonderful approach for both the stimulation of probiotic bacteria and the treatment of colonal ailments by the controlled release of drug-loaded microspheres (data not shown).

3.4.12 Light Transmittance Properties of Starch Samples

One of the major attributes of starch as a food ingredient is to impart viscosity and opacity to foods. Starch is used to thicken and impart opacity to spoon able salad dressings. Opacity varies considerably with the source and method used for starch modification upon storage and with the effect of refrigerated storage on the light transmittance of native and enzymatically produced starch pastes (Supplementary data, Table S3). A significant reduction in the light transmittance of both native maize and enzymatically produced resistant starch from maize flour was observed when subjected to 120 hours of refrigerated storage. This decrease is mainly related to changes in different properties of starch gels, including granule swelling and its

remnants, amylose and amylopectin contents and their leaching during storage, all of which contribute to the development of turbidity and retrogradation [50].

Native maize flour has a loose structural conformation and has amylopectin branches intact in a freely widespread structure. When it is subjected to a spectrophotometer, light gets absorbed in a loose structure, leading to high absorbance but less transmittance.

At any specific storage period, the resulting trend is that the transmittance value ofα-amylase-treated maize flour has greater transmittance primarily based on the nature of the enzymes used in resistant starch production, which influences its retrogradation tendency. α-Amylase-treated maize flour hydrolyzed 1,4 glycosidic bonds, thereby releasing amylose chains, increasing the amylose content. Upon retrogradation, the amylose content recrystallizes into a tightly compact resistant structure compared to the native structure and results in higher transmittance values. This decreased light transmittance of starch samples with the increase in refrigerated storage time can be due to the retrogradation ability of starch during which the initial broken bonds reassociation into a compact, organized structure [50]. Jyothi *et* al. reported similar results of light transmittance on arrowroot starch gel, wherein the transmittance of treated, modified, or resistant starch was higher than that of their native counterparts after equal storage periods [51].

3.4.13 DPPH Radical Scavenging ability of Enzymatically Treated and Native Maize Flour

The antioxidant potential of enzymatically treated maize flour was higher than that of native maize flour, as indicated by the decreased 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability and reducing power. The decrease in the antioxidant ability of untreated maize flour could be due to the presence of fewer reducers/OH bonds freely available to interact with DPPH free radicals and the Fe^{+3}complexes. The structural network of native flour might hide the reducing groups inside the helical structure surrounded by intact amylopectin branches, thereby showing the least antioxidant potential indicated by reduced DPPH radical scavenging ability (31.4%) as well as light green color in reducing power assay, whileα-amylase-treated maize flour showed an increased free radical scavenging activity of enzymatic resistant starch (35.3%) and significant reducing power proportional to the increased absorbance shown by resistant starch samples at 700 nm that were a darker green color. The increase in antioxidant activity of amylase-treated maize flour in

comparison to native flour can be associated with the exposure of a greater number of hydroxyl groups resulting from enzyme hydrolysis. The increased antioxidant potential of α-amylase-treated maize flour renders it suitable for use in the manufacture of anticancer, anti-aging, and anti-inflammatory formulations.

Table 3.2: Physicochemical properties of native maize flour (NMF) and α-amylase-treated maize flour (ATMF)

Parameters	Samples	
	Native Maize Flour (NMF)	**Amylase-treated maize Flour(ATMF)**
RS (%)	1.8	14
Non-RS (%)	69.79	56.87
Total starch (%)	71.59	70.87
Amylose content (%)	19.87	30
OAC (%)	90.91	75.76
MAC (%)	360	390
DPPH (%) scavenging activity	31.4	35.3
Reducing power (700 nm)	0.38	0.76

3.4.14 *In-vitro* Digestibility of Enzymatically Produced Resistant Starch from Maize Flour in Different Compartments of the Gastrointestinal Tract

α-Amylase-treated resistant starch from maize flour showed less release of reducing sugars in the mouth and higher gastric pH stability due to its compact resistant structure (high resistant starch 14%) resulting from treatment with α-amylase. Therefore, it can be inferred that enzymatically prepared resistant starch from maize flour is resistant to gastric digestion (acid hydrolysis)compared to untreated native maize flour because of the greater resistant starch content.

α-Amylase-treated maize flour showed lower in vitro digestibility owing to the increased resistant starch (14%) and amylose contents (30%), which leads to compact, crystalline structures that resist enzymatic hydrolysis compared to the loose, intact amylopectin containing native maize with decreased resistant starch (1.8%) and amylose levels (19.87%) (Supplementary data, Table S4).

Therefore, it can be inferred in the light of the results obtained by the digestion of enzymatically prepared maize flour in the different compartments of the GI tract that α-amylase-treated maize flour significantly resists digestion compared to native maize flour and by passing the small intestine reaching the large intestine in considerable amounts. Therefore, site-specific digestion, i.e., digestion in the colon by bacterial fermentation, may be a potential candidate for its use as a prebiotic and drug carrier to the colon for the treatment of inflammatory bowel diseases.

3.4. 15 Microscopy of the Pellet Obtained after in vitro Enzymatic Digestion of Starch Samples

The light micrograph and scanning electron microscopy images of the native maize pellet in Fig. 5A show a loose, deformed appearance with wide spaces, indicating loss of the original structure due to enzymatic digestion after complete digestion in the gastrointestinal tract. Fig. 5B shows a light micrograph of α-amylase-treated maize flour with a compact, flat, sheet-like structure with fewer spaces compared to native maize flour. This slightly compact structure of α-amylase-treated maize flour in comparison to native wide spaces is indicative of the greater resistance of α-amylase-treated maize flour, which has prevented enzymatic attack and hence lowered in vitro digestibility when exposed to pancreatic α-amylase and amyloglucosidase even for a period of 8 hours of complete gastric intestinal digestion.

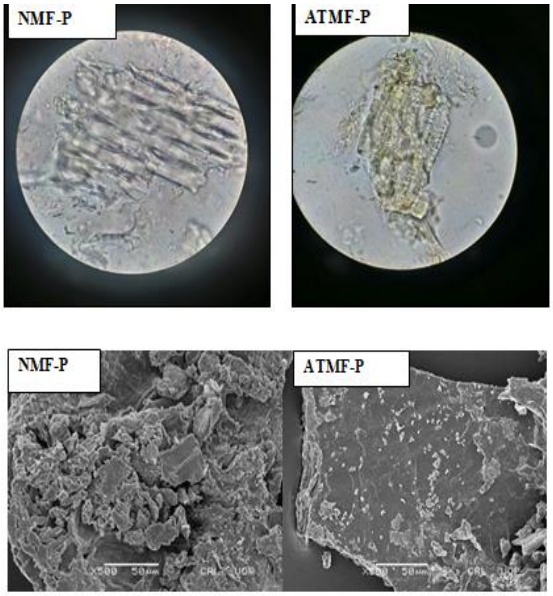


Fig. 3.5: Scanning electron micrograph and light microscopy of native pellet (NMF-P) and α-amylase-treated pellet (ATMF-P) after digestion in gastrointestinal conditions.

3.5 Conclusions

Dietary carbohydrates including resistant starch are resistant to rapid digestion, providing opportunities to mitigate the global rise in type II diabetes and related noncommunicable diseases. The characterization of nutritionally relevant features and end-use applications is markedly influenced by resistant starch synthesis processes and their physicochemical properties. Starch hydrolyzing enzymes play essential roles in the production of resistant starch, the most important of which isα-amylase, as revealed by the current study. The enzymatic treatment of starch prior to retrogradation aligns the short amylose chains for the formation of double helices, ultimately enhancing the degree of retrogradation. In the current study, a transformation in the crystalline structures contributed to an increased resistance of α-amylase-treated maize flour. According to the current results, the increased antioxidant potential of enzyme-treated maize flour could be used as a potential candidate in the manufacture of anticancer, anti-aging and anti-inflammatory formulations. The other desired properties, such as low water binding ability, swelling power, and oil and water absorption capacity and increased milk absorption capacity of enzymatically prepared maize flour, provide better handling in processing leading to crispiness and crunchiness of the products formed, usually in cookies, crusts,

breakfast cereals and bakery products. Enzymatically treated maize flour showed lower in vitrodigestibility compared to untreated native maize flour. The increased complexing ability of enzyme-treated maize flour with iodine as well as with fatty acid compared to that of native maize flour makes enzyme-treated maize flour suitable for encapsulating material for site-specific drug delivery.

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Conflict of Interest Declaration

We declare no conflict of interest.

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Chapter 4: Preparation of Resistant Starch using Pullulanase

Paper 2:

Title: Enzymatic Modification of Maize Flour Improves its Functional Properties, Digestion Resistibility, and Antioxidant Potential

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4.1 Abstract

Starches resistant to digestion have been widely used in food and pharmaceutical applications to develop new products, but naturally theses starches are not present or present in very little amount such as in maize flour. The information regarding functional, physiochemical properties and digestion resistivity of these starches is also sparse. Therefore, objective of the study was to enzymatically modify the maize flour by novel pullulanase from indigenously isolated *Bacillus licheniformis* to enhance the yield of resistant starch. The yield of resistant starch was increased by 20 folds at optimized conditions. Scanning electron microscopy and XRD revealed transformation of granular amorphous structure of starch into dense network of crystalline form. Thermogravimetric analysis revealed increased amylose content while ^{1}H NMR and ^{13}C NMR indicated debranching pattern of pullulanase treated starch compared to untreated starch. DPPH radical scavenging assay and reducing power illustrate significant increase in antioxidant activity of maize flour after pullulanase treatment which was explored for the first time to confer its potential as cancer preventing agent. Moreover, enzymatically modified resistant starch showed slow digestibility and increased complexing ability with iodine and fatty acid. The increased complexing ability with iodine and fatty acid of resistant starch could be exploited as safe coating material for targeted delivery of active sensitive drugs to the colon.

Keywords: Maize flour, Resistant starch, Pullulanase, Antioxidant activity, Digestion Resistibility, Iodine Complex

4.2 Introduction:

Maize (*Zea Mays*) is one of the most important staple foods for more than half of world's population (1). Starch is a main constituent of the maize grain and is considered as the key ingredient that has been used in food formulations (2). Starch is mainly composed of two homo-polysaccharides of D-glucose; amylopectin and amylose, the ratio of both molecules varies according to the type and botanical origin of starch (3). It is the most important energy source for human diet all over the world. On the basis of digestion rate, starch is categorized into three main groups: Rapidly digesting starch (RDS), which is instantly hydrolyzed within 20 minutes of ingestion; Slowly digestible starch (SDS), which is digested within 120 minutes of ingestion; and Resistant starch (RS), which is undigested even after 120 minutes of ingestion and passes from small intestine and reach large bowel undigested where it may be fermented by resident bacteria (4). The undigested resistant starch have many potential health benefits including reduced risk of diabetes mellitus type II (5), cardiovascular diseases, colorectal cancer (6, 7) , gastrointestinal abnormalities (8), and also take part to assist in metabolism of lipids and glucose (9-11).

Resistant starch on the basis of physical characteristic and extent of resistivity classified into four groups i-e RS-I, RS-II, RS-III and RS-IV. RS-I; is physically inaccessible starch and present in whole milled grain and seeds, RS-II; ungelatinized native granular starch and present in green banana and potatoes, RS-III; crystallized and retrograded starch present in cooked and cooled potatoes, rice, maize, bread and puddings and RS-IV; include chemical modified starch with substitution, conversion or cross linking of some functional groups like ether or ester groups.(12). Among four types of resistant starch RS-I and RS-II lose their resistivity during chewing and food processing before reaching to colon, while RS-III have potential to retain its resistant ability because of its thermal stability during food processing and acidic stability in gastric environment and having compact crystalline structure while RS-IV has some security concerns (chemical nature) due to which RS-IV use is limited in functional food products. Therefore, RS-III is the only form of resistant starch which is most commonly used in many food formulations. It is mainly comprised of retrograded amylose because of its high affinity to re-associate. It can be synthesized by thermal, acidic, or enzymatic treatment (13). But among these, enzymatic synthesis is preferred due to its environmental friendly or bio-based nature and make resistant

starch free of any toxic residues as compared to chemically synthesized resistant starch because biocatalyst are highly selective and specific for their substrate and after reaction it can easily breakdown into simple nontoxic compounds and become inactive (14).

Therefore, the most preferred way to further increase the quantity of RS-III in maize flour is by enzymatic debranching, which results in all linear glucans that have strong tendency to re-associate. Previously our research group reported 16 fold increase in RS-III with amylase (act only on α-1, 4-glucosidic bonds) (15). So, to move this work forward and to explore the effect of debranching enzymes on the hydrolysis of branching points and its role in the preparation of resistant starch pullulanase was implied in the current study. Debranching enzymes like pullulanase have drawn increased attention in starch conversion processes (16). It specifically hydrolyzes α-1, 6-glucosidic bonds and produce linear polymer linked by α-1, 4-glucosidic bonds from amylopectin molecule into condensed double helical structure, which facilitate starch retrogradation. This structural modification renders α-1,4-glucosidic bonds inaccessible to amylase hydrolysis in gastrointestinal environment (17). However, synthesis of resistant starch totally depends on the ratio of substrate, amylose and amylopectin (18), type and arrangement of crystalline structure of starch (19), degree of polymerization of amylose chain, autoclaving (gelatinization), retrogradation temperature and most critical is percentage of protein content in substrate (in case of maize flour 7.2/100g) (20, 21) which hinders rearrangement of amylose chain decreasing yield of resistant starch. Therefore, pretreatment and optimization of processing conditions with specific substrate for increased synthesis of resistant starch from maize flour is highly essential and the main focus of current research. The pretreatment contributes a significant role in preparation process as elaborated in our previous paper and resulted in significant yield of resistant starch content with addition of α-amylase(15). However, maize flour and starch both are interchangeable in some food formulations, but from nutritional point of view maize flour has more benefits over maize starch. Maize flour has additional fibers, vitamins, and minerals as compared to maize starch. Additionally, the caloric count of maize flour is less than maize starch, as 1 tablespoon of starch has 30 calories more than maize flour. Therefore, focus of current study is to enhance the resistant starch content of maize flour instead of maize starch.

The specific objective of the present study was enlightening the essential potential of debranching enzyme pullulanase from indigenously isolated *Bacillus licheniformis* for enhancing the synthesis of resistant starch (RS-III) from maize flour, to optimize the enzymatic reaction parameters and pretreatment process. Furthermore, characterization of resistant starch with respect to its physicochemical, functional properties has been investigated in current study.

4.3. Materials and Methods

4.3.1 Materials:

Maize flour was purchased from the local market of Mansehra (KPK, Pakistan). Protease was purchased from sigma, (St. Louis, MO, USA). Other chemicals and solvents used in this work were purchased from Merck Germany and were of analytical grade. Kit for the determination of resistant starch concentration was purchased from the Megazyme International Ireland Limited. Pullulanase (190 U/mg) was prepared and purified in the laboratory from *Bacillus licheniformis* which is indigenously isolated from less explored hot spring at Skardu Pakistan.

4.3.2 Cloning of the Pullulanase Gene from *Bacillus licheniformis* C-1

The gene encoding pullulanase was PCR amplified from genomic DNA of indigenously isolated *Bacillus licheniformis* C1. Primers (F 5' CCATGGTGCCGGGTATCAGCC -3' and the downstream primer R 5' GGATCCCTAAAAAATCCCTTTTGGTTC -3') were used to amplify the coding sequence of pullulanase from the strain submitted to the NCBI server (accession number: AF438149). The optimized PCR conditions for complete amplification of pullulanase were 30 cycles at 94°C for 45 Secs, 51°C for 1 min and 72°C for 2 min. The PCR products were analyzed on an agarose gel, purified, and sequenced. The amplified gene was inserted into the vector by cloning pGEX-2T by recombination between *EcoR1* and *BamH1* under the control of Tac/lac promoter and incorporating the C-terminal GST-tag. The resulting recombinant plasmid was transformed into *E. coli* BL21 (DE3) by the heat shock method. After transformation, the positive clones from LB-agar plates were further cultured into LB broth with antibiotic supplementation (50 µg/µL ampicillin) and placed at 37°C for 16 hours.

4.3.2.1 Expression and Purification of Pullulanase

The pullulanase was produced in a 1 L batch reactor at 37°C, pH 7, using a defined medium with 50 µg/mL ampicillin and a dissolved oxygen tension above 40%.

Expression of the pullulanase gene was induced at OD_{600nm} = 0.6 by supplementation with 0.5 mM IPTG (isopropyl beta-D-thiogalactopyranoside); temperature was reduced to 20°C after induction and incubated overnight at 180 rpm. After that, the reactor was terminated, and the culture was centrifuged for 10 min at 4°C at 6000 rpm. The biomass was collected, and the cells were resuspended in lysis buffer (20 mM sodium acetate, pH 5) and disrupted by sonication for 3 x 30 Sec with a 3mm titanium probe using a sound intensity of 60% (UP400S: Dr. Hielscher, (Sartorius, Göttingen, Germany). Thereafter, the mixture was further centrifuged at 15000 rpm for 50 min to separate soluble proteins from cell debris. The supernatant was filtered through a Minisart high-flow filter (0.45 µm), and degassed and purified on an anion exchange column. The protein was loaded on a column (1.5 × 50 cm) of Q-Sepharose pre-equilibrated with 20 mM sodium acetate buffer (pH8). The column was washed with 200 mL of the same buffer at flow rate of 0.5 mL min^{-1}. After washing, column was eluted with linear gradient of (0.70 mM) NaCl in sodium phosphate buffer. The active fractions were analyzed for enzyme activity and run on SDS-PAGE for analyzing the purity of samples. The fractions containing the purified protein (pullulanase) were pooled together and further dialyzed against 50 mM citrate buffer, pH 5, using a Spectra/Pro dialysis membrane with a 35 kDa molecular weight cut-off (Spectrum laboratories, Rancho Dominguez, CA, USA) overnight. Enzyme homogeneity and the molecular weights of purified pullulanase were estimated on a 12% SDS-PAGE gel with protein ladder (BIO-RAD = 161-0377).

4.3.3 Protein Analysis

The total protein concentration of the pooled fraction was measured using Bradford method (Sigma, Steinheim, Germany) using bovine serum albumin (Sigma-Aldrich) as a standard.

4.3.4 Application of Pullulanase for synthesis of resistant starch:

For the enhancement of resistant starch content, maize flour was made resistant to digestion according to the method of (Englyst and Hudson, 1987) with slight modifications. Maize flour 5 g was dispersed in 0.5M citrate buffer (pH 5) and the mixture was then pregelatinized by autoclaving at 121 ^{o}C for 20 min. After cooling the mixture, protease (200 U/mL; 50 µL) was added and incubated for 30 minutes at 37^{o}C. The sample was kept in a boiling water bath for 15 min to inactivate the protease. Afterwards the gelatinized slurry was then cooled to 60 ^{o}C, purified pullulanase (200U/mL; 150µL) was added and incubated at 50 ^{o}C shaker for 16 hours

followed by heating the starch slurry at 99 ^{o}C for inactivation of pullulanase. Second cycle of autoclaving (121 ^{0}C, 15 psi) was carried out for 1 hour and then subjected to refrigeration at -4 ^{o}C for 24 hours. Lastly, the treated starch sample was crushed to powdered form and stored for further analyses. Negative control without any enzymatic treatment was run in parallel.

4.3.5 Quantification of Resistant Starch, Non-Resistant Starch, and Total Starch

The maize flour after enzymatic digestion contains different types of starch in different ratio which is calculated as follows.

Resistant Starch

The Resistant Starch was quantified by using the following formula

Resistant Starch (g/100 g sample) = $\Delta E \times F/W \times 90$

Where:

ΔE = absorbance of reagent blank = measured absorbance for 100 µg of D-glucose using GOPOD reagent into microgram

W = dry weight of sample analyzed

For total starch quantified by using following formula

TS = RS +NRS

Where:

TS = Total Starch

RS= Resistant Starch

NRS = Non-Resistant Starch

4.3.6 Optimization of reaction conditions for enhancement of RSIII content of maize flour by pullulanase:

For the determination of the optimum conditions for the synthesis of RSIII with highest RS content, the enzymatic reactions were carried out at variable parameters including different temperatures (45, 50, 55,60,65,70 °C), pH (4.5, 5.0, 5.5, 6.0, 6.5, 7.0), time (4, 8, 12, 16, 20, 24 hours), amount of pullulanase (0,150, 200, 250, 300, 350 U/mL), retrogradation temperature (-4, 8, 15, 30°C) and protease pretreatment (40U/g of dry starch).The formation of RS-III produced under these conditions was determined using Megazyme Resistant Starch Assay Kit. Native maize starch demonstrated as NMF was used as control and synthesized resistant starch (RS-III) as PTMF i.e. pullulanase treated maize flour during the characterization description.

4.3.7 Physicochemical characteristicsof native and pullulanase treated maize flour:

Different physicochemical characteristics of native and enzyme treated maize flour were determined**.**

4.3.7.1 Water absorption capacity:

The water absorption capacity was estimated by mixing 0.25g of pullulanase treated maize flour and native maize flour in 12.5 ml of distilled water and the mixture was heated at 30 ^{0}C in water bath for about 30 min by shaking after every 5 min. The samples were then chilled at room temperature and centrifuged for 20 min at 3000×g. The pellet was dried in an oven for 4-5 h at 110ºC and weighed afterwards. Water absorption capacity was determined by the formula:

$$\text{WAC (\%)} = \frac{\text{(sediment) pellet wt}}{\text{sample wt}} \times 100$$

4.3.7.2 Swelling power Swelling Power

Swelling power was estimated according to the method of (Nattapulwat*et al.,* 2009) with slight modifications. Starch slurry was prepared by mixing and heating the native and enzymatically treated maize flour as in case of water absorption capacity except that sample weight was 0.5 g in 25 ml distilled water and swelling power was calculated at 30 ^{o}C as follows.

$$\text{SP (\%)} = \frac{\text{wt of sediment}}{\text{wt of dry starch} - \text{wt of dissolved starch}} \times 100$$

4.3.7.3 Oil Absorption Capacity

OAC was estimated by mixing was 6 ml of olive oil with 1 g of each sample (native and enzyme treated maize flour) in falcon tube. The mixture was vortexed for 5 min and left for 30 min to facilitate oil absorption. Afterwards, samples were centrifuged for 20 min at 3000 rpm, unabsorbed oil was removed, and OAC was determined by gain in weight as below:

$$\text{OAC (\%)} = \frac{\textit{weight of starch with oil} - \textit{original weight of starch}}{\textit{original weight of starch}} \times 100$$

Where weight of starch with oil absorption = Wt. of tube with pellet- Wt. of empty tube.

4.3.7.4 Milk Absorption Capacity

Milk absorption capacity was determined in the same way used for the determination of oil absorption capacity except that sample was 0.1 g in 2 ml of raw milk and calculated by the following formula

$$\text{MAC (\%)} = \frac{\textit{weight of starch with milk} - \textit{original weight of starch}}{\textit{original weight of starch}} \times 100$$

Where weight of starch after milk absorption = wt of tube with pellet- wt of empty tube.

4.3.7.5 Iodine Staining Index and Fatty Acid Binding Properties

The quantification of iodine complexing ability of native and debranched starches was modified from that described for amylose determination by Knutson (1986).Enzymatically treated starch sample was suspended in 1ml of 90% DMSO and cooked in boiling water bath for about half an hour, to which 4 ml of distilled water was added to attain three different concentrations i.e.., 1,3 and 5%w/v. In contrast, native maize flour was dispersed in 5ml of 90% DMSO to obtain the above three final concentrations and directly cooked in a boiling water bath for half an hour. Afterwards, 50 µl from both sample solutions was transferred into aluminum foil covered test tubes for light prevention then 5ml of 0.6 mM iodine solution in 10%DMSO was added and incubated for half an hour. The absorbance of iodine complexes with starch samples was spectrophotometrically measured at both 460 and 570nm. For the measurement of fatty acid binding ability, the starch suspensions for both samples were prepared in the same way as done for ISI determination. To each solution 0.25ml of butyric acid was added and incubated the solutions in boiling water bath for 15 min with intermittent mixing. Mixture was then cooled at room temperature for 30 min to increase the opportunity for complex formation. Finally, 50 µl of this solution was processed in the same way as done for ISI measurement with fatty acid complexation. The iodine staining index of iodine complex was determined at absorbance of 460 nm while at 570 nm was used for butyric acid complexing ability.

4.3.7.6 Light Transmittance Properties

A starch suspension of 1% of native and enzymatically treated maize flour was prepared by mixing 0.1g of each sample in 10ml of distilled water and cooked in a boiling water-bath for 30mins at 75 rpm. The resultant suspension was cooled for 1 hr and subjected to refrigeration for 5 days. Transmittance was spectrophotometrically measured after every 24hrs at 640nm.

4.3.8 Structural Characterization of Native and Pullulanase treated maize flour:

4.3.8.1 Scanning Electron Microscopy

Structural characterization of native and enzymatically treated maize flour was determined through scanning electron microscopy. Samples were dried in fine powdered and fixed on an aluminum stub which is coated with a film of gold via double sided stick tape. Finally, samples were observed at an increasing voltage of 20 kV.

4.3.8.2 Structural Characterization of Native and Pullulanase Treated Maize Flour

Structural characterization was carried out using Fourier transform infrared spectroscopy (FTIR). Samples were prepared by taking 2 mg powder of pullulanase treated maize flour and native maize flour and mixed with potassium bromide (KBR) pressed under the sheet. The samples were scanned for generating the spectra using Nicolet Avatar 360 FR-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The spectra were recorded at room temperature at a resolution of 4 cm^{-1} in transmission mode from 400 to 4000 cm^{-1}.

4.3.8.3 Thermal Analysis

Thermogravimetric Analysis (TGA) was determined at heating rate of 10˚C/min till 300 ˚C (Perkin Elmer 8000 TGA apparatus). The sample amount was 2–3 mg in each case and the experiments were accomplished in the presence of nitrogen as the inert carrier gas.

4.3.8.4 X-ray Diffraction

X-ray diffraction (XRD) was determined through X-ray diffractograms (D/Max-2200 X-ray diffractometer, Rigaku Denki Co., Tokyo, Japan). The powder samples were placed in a close desiccator at a room temperature to equilibrate the moisture content before analysis. The samples were then subjected to strong radiation of Cu Kalpha at a speed of 2^oC /minute. The radiations were generated from X-ray diffractometer with the angle of 2 theta (2θ) and temperature range 5 ^oC to 55^oC at 40 kV and 30 mA.

4.3.8.5 Nuclear Magnetic Resonance Spectroscopy

The chemical structures of native and pullulanase treated maize flour were characterized using Bruker Ascend Aeon WB 400 (*Bruker BioSpin AG, Fällanden, Switzerland*) NMR spectrometer. DMSO was used as a solvent for the experiments.

The working frequency was 400.21 MHz for ^{1}H and 100.63 MHz for ^{13}C. Data were processed using Bruker Topspin 3.5 software.

4.3.9 Applications of Pullulanase treated Resistant Starch (III) as compared to native starch:

4.3.9.1 In-vitro Digestibility:

In-vitro digestibility of native and pullulanase treated maize flour was carried out by mixing.10 mL of pancreatic α-amylase solution (300 U/mL) with each pullulanase treated maize starch and native starch in a dialysis bag (length 13 cm, width 4.5 cm and molecular cutoff value 14,000). The dialysis bag was positioned into a beaker containing phosphate buffer (350 mL at 37 ^{o}C) with gentle agitation (75 rpm). 0.2 mL dialysate was collected after 1, 2, 3, 4, 5, and 6 hours of incubation. The tube was then placed in boiling water bath (99.9^{o}C) to inactivate the enzyme. Maltose concentration released after digestion from starch samples was quantified by dinitro salicylic acid method.

4.3.9.2 Antioxidant Potential:

The oxidative property of both samples was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. DPPH solution of 316 µM was prepared in methanol and its absorbance was spectrophotometrically measured at 517nm. Each sample (1mg/ml) was mixed with DPPH solution (1:1) and incubated at 37°C for 1 hour. Pure methanol and DPPH solution (1:1) was used as a negative control and ascorbic acid with DPPH solution used as a positive control.

Percentage radical scavenging was measured by the following formula:

$$\text{DPPH radical scavenging activity \%} = \frac{1 - absorbance\ of\ sample}{absorbance\ of\ control} \times 100$$

4.3.9.3 Reducing Power:

For the measurement of reducing ability of the samples, working solution with concentration of 5mg/ml was prepared for both native and pullulanase treated maize flour samples, 1ml was taken from these solutions to which 2.5 ml of 1% potassium ferricyanide was added followed by addition of 2.5 ml of sodium phosphate buffer (0.2M). Samples were then incubated at 50ºC for 20 min then 2.5 ml of trichloroacetic acid (10%) was added and finally centrifuged for 10 min at 750×g. 1ml of ferric chloride (1%) and 5 ml of distilled water was mixed with the supernatant. The absorbance was measured at 700 nm and distilled water treated in the same way was taken as a blank.

4.3.10 Statistical analysis

Origin Program 8.0 (Origin Lab Company, USA) and Excel 2010 Program (Microsoft, USA) were used to analyze and report the data. Mean values from the triplicate experiments were reported.

4.4 Results and Discussion

4.4.1 Cloning and Expression of Pullulanase from *Bacillus licheniformis* in *E.coli* BL21 (DE3)

In the present study, the raw starch-hydrolyzing pullulanase gene from an indigenously isolated *B. licheniformis* C1 strain was successfully cloned and overexpressed in *E. coli*BL21 (DE3),and the enzyme was successfully used in the green synthesis of resistant starch from maize flour. The pullulanase gene from *B. licheniformis* C1 is a 21 kb DNA fragment with 2140 bp, and the molecular weight of the expressed enzyme was 75 kDa. Most of the expressed recombinant protein was in the soluble fraction when checked by SDS-PAGE (Fig. 1). The overexpression of the pullulanase gene in *E. coli* BL21 (DE3) gave rise to 572.7 U/mL of enzyme activity with 190.9U/mg specific activity (Table 1).

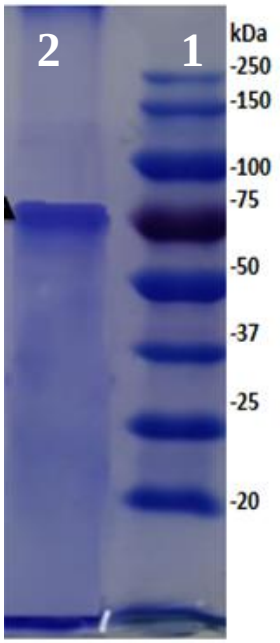


Fig. 4.1: SDS-PAGE for pullulanase Lane 1: Protein ladder, Lane 2: Purified Pullulanase

Table 4.1: Summary of the purification step of recombinant pullulanase expressed in *E.coli* BL21 (DE3)

Purification Steps	Total Activity (AU)	Total Protein (mg)[b]	Specific Activity (AU/mg)	Recovery (%)	Purification (fold)
Cell free supernatant	854	53.4	16	100	1
Purified enzyme	572.7	3	190.9	33.33	11.93

4.4.2 Green Synthesis of Resistant Starch with pullulanase

For the green synthesis of resistant starch reaction conditions were optimized as below.

4.4.2.1 Optimization of Reaction Parameters for Green Synthesis of Resistant Starch

The pretreatment and optimization of processing conditions with specific substrate for increased formation of resistant starch is highly essential and the main focus of current research. To accomplish this, current study focuses to enhance the yield of resistant starch from maize flour upon enzymatic treatment. The normal constituents of maize flour include carbohydrate, proteins, fats, vitamins, and ash (22). Protein and starch molecules have special association by hydrogen bond and greatly influence the physicochemical nature and nutritional quality of the protein-starch rich food products. These association and bonding leads to decrease *in-vitro* synthesis of resistant starch. Therefore, in order to overcome this bonding it is necessity to utilize protease to make available starch for starch hydrolyzing enzyme (23). The protease pretreatment was performed under optimized condition (pH7, temp 37 ^{o}C, concentration 200U/mL: 10U/reaction). Protease pretreatment increased the exposure of starch molecules to pullulanase action and resulted in increase of 7.1-fold yield as compared to heat and native control (Fig.4.1A and Fig.4.1B). Whereas, in our previous paper after protease pretreatment, 5.4-fold increase in RSIII was observed when maize flour was treated with α-amylase. Protease pretreatment followed by pullulanase debranching resulted in high yield of resistant starch(15).

In this study, pullulanase notably reduced branching points in amylopectin resulted in increased linear amylose content, which contributed greatly to the higher yield of

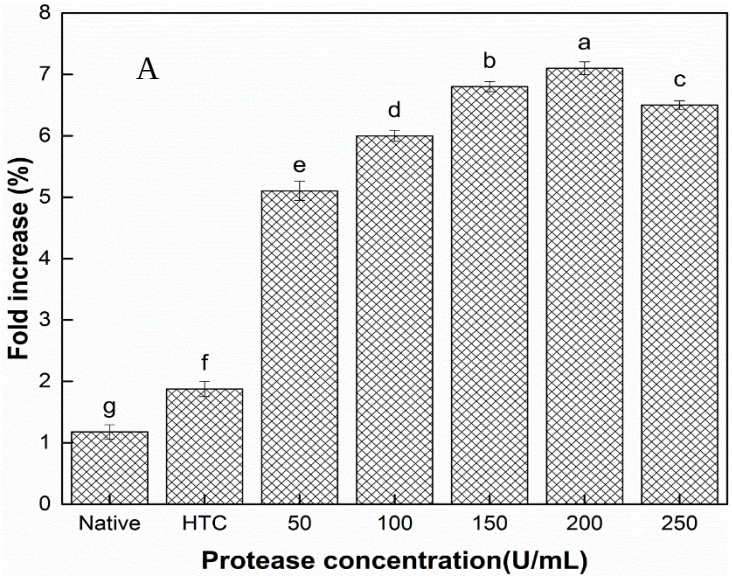
resistant starch. The yield was increased by 8.93-foldusing pullulanase for 16 hours of incubation (Fig.4.1B). However, the reaction time longer than 16 hours resulted in a reduction in the resistant starch yield. This indicates that many of the amylose chains were hydrolyzed into glucoside micro-molecules and unable to form double helix.

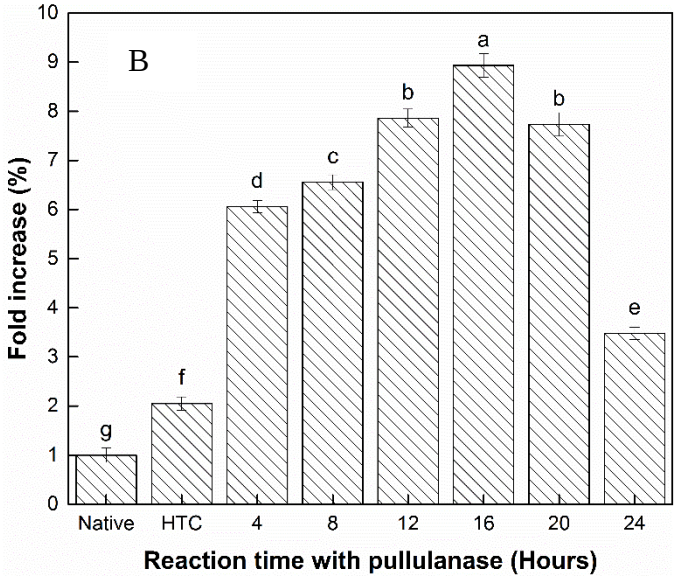
Temperature has direct influence on the pullulanase activity, 16.78-fold increase in yield of RSIII was observed at60°CFig.4.1C, when the temperature was too low, enzymatic hydrolysis would slow and incomplete. This would lead to reducing the RSIII yield. Therefore, optimum temperature for enzymatic action is necessary and contributes significantly in preparation process of resistant starch.

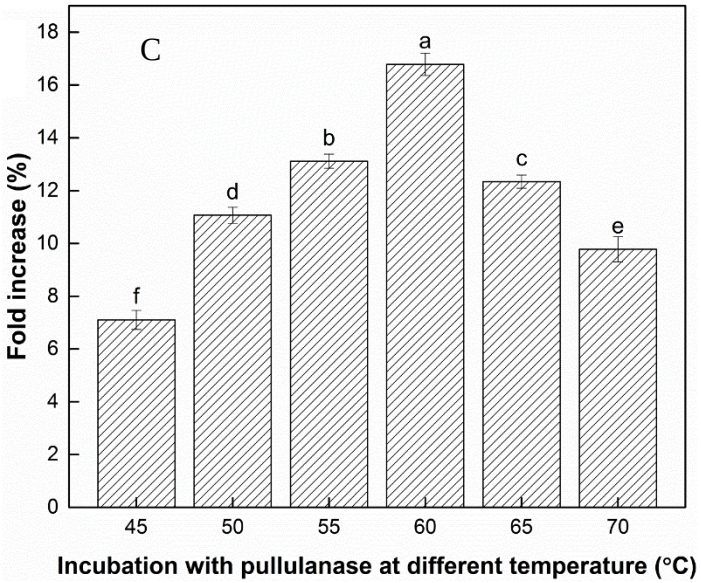
In case of pH the maximum resistant starch yield was increased by 18.12 folds over control at pH 5, Fig.4.1D. While with increase in pH profile resulted in decrease of resistant starch content, which is linked with lower stability of pullulanase at higher pH (5).In the production of resistant starch pullulanase essentially linked with decreasing the chain length of glucose in amylopectin by hydrolyzing the α-1,6 glycosidic bonds which is positively associated with increase in resistant starch content. Pullulanase concentration resulted in 15.7-fold increase of resistant starch content compared to control under optimized conditions (PUL: 20U/reaction; 200 U/mL). The production of RSIII gradually increased as the amount of pullulanase increased from 0 to 200 U/mL while further increase in enzyme concentration resulted in decrease of resistant starch synthesis as shown in Fig.4.1E. This is because at high concentration of enzymes the starch molecule completely hydrolyzed into glucose molecules , which ultimately have negative impact during the synthesis process when the mixture is cool down (24). Previously **we reported 16.7-fold increase in the yield of resistant starch by treatment of maize flour with amylase, yield (15).**

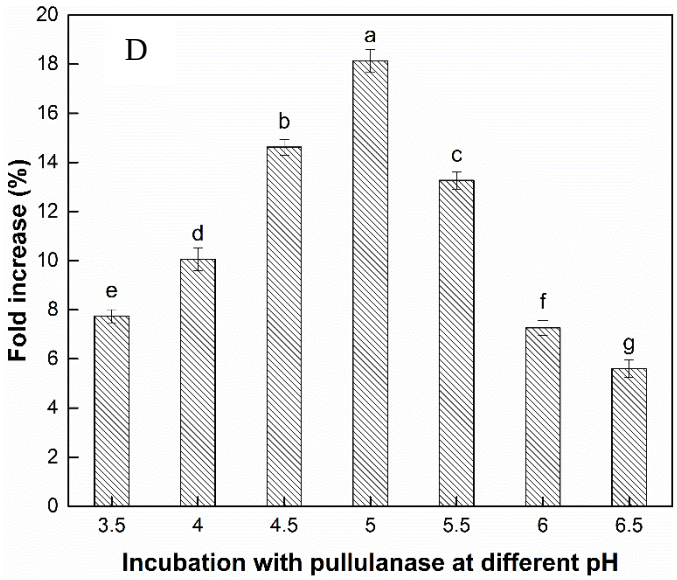
Storing temperature after gelatinization and debranching of starch has a major effect on the formation of RSIII (25). During retrogradation and recrystallization (26), linear short chain of amylose transform into an ordered and crystalline structure due to which it resistant to enzymatic digestion. High the degree of crystallization higher will be the amount of RSIII formed and hence it will be more resistant to gastrointestinal conditions (19). The effect of various retrogradation temperature (30, 15, 5, -4 ^{o}C) on the production of RS-III is shown in Fig.1F. It can be affected both by recrystallization temperature and chain length. The maximum production of RSIII was observed at a retrogradation temperature of -4 ^{o}C which was about 20.03 fold higher than control, while the lowest amount of RS-III was observed at 30 ^{o}C in

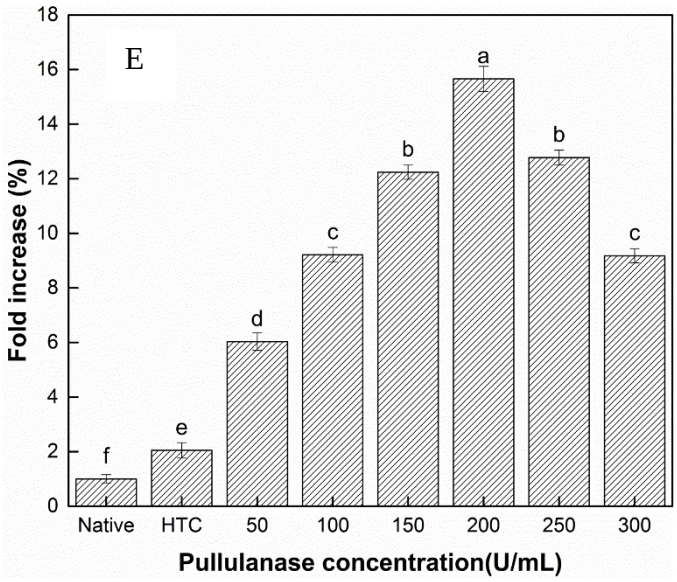
which the RSIII content was 10.6 fold as high temperature slow down recrystallization of amylose chains.






C
Fold increase (%)
Incubation with pullulanase at different temperature (°C)
45
50
55
60
65
70
a
b
c
d
e
f


D
Fold increase (%)
Incubation with pullulanase at different pH
3.5
4
4.5
5
5.5
6
6.5
a
b
c
d
e
f
g


E
Fold increase (%)
Pullulanase concentration(U/mL)
Native
HTC
50
100
150
200
250
300
a
b
b
c
c
d
e
f

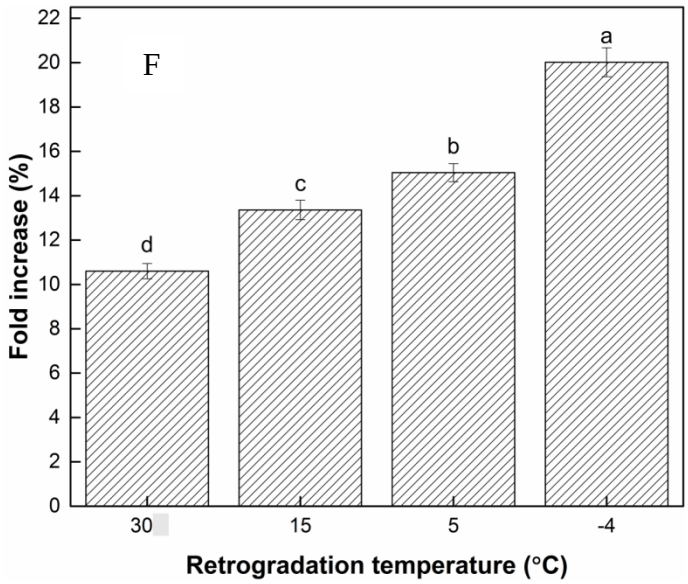


Figure.4.2: Optimization of different parameters for the synthesis of resistant starch (RSIII) A: Effect of protease concentration on RSIII yield, B: Effect of reaction time on RSIII yield, C: Effect of different temperature on RSIII yield, D: Effect of different pH on RSIII yield, E: Effect of pullulanase concentration on RS yield, F: Effect of Retrogradation temperature on RSIII yield.

4.4.3 Physicochemical Properties of Native and Pullulanase treated maize flour:

4.4.3.1 Swelling power and Water absorption capacity:

The swelling power is one of the most important characteristics depicting the ease with which starch will cook. It is ascribing the firmness of bonds in the crystalline region of starch granule (27). Starch granules with strong crystalline structure and greater tenacity in bonds when heated show less swelling power (28, 29). The gel forming capacity of crystalline region of starch granule is low because of the strong affinity of hydrogen bond to re-associate the amylose chains during retrogradation (30). The swelling power of pullulanase treated maize starch was 269%, significantly lower than untreated maize starch (414 %) (Table 4.1). Actually, the swelling power describes the degree of polymerization of amylose chains, debranching rate, crystalline and amorphous region, and ratio of amylose and amylopectin for conformation of starch molecules, which is an important factor to reveal the interaction of water and starch molecules. The observation of decrease in swelling power of pullulanase treated starch as compare to the untreated starch ascertains the crystalline structure formation and strong tenacity of bonds during retrogradation governing the synthesis of RS-III.

The water absorption capacity of starch granules was mainly dependent on the extraction procedure, source, thermal stability, and ratio of amylose/amylopectin (31, 32). The decrease in water absorption capacity of pullulanase treated starch (153%) as compared to untreated starch (180 %) ascribes the increased crystalline nature and conformation of starch granules during gelatinization temperature leading to reduced mobility of starch modules responsible for starch water interaction.

4.4.3.2 Effect of Light Transmittance:

The effect of refrigeration on light transmittance of native and pullulanase treated maize flour were analysed (Table 4.1). Considerable reduction in the light transmittance values of starch samples pastes was obtained during a period of 120 hours. The reduction in percentage of arrangements of amylose chains during retrogradation process. The light transmittance of enzymatically produced RS-III was comparatively higher than native starch gels after subjected to equivalent periods of storage. The reason for more absorbance of native starch is due to unorganized, loose and free amylopectin chains which are responsible for more absorbance and less transmittance as subjected by spectrophotometer. At any specific storage period, the transmittance value of ATMF(15) is lower than PTMF . ATMF has less debranching because of absence of use of pullulanase in its production, therefore, has low amylose and RS content, hence loose structure, lower levels of retrogradation ultimately less transmittance as compared to PTMF. RS-III starch sample involved the use of pullulanase enzyme which has ability of hydrolysing 1,6 bonds leading to greater amount of amylose and RS-III content, more compact structure formation (retrogradation) due to lack of branching points and ultimately low absorbance and greater transmittance as compared to native. This is mainly based on the nature of the enzymes involved in RS-III production which influences its retrogradation tendency and hence transmittance of starch samples (33).

4.4.3.3 Iodine Complexing Ability:

The preliminary efficiency of starch chain to interact and form inclusion complexes with iodine molecules demonstrate as a good tool for binding drug and other organic compounds and used as carrier molecule in pharmaceutical industries.(34). The iodine binding ability of the starches depends on chain length, degree of polymerization, concentration of starch and conformational structure of the starch molecules (35). The absorbance values at 460nm were indicative of presence of short chains (< DP 30) whereas absorbance values at 570nm were representative of longer chains (around DP

35–300).The iodine staining indices of native maize and enzymatically produced resistant starch at different starch concentrations at 460nm and 570nm are presented in (Table 4.2). The native maize flour depicted significantly lower values of light absorption at both 460 and 570nm even at high starch concentration of about 5%. Whereas, ISI of pullulanase treated resistant starches (debranched) at all the three starch concentrations exhibited higher absorbance indicating higher complexing ability. Native maize starch showed lower absorption values primarily because the inner branched chains of intact amylopectin molecules are embedded in the form of highly organized crystals making it difficult for native to form iodine complexes. It is also reported that with the increase in hydrolysis of starch with pullulanase there is an increase in the absorbance values of partially debranched RS-iodine complexes at both wavelengths. As reported by Khan *et al,* amylase treated maize starch showed comparatively higher values of iodine binding compared to native and lower than resistant starch produced by pullulanase. This is because hydrolysis by amylase enzyme releases amylose molecules making the inner helical structure accessible to iodine binding(15). Whereas, PTMF due to hydrolysis by pullulanase leading to release of smaller hydrolyzed /or hydrolysis products consisting of various sizes of branched and linear glucose chains. The greater iodine binding ability as own by greater values of absorption of RS-III-iodine complexes indicating its biocompatible with other biomolecules.

4.4.3.4 Milk absorption capacity:

Milk absorption ability of pullulanase treated starch is very important to make it an important ingredient in breakfast cereals with more nutraceutical values (36).Result indicated that native maize showed a lower MAC as compared to RS-III (Table 4.1). MAC is related to the interaction between amylose and milk proteins which form a complex and hence greater the amylose content of starch greater is the ability of milk absorption. Native maize showed a lower MAC of about 360% as compared to the MAC of enzymatically synthesized RS which demonstrated greater MAC (430%). A possible explanation of this increased MAC of RS samples is the use of enzymes leads to debranching of amylopectin and increased amylose content levels which results in greater amount of amylose available to form complex with milk proteins ultimately leads to more milk bound to starch samples and hence greater MAC. Moreover, native is untreated and has intact amylopectin and bound amylose, the available free amylose is less resulting in lowest MAC.

4.4.3.5 Oil absorption capacity:

The Oil absorption capacity (OAC) of enzymatically produced RS samples and native maize is presented in the Table 4.1. OAC of starch might include the physical entrapment of oil inside the porous structure of starch due to capillary forces and also by the development of complexes of amylose with lipids within the helical starch structures (33).The difference in OAC demonstrated the difference in chain length distribution of amylopectin and amylose ratio. Hydrophobic proteins also contribute majorly in oil absorption and signify the formation of stable food products (37, 38). Native maize starch showed an increased OAC of about 90.91% when compared with HTM and the enzymatically synthesized RS samples. The increased oil uptake of native starch might be because of the varietal differences in ratio of amylose and amylopectin, chain length distribution(39), larger surface area and porosity of the particles and presence of greater hydrophobic proteins which assist oil absorption. This is because native maize is untreated with enzymes (proteases, amylase, and pullulanase) therefore; it has a loose and porous structure and higher proteins contributing to high OAC. In comparison to the native maize enzymatically produced RS samples showed lower OAC in the order of HTM>ATMF>PTMF with values of the order 84.97%>75.76%>69.7%(15). This is primarily because debranching with amylase, pullulanase leads to greater realignment of starch linear amylose chains in more tight, compact crystallites making the double helices of the crystallites resulting in low porosity that restricted oil uptake. Moreover, protease pre-treatment during enzymatic synthesis leads to removal of hydrophobic protein hence low oil absorption. The oil absorption capacity of maize flour is vital factor for maintaining the flavour and enhancing the palatability of the food product. Therefore, low oil absorption capacity of pullulanase treated starch makes it ideal candidate for formulations of low caloric food products and improve crispiness and crunchiness to the end products like in biscuits, wafers, hush pubbies formulation etc.

4.4.3.6 Amylose content of Native and pullulanase treated Maize Flour

The apparent amylose content of native maize and enzymatically synthesized resistant starch samples is presented in the table 4.1. An increase in amylose with a consequent decrease in amylopectin content was observed for enzymatically produced resistant starch samples in comparison to native maize. This is because the increased RS and amylose contents which leads compact, crystalline structures which resist enzymatic hydrolysis as compared to the loose, intact amylopectin containing

native maize with decreased RS and amylose levels. Among the enzymatically produced starch samples PTMF showed the highest amylose content (36.11%) followed by ATMF (15) and Native maize had the least amylose content (19.87%). According to previous reported results the flour with highest RS content (9.0%) depicted high amylose content due to increased formation of amylose as the result of de–polymerization process leading to the formation of crystals contributing resistance (40).

Table 4.2: Physicochemical properties of native Maize Starch (A) and Pullulanase treated maize four (RS-III) (B)

Parameters	**Sample types**	
	Native Maize Starch (A)	**Pullulanase treated maize starch RS-III (B)**
[a]SP (%)	414	269
[b]WAC (%)	180	153
[c]OAC (%)	90.91	69.69
[d]MAC (%)	360	430
Amylose Content (%)	19.87	34.86
Reducing power	0.3	0.4
Light transmittance (%)	0.01	2.5
Antioxidant activity (%)	31.4	36.4
Relative Crystallinity (%)	18	34

[a]SP(%) = Swelling Power, [b]WAC (%) = Water Absorption Capacity, [c]OAC (%) = Oil Absorption Capacity, [d]MAC (%) = Milk Absorption Capacity and [e]RSIII (B) %= Pullulanase treated resistant starch III)

Table 4.3: Iodine binding capacity of native and RS-III at 460 nm and 570 nm

Sample Type	**Wavelength (nm)**					
	460nm			**570nm**		
Starch Concentrations %w/v	1%	3%	5%	1%	3%	5%
Native (A)	0.13	0.31	0.35	0.12	0.15	0.25
RSIII (B)	0.172	0.345	0.374	0.173	0.314	0.342

(RSIII (B) = Pullulanase treated resistant starch III)

4.3 Structural characterization of native and pullulanase treated maize flour:

4.3.1 Scanning Electron Microscopy:

The scanning electron microscopy results revealed that native maize starch granules have smooth surface with clear polygonal shape and have no sign of surface changeFig.4.2. However, the pullulanase treated starch granules have clear changes in morphology with irregular surface with dense matrix (Fig.4.2B) as compared to native maize starch (Fig 4.2A) and high amylose maize starch (HAMS) (Fig 4.2C). The morphological differences between treated and untreated starch is possibly due to re-association and double helical formation of amylose chains during pullulanase treatment and retrogradation. These characteristics depicted the increased resistivity of pullulanase treated maize starch to gastric conditions and are also responsible for dense crystalline structure.

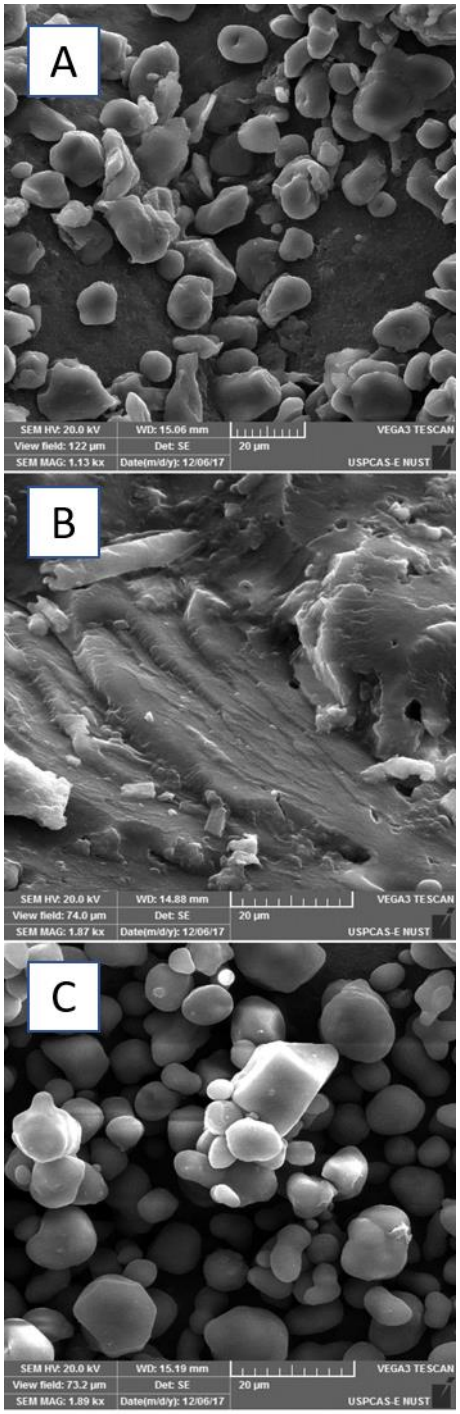


Figure.4.3: Scanning electron micrograph of (A) Native maize starch (B) Pullulanase treated starch sample (C) Industrial resistant (Hylon VII) starch

4.3.2 XRD and relative crystallinity:

The intensity of the XRD peak and relative crystallinity of pullulanase treated and untreated starch ascribe the changes in crystalline and amorphous region (41, 42). A sharp diffraction peak of pullulanase treated starch revealed complete crystalline surface and stretched crystal line while, untreated starch with wide diffraction peak

determined an amorphous structure because of incomplete crystal and small crystal line (Fig.4.3A). Native maize starch has shown strong diffraction peaks at 2θ with values around 15.38º, 17.1º and 19.6ºindicates that the native maize starch which is used for analysis is of cereal and exhibit A-type crystalline structure. However, the pullulanase treated samples have shown very strong and high peaks which are representative of the formation of a new crystalline structure after pullulanase treatment. For pullulanase treated recrystallized starch the reflection peak at 2θ= 14.58º, 20.12º, 25.15º and 22.5º reflects the B-type crystallinity pattern.

The change in crystalline pattern is consistent with previous research work from literature which demonstrate the intense peak at 20.12 attributed to the disappearance of amorphous region upon enzymatic treatment (3, 43). The reason for the crystalline shift from amorphous form is related to the re-association of amylose molecules its chain length which implied to promote B-type crystalline packaging of pullulanase treated maize flour. The relative crystallinity of pullulanase treated sample increased (34 %) as compared to the native (18 %), in accordance with the increase of percentage of resistant starch.

4.3.3 Fourier transforms Infrared Spectroscopy

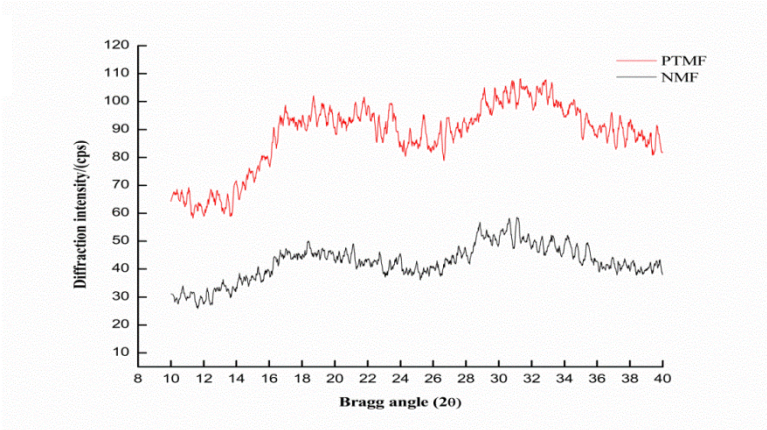
Infrared spectroscopy analysis illustrated the molecular conformation of starch granules specifically in terms of the short chain organized double helical structure. This ascribes starch chain conformation, retrogradation, and crystallinity at the exterior regions of starch granule with changes in peak intensity (44, 45). The variation in intensity of IR spectra depicts morphological changes in pullulanase treated and untreated starch. The IR peaks at 1241, 2926 and 3290 cm^{-1} revealed bending vibration of O-H and broad vibration of poly C-H and –OH respectively. The peaks in the region of 1365-1413 cm^{-1} matches to C-H bending vibration. Whereas, peaks at 1047 and 1022 cm^{-1} are indicative of the crystalline and amorphous structure of both pullulanase treated and untreated starch, respectively (Fig.4.3B). The peak intensity of pullulanase treated and untreated starch is increased at 1047 cm^{-1} comparatively because of more efficient rearrangement of double helical structure governing increased crystallinity.

4.3.4 Thermogravimetric analysis:

Thermal stability was investigated by TGA study as shown in Fig.4.3C. Both pullulanase treated and native maize flour samples showed a single step major weight loss in addition to the slight weight loss occurring below 150 °C which is attributed

due to loss of water molecule. The presence of water molecules in both samples were confirmed by ^{1}H NMR spectroscopy, as disused in later section. Below 150 °C the weight loss in native (7%) and pullulanase treated Starch (4%) is associated with the water evaporation leading to major weight loss at 280 °C and 265 °C respectively in both samples. The weight loss in pullulanase treated sample at slight lower temperature compared to native sample is due to increase percentage of crystalline amylose chains with respect to amylopectin chains. While, highly branched and long chains of amylopectin in native starch samples needs more energy to dissociate. However, the residual weight at 600 °C resulted in more weight loss in native sample (75 %) as compared to pullulanase treated samples (88 %). These results were in accordance to our previous publication in which more amylose percentage over amylopectin in amylase treated starch resulted in more residual weight compared to native starch.

A



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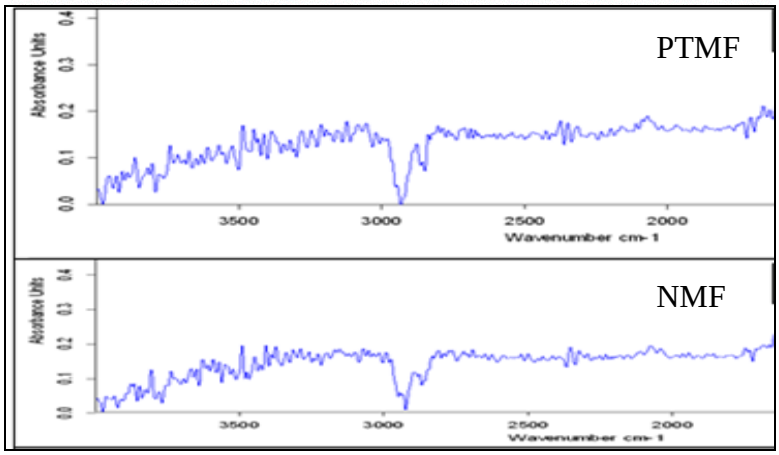

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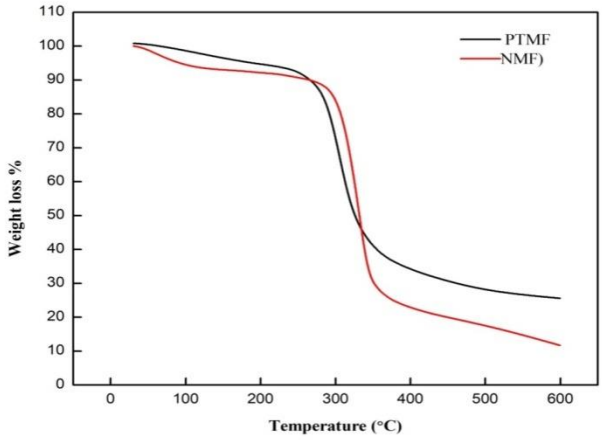


Figure4.4: Structural characterization of native and pullulanase-treated maize flour A: X-ray diffraction patterns of native control (NMF) and pullulanase treated sample (PTMF), B: FT-IR spectra of Native control (NMF) and Pullulanase Treated sample (PTMF), C: TGA thermogram of native control (NMF) and the pullulanase treated sample (PTMF)

4.3.5 Nuclear Magnetic Resonance Spectroscopy:

The chemical structure of a starch molecule is shown in Fig.4.4while Fig.4.5A shows the ^{1}H NMR spectra of maize starch before and after treatment with pullulanase. The anomeric protons with chemical shifts of 5.50 and 5.11 ppm are assigned to the internal α-1, 4 and α-1, 6 linkages, respectively. The resonance line at 4.59 ppm is due to the OH group while the resonance lines in the range of 3.70 ppm to 3.40 ppm are attributed to the proton atoms attached to C5, C3 and C2 carbon atoms. Some of these resonance lines may overlap with the broad line of the water present in the samples. In addition, there are few overlapping resonance lines in the range of 1.50 ppm to 1.20 ppm, which is the typical range of $-CH_2-$ protons. There are few lower intensity resonance lines at 0.86 ppm which represents the presence of $-CH_3$ protons. Both the ^{1}H NMR spectra are very similar indicating that no significant changes occur in the chemical structure of maize sample upon treatment with pullulanase. However, there exists additional very sharp resonance lines at 2.09 ppm and 1.24 ppm in the sample treated with pullulanase, which are absent in the control sample. These resonance lines in the pullulanase treated sample could be assigned to glucans with reducing ends, which were released by the attack of pullulanase on α-1, 6 linkages.

Fig.4.5B shows the ^{13}C NMR spectra of maize starch before and after treatment with pullulanase. The ^{13}C NMR spectra of control and the pullulanase treated sample are

very similar indicating that the chemical nature of the treating sample has not significantly changed upon treatment. The resonance line at 100.5 ppm suggests the presence of C1. The resonance lines in the range of 72 ppm to 74 ppm represent C_2, C_3 and C_5. The resonance line at 79.2 ppm is attributed to C4 while the resonance line at 60.9 ppm is assigned to C6.

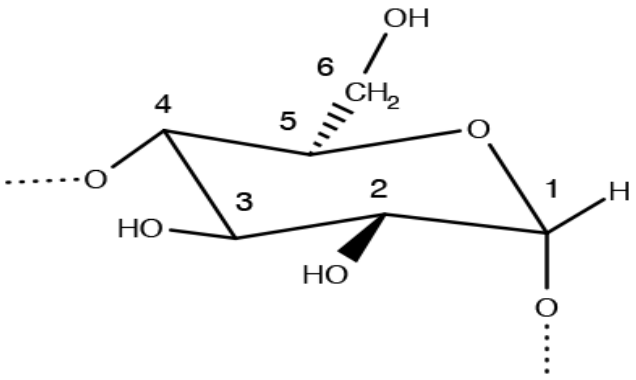
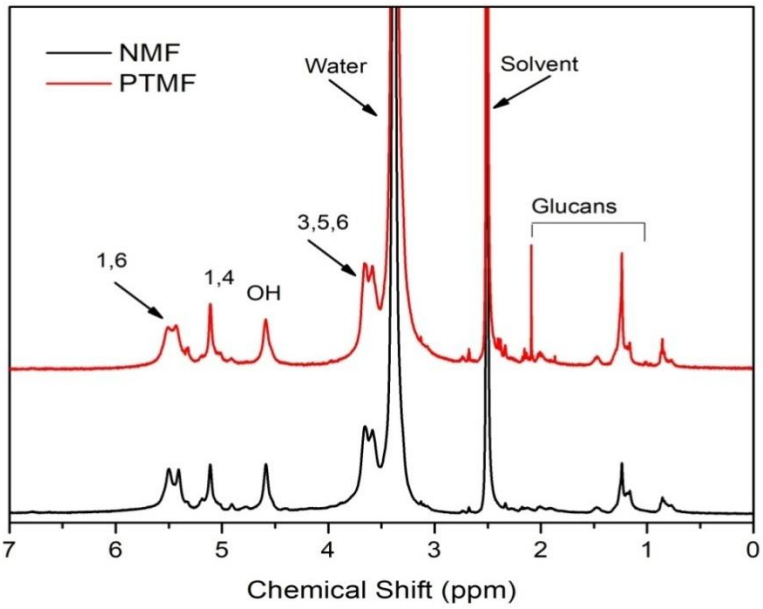


Figure .4.5 Chemical structure of a starch molecule

A



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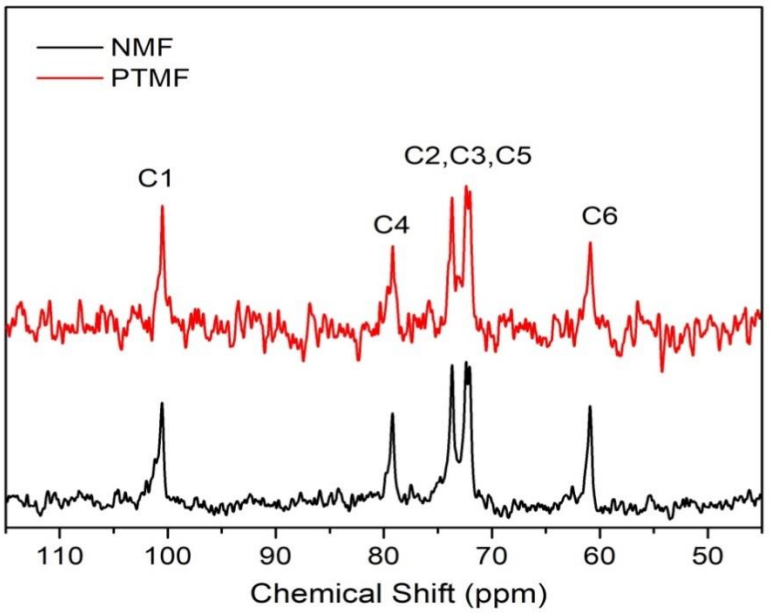

Figure.4.6. NMR spectra of native and pullulanase-treated maize flour,A: ^{1}H NMR spectra of control (native maize flour) and pullulanase treated maize flour in DMSO. The ^{1}H resonance line at 2.50 ppm is for the DMSO that was used as solvent while the resonance line at 3.38 ppm indicates the presence of water in both the samples, B: ^{13}C NMR spectra of control and the sample dissolved in DMSO.

4.4 Application of Pullulanase Treated Maize Flour (RS-III):

RS-III due to its slow digestibility has many applications; therefore *in-vitro* digestibility is measured.

4.4.1 *In-vitro* digestibility of pullulanase treated and untreated starch:

The Fig.4.6 shows the comparative concentration of maltose (mg) produced because of resistance to pancreatic alpha amylase digestion of both pullulanase treated and untreated starch over duration of 6 hours. In all the time intervals ranging from 1 to 6 hours, the untreated starch underwent greater enzymatic digestion and resulted in greater diffusion of reducing sugar concentration into the surrounding buffer. On contrary, the pullulanase treated maize starch sample showed resistance to the enzymatic digestion over a period of 6 hours indicated by lesser amount of reducing sugar produced and diffused into the buffer. This is because the untreated maize starch (native starch) has an A-type crystal structure which has pores through which the pancreatic-α-amylase binds at different regions leading to the greater digestion and hence greater release of reducing sugars. Whereas, pullulanase treated maize

starch has a compact B-type crystal structure acquiring a conformation that is resistant to significant gastric enzymatic digestion and therefore, enzyme attacks the surface but cannot hydrolyze properly and hence less maltose is released. After 4 hours of incubation, the maltose released in both pullulanase treated and untreated starch is nearly comparable in concentration even though the untreated starch has more maltose production but maltose produced by pullulanase treated starch in last 2 hours is significantly higher than that produced in initial 4 hours. This is particularly because as the time of contact with the enzyme increases, more enzyme binding occurs and hence more digestion leading to greater maltose concentration which is nearly comparable but still lesser than maltose released by untreated starch. Pullulanase treated maize flour due to slow digestibility attributes many functional benefits by controlling the glycemic index, help in body weight management, and prevent the onset of heart disease and diabetes. The property of slow digestibility attracted great attention in treatment of diabetes type II.

Hence this experiment proved that pullulanase treated starch is more resistant to invitro digestibility with pancreatic-α-amylase as compared to the less resistant native maize starch.

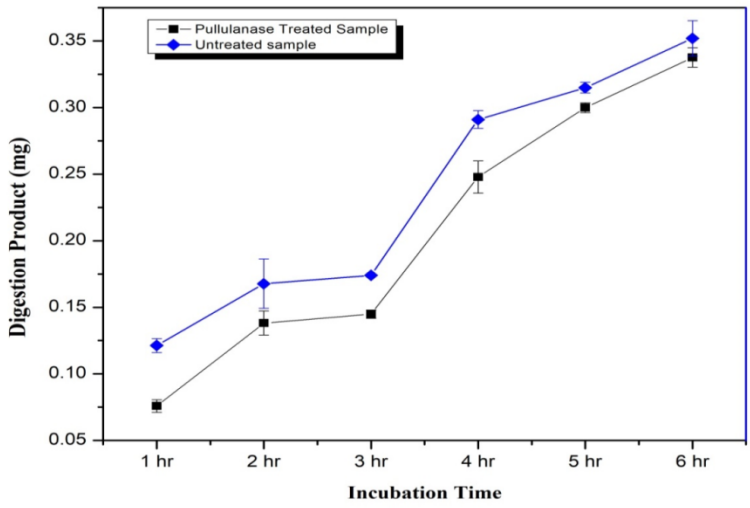

Figure .4.7: *In-vitro* digestibility of Native (NMF) and Pullulanase treated maize flour (PTMF) at different time intervals

4.4.2 Antioxidant Potential of pullulanase treated and untreated starch:

Natural dietary food items have attracted great attention due to their tendency to prevent cancers and to lower the risk of cancer development by reducing oxidative

stress (46, 47). Oxidative stress has more prominent role in activating the pathogenesis of numerous pathophysiological disorders including cardiovascular diseases, diabetes, and cancer. So, addition of antioxidant rich ingredient in daily diet can be helpful to overcome such chronic diseases (48). According to the current research the exposure of OH –group after enzymatic treatment as confirmed by FTIR analysis due to increase in its peak intensity (Fig. 4.3 B) leads to more antioxidant potential (35.3 %).The extra glucans resonance in ^{1}H NMR also indicative of its antioxidant potential as shown in Fig 4.5A. While in case of native starch (31.4%) complex amylopectin chains reduces the exposures of free bonds to react as ascribed in the assay with DPPH free radicals and the Fe^{+3} complex. The results were in accordance to the Shah *et al*.,(49) and Khan *et al*.,(50)research about investigation of effect of irradiation effect on polysaccharide claimed high antioxidant activity because of exposure of hydroxyl groups upon treatment. This property of pullulanase treated sample makes it an important ingredient in anti-aging cosmetics product ingredient.

The conclusion of present research work is to explore the effect of pullulanase debranching and processing condition optimization, *in-vitro* digestibility, and antioxidant potential of enzymatically synthesized resistant starch. The morphology and crystal structure of pullulanase treated starch were changed with significantly enhanced thermal properties. With increasing digestion time, the average digestion rate of synthesized RS-III decreased. The antioxidant potential and reducing power of pullulanase treated starch increased with agreement of resistant starch content and therefore improve the desired functional properties of maize starch.

Conflict of interest statement

We declare no conflict of interest.

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Chapter 5: Preparation of Resistant Starch using Amylase and Pullulanase

Paper 3:

Title: The effects of enzymes on resistant starch content of maize flour, its physicochemical properties and using this flour in cookies formulation

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5.1 Abstract

The current study was aimed to enzymatically modify maize flour by using amylase and pullulanase in combination for increasing the yield of resistant starch type III (EM-RSIII). The enzymatic treatment imparts unique desired physicochemical properties to the maize flour including resistant starch content, amylose content, milk oil and water interaction ability while the sensory analysis was performed by distributing questioners among individual and results were interpreted by five-point hedonic scale. Result indicated increase in the stability, amylose content and milk absorption capacity of maize flour while decrease in swelling power, water absorption and oil absorption capacity. The enzymatic treated maize flour led to transformation of amorphous native structure into a crystalline structure which was confirmed by light microscopy, scanning electron microscopy, X-ray diffractograms analysis and invitro digestibility of EM-RSIII. The EM-RSIII rich maize flour was used in cookies preparation and a significant difference in palatability and color parameters was observed. Crispiness and texture of EM-RSIII containing cookies was increased due to its low swelling power, water absorption and oil absorption capacity. The sensory analysis showed that consumers depicted highest acceptability of EM-RSIII supplemented cookies specifically for its appearance, sharp aroma, texture and accepted as nutritionally important food item for consumers.

Keywords: Resistant starch, Maize flour, Digestibility, Nutritional properties, Resistant starch containing cookies

5.2 Introduction

The inclusion of prebiotics in industrialized foods has become a viable and healthy alternative, since there is a great demand of consumers for functional foods that can help in maintaining good health. Moreover, the food industry can obtain numerous advantages from the addition of prebiotics in food products, such as improvement of sensory characteristics, better balance of the nutritional composition, and longer shelf-life. The importance of the consumption of prebiotics is unquestionable and they should be part of healthy diet. Recently, resistant starch has attracted much attention in societies because of its functional and physiological properties [1]. RS has the potential to mend human health by preventing the risk of chronic diseases such as diabetes mellitus type II [2], colorectal cancer [3], obesity [4], and cardiovascular diseases[5]. Furthermore, RS escape digestion in the stomach and small intestine and reach the colon undigested [6], where RS is fermented by colonic bacteria and acts as a prebiotic [7]. Additionally, RS also has positive effect on the palatability of food compared to traditional dietary fibers. The crispness of the final fried crust is an important quality issue in battered, fried food. In this regard, low oil absorption and swelling power of RS has been associated with greater crispiness of the product during frying[8]. The increased complexing ability [8a] and lower digestibility of RS under similar GIT conditions during in vitro examination [8a, 9] make it also feasible as coating material for site specific delivery of drug. It has pronounced significance in pharmaceutical and food industries.

In human body, to comprehend the physiological effectiveness of resistant starch, it is recommended that daily carbohydrate intake must contain 10 to 20% resistant starch, whereas most of the marketable foodstuffs have less than 5%, and most of the conventional food products lose resistant starch content during processing[10].Therefore, it is essential to design a process for improving the resistant starch content of food with consumer safety and acceptability in mind. To cope with this, the focus of present study was to increase the resistant starch content of maize flour by enzymatic hydrolysis to improve its physicochemical properties for its use as important food ingredient[11].

Native resistant starch loses its resistivity in food during cooking and chewing process and become accessible to digestive enzymes therefore, there is need to mend a method for preparation of resistant starch with more stable structure which becomes resistant to digestive enzymes.In recent times, researchers mostly focused on the physical,

chemical and enzymatic treatments to enhance the resistant starch content of starch from numerous botanical sources [12]. Among them, enzymatic treatment is favored because of its bio-based or environment friendly nature. Enzymatically prepared resistant starch is preferred over chemically synthesized resistant starch because biocatalysts have stereospecificity, easily biodegradable and free of any toxic by-products [8a, 13]. For this purpose, pullulanase and α-amylase were selected to specifically hydrolyze α-1-4 and α-1-6 glycosidic linkages in starch macromolecules in order to produce short, linear oligosaccharides of varying length for the formation of resistant starch with more compact crystalline structure. To get the required chain length of amylose (10 to 100 glucose units) after hydrolysis concentration of amylase and pullulanase needs to be optimized. Combination of starch hydrolyzing enzymes instead of single enzyme is preferred in order to increase the opportunity of alignment and aggregation of amylose chains in less time to form compact crystalline lamellae which have ability to resist the gastric digestion. [14]. Englyst *et al*. [15] reported that for improving resistance to gastric digestion amylose chains are prone to form double helices which strengthened the tightly packed crystalline structure in starch molecule. Therefore, the current study emphasized on improving the resistant starch content and physicochemical properties of maize flour in place of maize starch and to use it in formulation of cookies.The reason to use maize flour instead of maize starch is that very little information is available on the use of enzymatically prepared RS III (EM-RS III) from maize flour in cookies formulation which also signifies additional benefits for increasing vitamins and fiber content of prepared cookies.

The specific objective of the present study was enlightening the essential potential of debranching enzyme pullulanase and amylase from indigenously isolated *Bacillus licheniformis* for enhancing the synthesis of resistant starch (EM-RS-III) from maize flour. To optimize the enzymatic reaction parameters for increasing the yield of RS, characterization of resistant starch with respect to its physicochemical and functional properties were carried out. Finally, enzymatically synthesized EM-RS-III was used as a food hydrocolloid, by supplementation it in cookies ingredients to substitute the native flour with EM-RS III rich functional fiber. Sensory evaluation of the cookies was carried out to check its consumer acceptability.

5.3 Materials and methods

5.3.1 Materials

Maize flour was purchased from local market of Mansehra (KPK Pakistan). Protamex® **(**endoprotease with a broad specificity from *Bacillus* Sp. P0029) was purchased from Sigma-Aldrich, USA. Other solvents and chemicals used in this work were obtained from Merck (Germany) and were of analytical grade. The kit for the estimation of resistant starch concentration was purchased from Megazyme International Ireland Ltd. Pullulanase and amylase were expressed and purified from indigenously isolated strain of *Bacillus licheniformis*[8a].

5.3.2 Preparation of Resistant Starch from Maize Flour

Resistant Starch was synthesized from maize flour. Five grams of maize flour in 0.5 M citrate buffer (pH 5) was dispersed, and the mixture was pregelatinized by autoclaving (121°C for 20 min). The gelatinized mixture was cooled to 37°C and protease (200U/ mL: 250 µL) was added and incubated for 40 minutes. The enzymatic reaction was terminated by boiling the mixture in water bath at 100°C for 5 minutes to denature protease. After cooling the gelatinized slurry amylase and pullulanase were added to the protease treated maize flour, and was incubated at 50°C, 100 rpm in shaking incubator for 16 hours. Enzymes inactivation was achieved at 99°C in water bath for 10 minutes. Then, a cycle of autoclaving (121°C, 15 psi) for 1 hour was carried out, and the paste was cooled at -4°Cfor 24 hours. A negative control without any enzyme treatment was run in parallel. Dried resistant starch was grinded into fine powder of approximately less than 0.1 mm and stored in the airtight container for further analysis. Resistant starch content of native and enzyme treated maize flour was determined by Megazyme Resistant Starch Assay Kit and the sample codes used for native maize flour were NMF and enzymes treated maize flour as EM-RS III during characterization.

5.3.2.1 Optimization of Reaction Conditions

In order to enhance the resistant starch content of maize flour and to optimize the reaction conditions, samples were pretreated with different concentration of protease (0, 50,100,150,200,250 and 300 U/mL) followed by pullulanase and amylase treatment(0,50,100,150, 200,250 and 300 U/mL) and finally exposed to retrogradation temperature (-4,5,15 and 30 °C). Megazyme Resistant starch assay kit was used to analyze the prepared resistant starch content under these conditions.

5.3.3 Morphological Characterization

5.3.3.1 Light Microscopy

Structural and morphological properties of native and enzymatically treated maize flour were determined through light microscopy. Samples were mixed with Glycerol/water (v/v) of 1:1 to form suspension and analyzed under microscope with 40 X resolution after 30 minutes.

5.3.3.2 Scanning Electron Microscopy

Scanning electron microscope (VEGA-3, Oxford instrument by TESCAN Czech Republic) was used to analyze the native and enzymatically treated maize flour. The samples were freeze dried and fixed on gold coated aluminum stub through double sided stick tape. Samples were analyzed at increasing voltage of 20 kV.

5.3.4 Structural characterization

Fourier transform infrared spectroscopy (FTIR) of Nicolet Avatar 360 FTIR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to analyze the structural characteristics of native and enzymatically treated maize flour. Samples (2 mg) were pressed under sheet with potassium bromide (KBr) for dehydration. The FTIR spectra were generated in order to scan the samples at room temperature at resolution of 4 cm^{-1} from 400 to 4000 cm^{-1} in transmission mode.

5.3.5 Thermal analysis

Thermogravimetric analysis (TGA) was performed by using Perkin Elmer 8000 TGA apparatus at heating rate of 10°C/min to 300°C. The experiment was performed in the presence of nitrogen gas using 2-3 mg of samples.

5.3.6 X-ray diffraction

X-ray diffractogram (D/Max-2200, Tokyo, Japan) was used to analyze the X-ray diffraction (XRD) of native and enzymatically treated maize flour. Before analysis, the samples underwent drying in a desiccator at room temperature and were exposed to strong radiation of Cu-Kalpha at speed of 2°C/min. X-ray diffractometer produced the radiation with an angle of 2 theta at 40 Kv and 300 mA at the temperature range of 5 to 55°C.

5.3.7 Physicochemical Properties

Physicochemical properties of enzymatically treated and native maize flour were determined as follow.

5.3.7.1 Amylose content

The amylose content of enzymatically prepared dried resistant starch (EM-RS III) and untreated maize flour (NMF)was quantified. Starch samples (0.1 g) was dissolved in 1 mL of absolute ethanol to which 10 mL of 1 N NaOH was added; the volume was made up to 100 mL with distilled water followed by overnight incubation at 25°C. Distilled water (20 mL) with three drops of phenolphthalein indicator was mixed with around 2.5 mL of each sample followed by titration against 0.1 N HCl until the observation of disappearance of pink color. Iodine reagent (1 mL) was added to the above mixture followed by the making the final volume to 50 mL, and the absorbance was spectrophotometrically measured at 590 nm. The amylose content of the samples was quantified from the **amylose standard curve. 1 mL of iodine reagent in 50 mL distilled water was taken as a blank**

5.3.7.2 Oil Absorption Capacity

Enzymatically prepared dried resistant starch (EM-RS III) and untreated maize flour (NMF) (1 g) was mixed with 6 mL of olive oil in centrifuge tubes using constant stirring. The tubes were then vortexed for 5 min, left for 30 min to permit oil absorption and then centrifuged for 20 min at 3000 rpm. The pellet was separated by decanting the supernatant in a petri plate, dried and later weighed. The gain in weight is represented as the oil absorption capacity and calculated as

$$OAC\ (\%) = \frac{weight\ \text{of starch with oil} - \text{original weight of starch}}{\text{original weight of starch}} \times 100$$

5.3.7.3 Milk Absorption Capacity

Milk absorption capacity (MAC) was quantified by following the method used for oil absorption capacity and 0.1 g of each starch sample EM-RS III and NMF was added into 2 mL of raw milk. Formula used for MAC was:

$$\textbf{MAC (\%)} = \frac{weight\ of\ starch\ with\ milk - original\ weight\ of\ starch}{original\ weight\ of\ starch} \times 100$$

5.3.7.4 Water Absorption Capacity

To determine the water absorption capacity 0.25 g of starch samples were mixed in 12.5 mL of distilled water and was heated at 30, 60 and 90ºC respectively in water bath for 30 min on continuous stirring. The samples were then cooled at 25ºCfollowed by centrifugation for 20 min at 3000 rpm. The separated supernatant and pellets were then taken and dried by decanting it in a petri plate by placing in an

oven for 4-5 h at 110ºC and later weighed. WAC was then determined by the formula:

$$WAC\ (\%) = (sediment)\ pelletwt\ /\ Samplewt \times 100$$

5.3.7.5 Swelling Power

Swelling power was quantified by adding 0.5 g of enzymatically prepared dried resistant starch (EM-RS III) and untreated maize flour (NMF)in 25 mL distilled water followed by heating the samples at three different temperatures (30, 60, and 90 °C) as in case of water absorption capacity. Dried samples and the swollen starch sediment (pellet) obtained after centrifugation were weighed. Swelling power was calculated as follows:

$$SP(\%) = \frac{wt\ of\ sediment}{wt\ of\ dry\ starch - wt\ of\ dissolved\ starch} \times 100$$

5.3.7.6 Water Binding Capacity

Samples were prepared in the same way as incase of water absorption capacity and 0.25 g of enzymatically prepared dried resistant starch (EM-RS III) and untreated maize flour (NMF)sample was dissolved in 5 mL distilled water, and the binding capacity was calculated from the following equation:

$$\text{WBC (\%)} = \frac{(M2 - M1 - Mo)}{Mo} \times 100$$

where, Mo was the weight of initial starch taken, M1 empty centrifuge tube, and M2 the weight of centrifugation tube containing the dried pellet.

5.3.8 *In-vitro* digestibility of EM-RS III in different compartments of the gastrointestinal tract

In vitro digestibility of enzymatically prepared dried resistant starch (EM-RS III) and untreated maize flour (NMF) was performed as described by Dona *et al*[31] with certain modifications. About 1 g of each starch sample was mixed to 100 mL of water from which 2 mL was taken and treated with α-amylase (3 U/mL) in 5 mL of potassium phosphate buffer (pH 7). To mimic the mouth conditions, it was incubated at 37°C for about 2 min in a shaking water bath. Samples were withdrawn every 30 sec, centrifuged and supernatant was collected for quantification of sugars released because of enzymatic digestion. To imitate the stomach conditions, to each sample 6 mL of 0.1 N HCl solution was added, and the pH was adjusted to 1.3 to mimic the gastric pH; followed by incubation in a 37°C shaker incubator for 2 h. Samples were

withdrawn every 40 min, centrifuged, and the supernatant was collected. For creation of small intestinal conditions, the starch sample was treated with 10 mL pancreatic α-amylase (300 U/mL) in a dialysis bag with a molecular weight cut of 14 kDa. The dialysis bag was placed into a beaker containing phosphate buffer (350 mL at 37°C) with gentle stirring (75 rpm). Dialysate (2 mL) was collected every 1h up to 6 h of incubation. The supernatants containing tubes from mouth and intestinal compartments were processed by heating in a water bath (99.9°C) to denature enzyme. The digestion rate of EM-RS III and NMF from each compartment of the GI tract was determined by the amount of reducing sugars quantified by dinitro salicylic acid method.

5.3.8.1 Microscopy of Samples After *In-vitro* Digestion

After *in vitro* enzymatic digestion light microscopy and scanning electron microscopy were performed for EM-RS III and NMF. On a glass slide samples were mixed with 50 % glycerol and analyzed under 100X microscopic resolution. For scanning electron microscopy, samples were dried to a fine powder and fixed on an aluminum stub coated with a film of gold via double-sided stick tape. Finally, the samples were observed at an increasing voltage of 20 kV.using scanning electron microscope (VEGA-3 by TESCAN Czech Republic with oxford EDS detector, Oxford Instruments).

5.3.9 Synthesis of Resistant Starch Supplemented Cookies

Five different types of cookies were baked with different composition of EM-RSIII. The ingredients which were common in all five samples (total weight 140 g) were a) butter 60g b) brown sugar 45g c)1egg d) salt 0.1g, e) sodium bicarbonate (baking powder) 0.3g and f) 0.15 mL vanilla extract. The concentration and type of flour used in the five different cookie samples is shown in Table 5.1.

Table 5.1: Type and concentration of flour used in cookies

Cookies	**Concentration of carbohydrates (g)**
Native maize	30
Cookie baking flour	30
Resistant starch	30
Native maize + RS (1:1)	15+15
Cookie baking flour + RS (1:1)	15+15

The native maize, EM-RS III and cookie baking flour were separately mixed with grinded brown sugar and sieved in separate bowls. Butter was creamed in a mixer for about 5-10 min. Afterwards homogenized mixture was formed by adding egg and vanilla essence. Furthermore, all ingredients were mixed to form dough which was then kept aside for 30 minutes at 4°C. Cookies of multiple combinations mentioned above were made into circular shape and were baked in a pre-heated oven at 180°C for 10-15 min. After cooling, cookies were packed and stored in sealed bags for sensory evaluation within 24-72 hours of baking. Moreover, a questionnaire was also developed for the sensory evaluation of cookies (supplementary data).

5.3.9.1 Color Estimation of EM-RSIII Supplemented Cookies:

For measurement of color of prepared cookies with different proportion of EM-RSIII a Konica Minolta Chroma Meter CR-400 (Tokyo, Japan) was used. The color meter was calibrated prior to color measurement on white tile as blank. Samples were measured for color estimation in triplicate using samples in transparent petri dish. CIEL a*b* color space was used for color estimation. Parameter L* represent the brightness from black to white, a* measured the red green color with negative values representing greenness and positive values indicating redness. While parameter b* presenting yellow-blue color with negative values indicative of blueness and positive values representing yellowness.

5.3.9.2 Sensory Evaluation of EM-RSIII Supplemented Cookies

For sensory analysis freshly baked cookies were presented to fifteen members (lab mates). Cookies supplemented with different proportion of EM-RSIII were evaluated for sweetness, flavor, color; overall quality and texture (hard, porous, crisp and uniform). In order to improve reproducibility, the orientation session was conducted, and accuracy of evaluation were assessed. For each sample, panelists scored their liking of these characteristics using the 5point hedonic scale (1 dislike extremely, 2 dislike, 3 neither like nor dislike 4 like, 5 like extremely).

5.3.10 Statistical Analysis

The results are described by the mean values ± standard deviations (SD). One way of variance (ANOVA) was performed by SPSS 22.0 (IBM, SPSS statistics), followed by the difference in the samples was compared by Duncan's multiple range tests ($p < 0.05$) and expressed as mean values based on three replicates. Figures were drawn using Origin 9.1 (Origin Lab Corp., Northampton, MA 01060 USA) software package.

5.4 Results and discussion

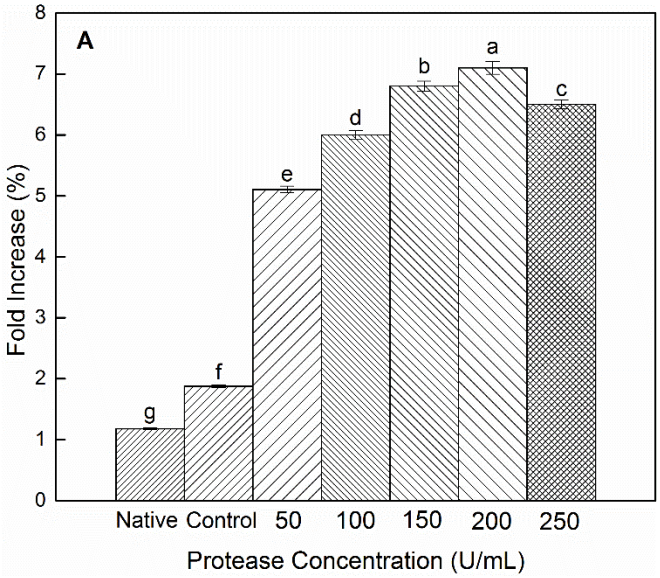
5.4.1 Optimization of Reaction Parameters for Preparation of Resistant Starch

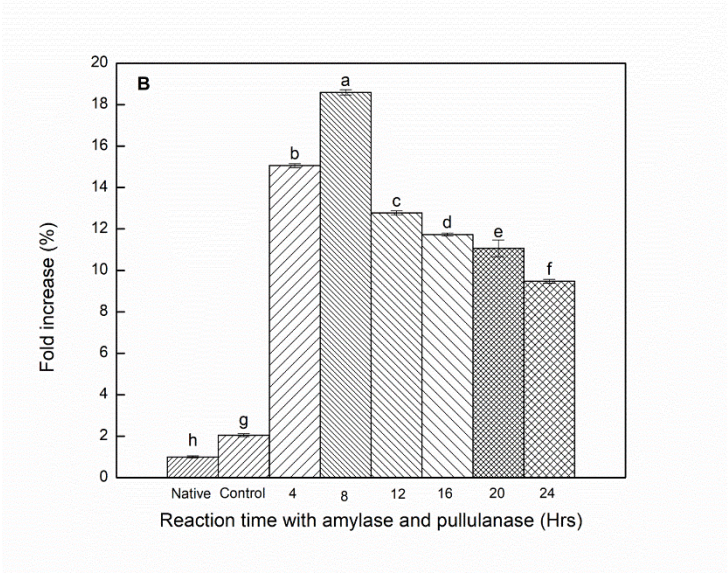
Maize flour is important staple food in Asia and rich source of proteins, carbohydrates, fats, and vitamins[16]. Physicochemical and nutritional properties of maize flour depend on their arrangement of protein and starch molecules. Protein and starch interact together by hydrogen bonds and resist the *invitro* preparation of resistant starch because of protein entrapment[17]. Therefore, with the aim to enhance the content of resistant starch in maize flour it was pretreated with optimized concentration of protease (200 U/mL) at 37°C pH 7 (Fig 5.1: A).Protease pretreatment exposed the starch chains for easy hydrolysis by starch hydrolyzing enzymes i.e. pullulanase and α-amylase. Starch hydrolysis resulted in shorter chain length of amylose and amylopectin facilitating the rearrangements upon retrogradation[18]. The amylase and pullulanase were used in combination with the ratio of 1:1 (200U/mL) respectively, as optimized after one factor at a time approach. Because of its ease and convenience, one-variable-at-a-time approach has been the most preferred choice among the researchers for designing the medium composition and used in the initial stages in diverse fields. Both the enzymes were used sequentially i.e. pullulanase was added first incubated for 1 hr at optimized conditions. Pullulanase catalyzes hydrolysis of α-1,6 glycosidic bonds specifically. Pullulanase was added which notably reduced the highly branched chains of amylopectin at α-1-6 linkages and resulted in long linear chains of amylose. In the current study after pretreatment, pullulanase was added which notably reduced the highly branched chains of amylopectin at α-1-6 linkages and resulted in long linear chains of amylose. A linear and compact amylose chain contributes greatly in increasing the resistant starch content. In order to organize the amylose into optimum chain length α-amylase was applied to specifically target the α-1-4 glycosidic linkages of amylose and amylopectin[19]. The reaction time for pullulanase and α-amylase hydrolysis was investigated from 0 to 24 hours, and a maximum increase in the yield of resistant starch was found to be 18.56-fold compared to heat treated and native control after 8 hours (Fig. 5.1 B). However, significant decrease in resistant starch yield from 18.56-foldto 9.48-fold was observed with increase in reaction time when preceded from 8 hours to 24 hours. The decrease in RSIII yield depicted the shorter chain length of amylose into glycosidic macromolecules (less than 10 glucose units) which were

necessary to form double helix upon retrogradation for crystalline structure. When amylase was used individually it resulted in 16.72-fold increase after 16 hours of incubation[8a]. Consequently, combination of enzymes resulted in dual benefit i.e. increase in the yield of resistant starch in less time. In that respect, both ratio and sequence of enzymes have unprecedented role in the production of resistant starch.

Retrogradation temperature after gelatinization and enzymatic hydrolysis of starch has prominent role for increasing resistant starch yield[20]. In fact, retrogradation facilitates the rearrangement and organization of amylose chains to make them resistant to enzymatic hydrolysis[19]. Higher the yield of resistant starch more will be the crystallinity and higher gastric resistivity[21]. The effect of various retrogradation temperature (30, 15, 5, -4^{o}C) on the production of RSIII is shown in Fig.5.1D. It can be affected both by recrystallization temperature and chain length. The maximum production of RSIII was observed at a retrogradation temperature of -4^{o}C which was about 24.03-fold ($p<0.05$) higher than control, while the lowest amount of RSIII was observed at 30^{o}C (10.6-fold) as increase in temperature slow down recrystallization of amylose chains.

The RS content of untreated native maize as well as enzymatically treated starch samples and gelatinized maize control was quantified according to the standardized protocol of Megazyme Assay Kit (Ireland). The Resistant starch content of enzymatically produced starch was found out to be 24.03% (Table 5.4),the higher RSIII content could be due to high amylose content which is produced as a result of both enzymes. The enzymatic de-branching resulted in linear amylose which recrystallize into a more tightly packed resistant structure having the highest RSIII content compared to HTM where low RS content is due to formation of destabilized crystalline structure of starch after retro-gradation. Industrial RS obtained from Megazyme International was used as a positive control with the highest RS content of 44%.


A
Fold Increase (%)
Native Control 50 100 150 200 250
Protease Concentration (U/mL)


B
Fold increase (%)
Native Control 4 8 12 16 20 24
Reaction time with amylase and pullulanase (Hrs)

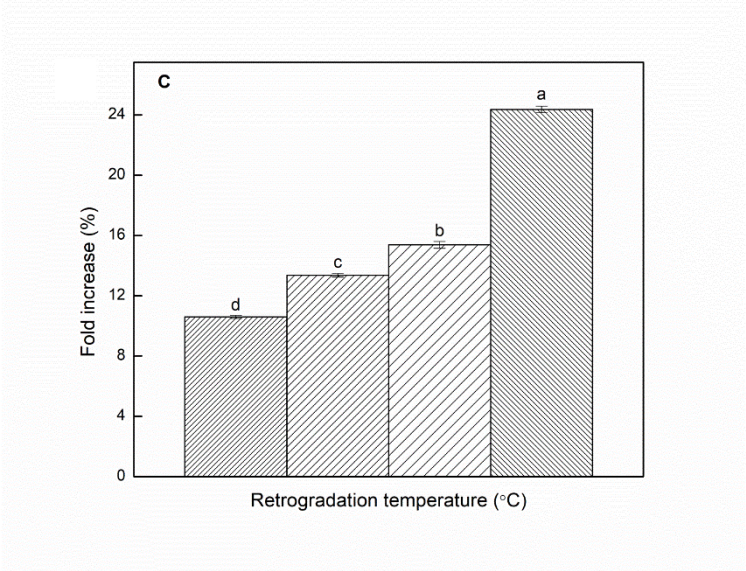

C
Fold increase (%)
Retrogradation temperature (°C)

Figure 5.1: Optimization of different parameters for the enzymatic preparation of resistant starch type III (EM-RSIII). A: Effect of protease concentration on RSIII yield, B: Effect of pullulanase and α-amylase concentration on RSIII yield C: Effect of different retrogradation temperature on RSIII yield

5.4.2 Non-Resistant Starch Content

The Non-Resistant starch (solubilized starch) content of enzymatically produced maize flour was significantly lower than gelatinized HTM and control as shown in Table 5.2. Enzymatically produced starch EM-RS III showed significantly lower Non-RS contents of 46.45% as compared to the native maize flour (69.79%) because of arrangement of amylose chains into RSIII chains instead of soluble starch after retro-gradation and this phenomenon had lowered the Non-RS while improved the RSIII content. Industrial RS obtained from Megazyme International was used as a negative control with the lowest Non-RS content of 26.5% corresponding to the highest RS content.

5.4.3 Total Starch Content

Starch is the major constituent of maize kernel and makes up about 70 to 73 percent of the kernel weight. Carbohydrates are present in highest amounts in maize kernel[22].Carbohydrates other than starch include glucose, sucrose and fructose contributing 1-3 percent in the maize kernel[23]. Total starch is the combination of both the solubilized starch (Non-RS) as well as the resistant portion (RS). RS and Non-RS contents were determined by using the standardized protocol of Megazyme Assay kit (Ireland) and the sum of these corresponds to the total starch content of the starch. RS, Non-RS and total starch contents are given in the Table 5.2.

5.4.4 Morphological characterization

There are clear differences in the shape, physical and structural appearance of gelatinized and enzymatically gelatinized maize flour as shown in Fig 5.2. Heat-treated gelatinized maize flour was smooth, flat, single layered and undistributed (Fig 5.2: A). Enzymatically gelatinized maize flour had apparent changes in physical appearance (Fig 5.2: B) viscosity and color. Treatment of starch with amylase and pullulanase lead to increase of crystalline structure and hence different physical appearance as compared to gelatinized maize control Fig 5.2C-F. Light microscopy and scanning electron microscopy demonstrated that native starch appears round, bell-like oval or pear shaped, and no structural deformity was present at the outer surface of native granules, while enzymatically treated maize four looked roughly flat block-

like in terms of the outer shape. These observations are consistent with those reported for chickpea starch [24] and corn starch [25].

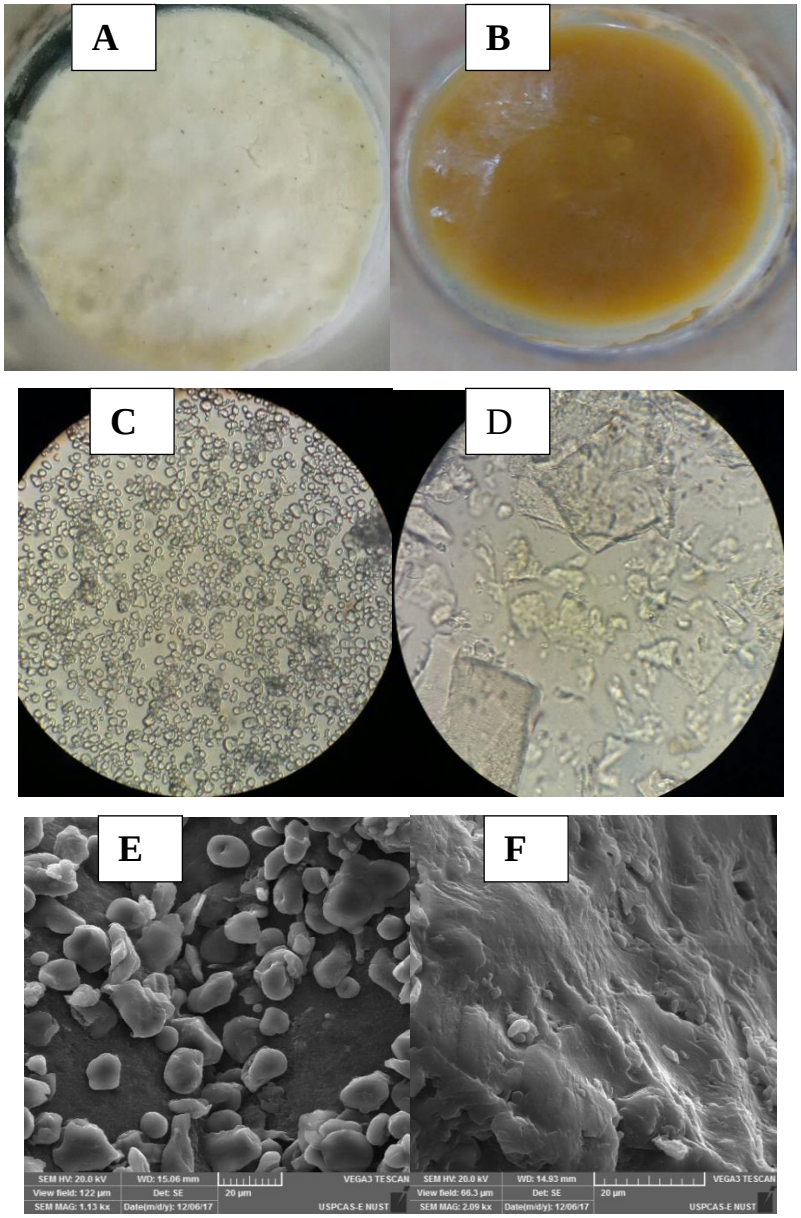


Figure 5.2: Morphological characterization of native and enzymatically treated maize flour (A) Gelatinized heat treated native maize flour, (B) Gelatinized enzymatically modified maize flour, (C) Light micrograph of native maize flour, (D) Light micrograph of enzymatically modified maize flour, Scanning electron micrograph of (E) native maize flour (F)enzymatically modified maize flour

5.4.5 Color analysis

Enzymatically prepared resistant starch after drying had different color than untreated native maize flour and gelatinized (heat-treated) maize controls as shown in Fig 3. Heat treated maize flour had light brown color while enzymatically treated starch showed dark brown color. More dark brown color was an indication of more glucose

production through starch granules indicating the malliord reaction[26](chemical reaction between amino acids and reducing sugars that gives browned food its distinctive flavor and new aroma).

In Table 5.2 color values of EM-RSIII samples against the heat-treated maize (HTM) and untreated native maize (NM) controls obtained from color flex colorimetry are listed. EM-RS III samples depicted differences in values of L*, a* and b* showed greater variation in color values in comparison to the values of untreated native maize control. A considerable reduction in the L* values (lightness) of all treated starch samples was observed through treatment including gelatinization alone as well as gelatinization followed by enzymatic treatment. Ozturk, Koksel, and Ng [27] also observed decreased in crust color by addition of 30% CrystaLean-supplemented flour samples in RSRI-supplemented breads. For HTM, reduction in L* may be due to maillard reaction at high temperature of autoclaving. Moreover, a* (redness), b* (yellowness) values of EM-RS III also showed minor increased values with enzymatic treatment as shown in Table 5.3.

Figure 5.3: Physical appearance of (A) Native maize flour (NMF), (B) Heat treated maize flour (HTM) and (C) Enzymatically prepared dried resistant starch (EM-RS III)

5.4.6 X-ray Diffraction of Starch Samples and Change in Crystallinity

The X-ray diffractograms of enzymatically treated and native maize flour are shown in Fig 5.3A. The crystallinity and amorphous nature of starch molecule dependent on the chain length and rearrangements of amylose and amylopectin. Mostly, double helices of amylose resulted in the semi crystalline region of starch molecules while long amylopectin molecules contribute into amorphous region[28]. Katz and Itallie categorized the crystalline region of starch into three main groups: A-type includes shorter chains, B-type includes longer chains while A and B types depicted the mixtures of shorter and longer chains and designated as C-type[29]. In the present study, the raw or native showed strong diffraction peaks at 13.21°, 18.42°, 28.85°, and 30.69°, which describing the A-type crystalline group for cereals or maize.

Nevertheless, the very strong and high peaks at 2θ= 17.21°, 20.03°,19.73°, 21.57° and 25.25° demonstrated the transformation of amorphous to crystalline structure after enzymatic treatment. Similar reports showed the presence of a peak at 20.03° 2θ described the occurrence of crystallinity due to amylose chains rearrangement after enzymatic treatment and retrogradation[30]. The possible reason for differences in crystalline pattern of samples are related to portion of crystalline region, chain length of amylose and amylopectin and their orientations and rearrangements as all these factors greatly influence the crystallinity and resistant starch yield[31]. Miao et al.[32]showed that X-ray diffraction of resistant starch correspond to crystallinity and amorphous region which is dependent on the double helices of amylose. Guraya *et al*.[33] used pullulanase to debranch nonwaxy or waxy starches, and found that an increased in the degree of debranching would result in higher molecular alignment and aggregation of starch which provide opportunities to form perfectly crystallized structures. Thus, the crystallized RS structures led to the differences in their resistance to starch-degrading enzymes that are used with a combination of α-amylase and pullulanase.

5.4.7 Fourier Transform Spectroscopy for Structural Analysis

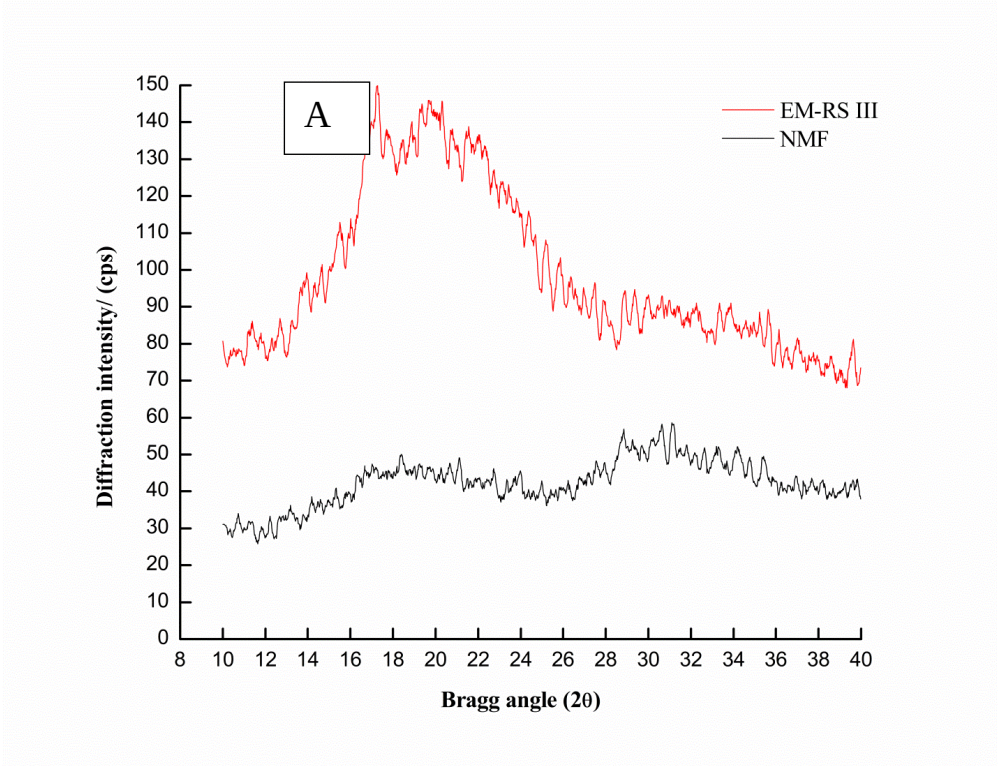
The FTIR spectra of enzymatically treated maize flour is shown in Fig 5.3B. Slight variations in the intensity of spectra of enzymatically treated maize starch was observed compared to native starch. These slight variations depicted only the physical modification and no chemical modifications were observed in the spectra after enzymatic treatment.

5.4.8 Thermal Decomposition Measurement by Thermogravimetric Analysis

Thermogravimetric analysis revealed the thermal decomposition of starch molecules as shown in Fig 5.3C. The native and enzymatically treated samples presented the single step major weight loss in addition of slight weight loss below 150°C. Due to evaporation of water molecules both the samples showed loss in weight. The enzymatically treated maize flour showed weight loss up to 5.25 % while native maize flour showed weight loss up to 7% at 150°C due to loss of water molecule. The major weight loss in case of enzymatically treated maize flour was observed at 253°C while for native starch weight loss observed was at 280°C a slightly higher temperature. Because of the presence of long chain of amylopectin in native starch as compared to enzymatically treated maize flour. When the temperature exceeds up to 600°C 88 % weight loss was observed in case of native maize flour and 76 % weight

loss in case of enzymatically treated maize flour. This major weight loss is due to oxidative conditions in which starch molecules underwent depolymerization when temperature starts to exceed from 300°C. At higher temperature, degradation of starch molecules started and resulted in the formation of pyro dextrin, furfural, levoglucosan, low molecular weight volatile products and finally carbon/ash remains as by product[34].

Starch molecule is composed of highly branched amylopectin and linear amylose chains, which mainly contributes to the physical and chemical properties of starch molecule. When amylose to amylopectin ratio exceed in starch molecules it become more prone to thermal decomposition because longer amylopectin chains can easily resist the thermal decomposition[35]



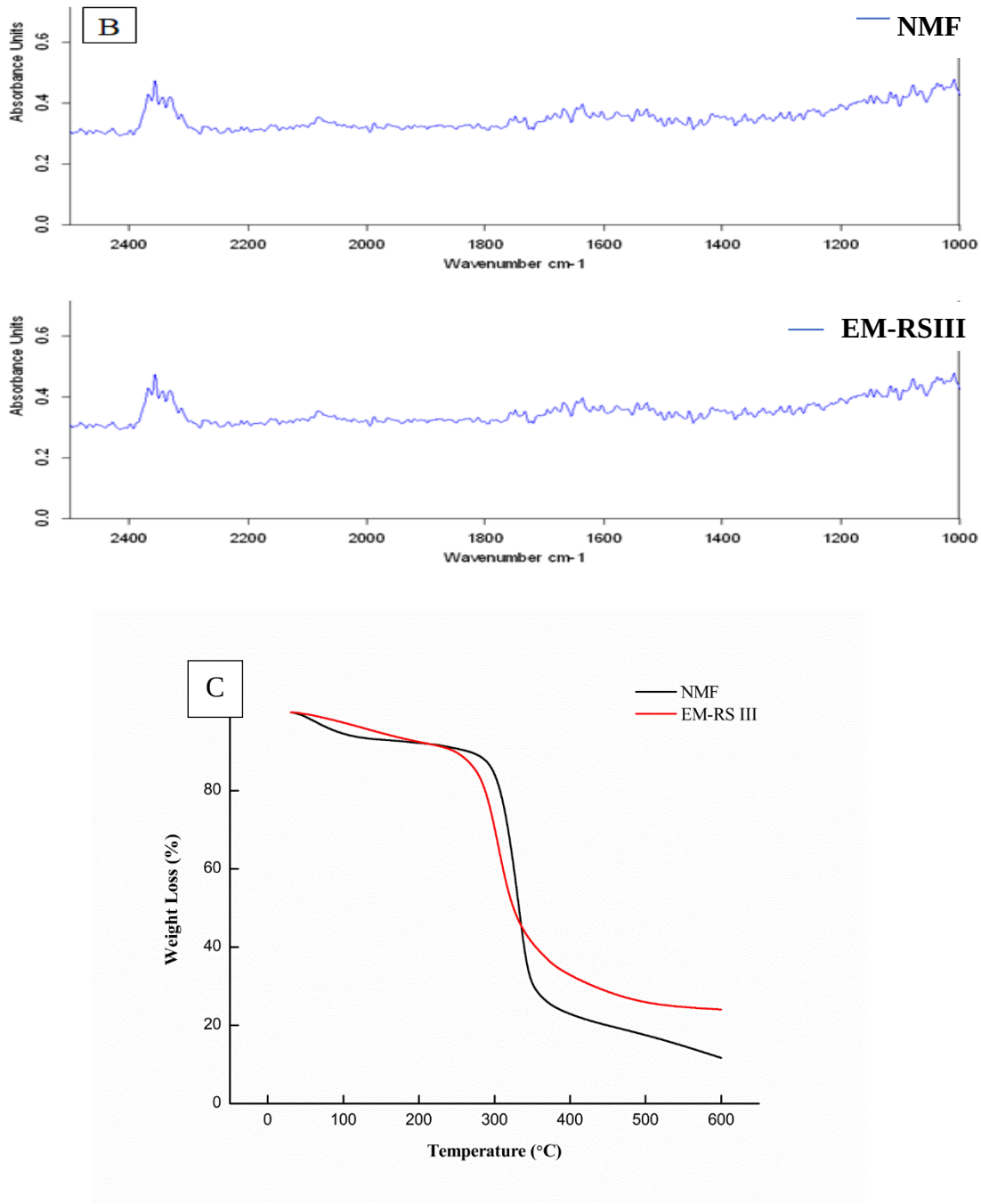


Figure 5.4 (A)X-ray diffraction patterns of native maize flour (NMF) and enzymatically treated sample (EM-RSIII),**(B)** FTIR spectra of native sample (NMF) and enzymatically treated samples (EM-RSIII) **(C)** TGA thermogram of native control (NMF) and enzymatically treated samples (EM-RSIII).

5.4.9 Physicochemical Properties of Native and Enzymatically Treated Maize Flour

5.4.9.1 Amylose content

Amylose content of starch samples were quantified and it has been noticed [8a] that amylose content in maize is highly influenced by cultivar, agronomic conditions and

climate[36]. Moreover, an increased amylose content was observed for enzymatically produced resistant starch samples as compared to heat treated samples. EM-RS III showed highest amylose content (36.11%) because of de-branching of α-(1–6) linkage of amylopectin by pullulanase enzyme, which produces small chain linear polysaccharides as amylose. While amylose content for gelatinized maize control (24.75%) and of untreated native maize flour (19.87%) has less variation because HMT underwent only physical treatment which resulted in less differences in overall chemical structure and composition of starch as shown in Table 5.2. These results were in agreement with Rosida*et al* in which the flour with highest RS content (9%) showed high amylose content due to increased amount of amylose as the result of de–polymerization process leading to the formation of crystals contributing to resistance[37].

5.4.9.2 Amylopectin content

Enzymatically prepared resistant starch (EMRSII) showed a considerable decrease in amylopectin content as compared to untreated native and gelatinized (heat-treated) maize controls. Enzymatically prepared resistant starch (EM-RS III) showed the lowest amylopectin content (63.89%) whereas untreated native maize had the highest amylopectin content (80.13%) as demonstrated in Table 5.2. Low amylopectin content of starch after enzymatic treatment is attributed to high amylose content because after pullulanase treatment amylopectin chains are dispersed and converted into amylose[38]. The increased RS and amylose contents results in compact, crystalline structures rendering it resistance to enzymatic hydrolysis compared to the loose, intact amylopectin in native maize which have low RS and amylose content[39].

5.4.9.3 Oil Absorption Capacity

Oil absorption capacity of untreated and gelatinized native maize and enzymatically produced EM-RS III starch sample is shown in Table 5.2**.** Native maize had significantly higher oil absorption capacity(90.91%) compared to EM-RS III samples having 66.67%.Particle size and oil absorption capacity of enzymatically synthesized resistant starch decreases because of compact structural rearrangement which shows that oil absorption capacity is probably inversely correlated with particle size. OAC of starch may be due to the development of amylose-lipid complexes within the helical structures of starch and by the physical entrapment of oil within the porous starch structure owing to capillary forces[40]. This is clear in case of hydrolysis with amylase; pullulanase which results in realignment of starch linear amylose chains into

tightly compact crystallites forming the double helices resulting in low porosity that restricted oil uptake [41]. Moreover, protease pre-treatment during enzymatic synthesis leads to removal of hydrophobic protein hence decrease oil absorption. The native starch showed increased oil uptake due to the varietal differences in ratio of amylose and amylopectin, chain length distribution [42], larger surface area, particles porosity and presence of greater hydrophobic proteins [43]which assist oil absorption.

5.4.9.4 Milk Absorption Capacity

Milk absorption capacity of untreated, gelatinized native maize and enzymatically treated resistant starch samples is shown in Table 5.2. Enzymatically produced resistant starch showed significantly higher MAC of about 550% ($p<0.05$) and the least MAC was that of native maize (360%). A possible explanation of this increased MAC of EM-RS III samples is due to the enzyme-induced de-branching of amylopectin and increased amylose content levels which leads to greater amount of amylose available to form complexes with milk proteins ultimately more milk is bounded to starch. Moreover, native is untreated and has intact amylopectin and bound amylose, the available free amylose is less resulting in lowest MAC. Addition of whey protein retards staling of bread by protein-amylose complexes formation leading to prevention of retro-gradation of amylose and amylopectin chains [44].

5.4.9.5 Water Absorption Capacity

Water absorption capacity (WAC) of starch samples depicted direct relation to temperature as shown in Table 5.4. Native maize flour demonstrated highest WAC at all investigated temperatures followed by gelatinized HTM, and EM-RS III respectively due to high content of amylopectin crystalline structure to take up water to reach a homogenized consistency in native maize flour.

5.4.9.6 Water Binding Capacity

Water binding capacity (WBC) of native maize flour and enzymatically prepared resistant starch samples at various temperatures (30, 60 and 90°C) revealed reliance on temperature as mentioned in Table 5.4. Native maize showed highest WBC at all the three temperatures when compared with the lower values of WBC exhibited by HTM and enzymatically produced EM-RS III samples. The lowest values of WBC at any temperature were of EM-RS III due to enzymatic and physiochemical modifications. It has been observed previously that enzymatic and chemical

modifications reduce WBC of starch presumably by blocking the water binding sites[45].

5.4.9.7 Swelling power

Swelling power (SP) of native maize and enzymatically prepared starch samples showed a significant variation at different temperatures (30, 60 and 90°C) as shown in Table 5.4. Native maize showed the highest swelling power at all the three temperatures as compared to the lower values of swelling power exhibited by enzymatically produced EM-RS III sample. The reduction in swelling power of EM-RS III starch is due to the disruption of side chains of amylopectin. In case of enzymatic treatment more disruption occurs due to action of pullulanase which further lower swelling power. Brown [46](1996) stated that granules of enzyme-resistant high-amylose maize starch are not completely gelatinized even at the boiling point of water, therefore after enzymatic treatment starch samples have low interaction with water molecule. However, increase in swelling power was noted with increased temperature from 30-90°C because high temperature could open starch structure which in turn increase WAC and WBC leading to the escalation of swelling power of various starch samples[47].

Table 5.2: Compositional characteristics determination of native, heat treated and enzymatically modified maize flour

Sample types	Parameters						
	RS (%)	**Non-RS (%)**	**Total Starch (%)**	**Amylose content (%)**	**Amylopectin content (%)**	**OAC (%)**	**MAC (%)**
Native Maize flour (NMF)	1.8 ± 0.05 c	69.79 ± 1.62 a	71.59 ± 1.25 a	19.88 ± 0.64 c	80.13 ± 1.65 a	90.91 ± 1.87 a	360 ± 2.74 c
Gelatinized Maize flour (HTM)	2.05 ± 0.08 b	68.86 ± 1.34 a	70.91 ± 1.38 a	24.75 ± 0.71 b	75.25 ± 1.59 b	84.97 ± 1.73 b	371 ± 2.98 b
Amylase and Pullulanase treated Maize flour (EM-RSIII)	24.3 ± 0.9 a	46.45 ± 1.04 b	70.48 ± 1.62 a	36.11 ± 0.93 a	63.89 ± 1.21 c	66.67 ± 1.49 c	550 ± 4.52 a

Table 5.3: Colour analyses of enzymatically prepared Resistant Starch (EM-RSIII) against Native Maize flour and heat-treated maize flour control

Starch samples	Color values		
	L*	**a***	**b***
NMF	83.35 ± 0.09 a	0.66 ± 0.11 c	12.29 ± 0.07 c
HTM	53.83 ± 0.08 b	5.4 ± 0.05 a	19.20 ±0 .12 a
EM-RSIII	48.63 ± 0.10 c	5.01 ± 0.04 b	15.76 ± 0.06 b

Table 5.4. SP, WAC and WBC of starch samples at different temperature

Starch samples	30°C	60°C	90°C
	Parameters		
	Water absorption capacity (%)		
NMF	180 ± 2.04 a	240 ± 2.64 a	387 ± 3.47 a
HTMF	171 ± 2.07 b	218 ± 2.53 b	343 ± 3.15 b
EM-RS III	87 ± 0.82 c	107 ± 1.29 c	153 ± 1.87 c
Water binding capacity (%)			
NMF	80 ± 0.92 a	140 ± 1.29 a	287 ± 3.22 a
HTMF	76.9 ± 0.86 b	109 ± 1.17 b	196 ± 2.67 b
EM-RS III	53 ± 0.51 c	67 ± 0.93 c	89 ± 0.91 c
Swelling power (%)			
NMF	268 ± 3.21 a	285 ±3.97 a	414 ± 4.97 a
HTMF	252 ±2.57 b	277 ± 3.21 b	389 ± 3.88 b
EM-RS III	144 ± 1.93 c	200 ± 2.75 c	230 ± 2.47 c

5.4.10 *In-vitro* Digestibility of Native and Enzymatically Treated Maize Flour in Different Compartments of Gastrointestinal Tract

Digestion of starch in different compartments of GI tract resulted in the production of maltose and other reducing sugars quantified by DNS method[48].Native maize underwent greater enzymatic digestion as indicated by greater amounts of reducing sugars released from both enzymatic hydrolysis (mouth, small intestine) and acidic conditions (stomach) due to less compact starch structure prone to enzymatic attack depicting lower resistivity and gastric stability of NMF. Whereas, enzymatically produced resistant starch sample resisted enzymatic as well as acid hydrolysis as shown by lower amounts of reducing sugars released. This is because the native maize flour has an A type porous structure with intact amylopectin and less RS and amylose levels favoring greater enzymatic digestion and hence greater maltose released[49]. Whereas, enzymatically produced RS samples has a compact B type structure with greater RS and amylose contents with compact conformation that is resistant to enzymatic and acid digestion and therefore, less maltose is released after enzymatic attack[50]. Thus, it can be inferred from the results attained by the digestion of enzymatically synthesized maize flour in the different GI tract compartments that

enzymatically prepared resistant starch (EM-RS III) significantly resists enzymatic hydrolysis compared to native maize flour.

5.4.10.1 Scanning Electron Microscopy of Starch Sample Retrieved after Digestion

SEM images of starch samples taken before and after 6hrofenzymatic digestion showed the morphological differences induced by *in vitro* enzymatic digestibility. Fig 5.5A designates untreated native maize flour before 6hr enzymatic digestion which appears as round, regular oval shaped without any degradation or structural deformity, whereas Fig 5.5B is native maize flour retrieved after *in vitro* digestibility, it underwent greater enzymatic digestion as shown by deformed, degraded appearance and loss of structural integrity because of susceptibility to *in vitro*digestion.

The SEM image of maize flour treated with combination of amylase and pullulanase EM-RS III which has the most compact, highly packed compact structure before digestion as shown in Fig 5.6A while Fig 5.6B represents the EM-RSIII obtained after 6hr enzymatic digestion retained the most crystalline compact structure with least retrogradation as compared to all other starch samples indicating its greater resistivity to *invitro* digestibility for 6hr with pancreatic-α-amylase.

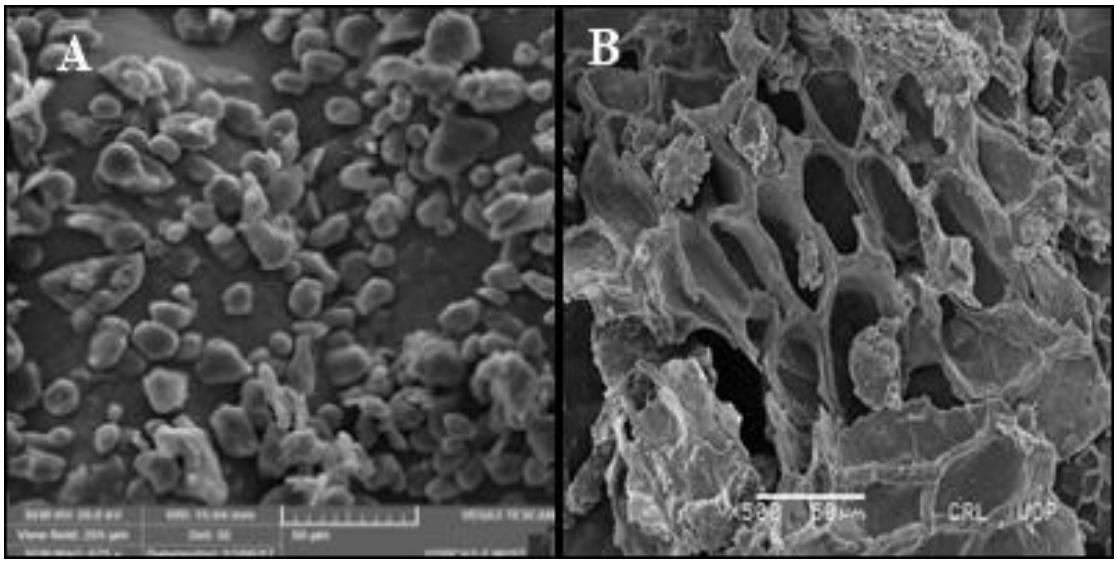


Figure 5.5: Scanning electron micrograph of Native Maize A) before digestion and B) after 6hr enzymatic digestion with pancreatic-α-amylase

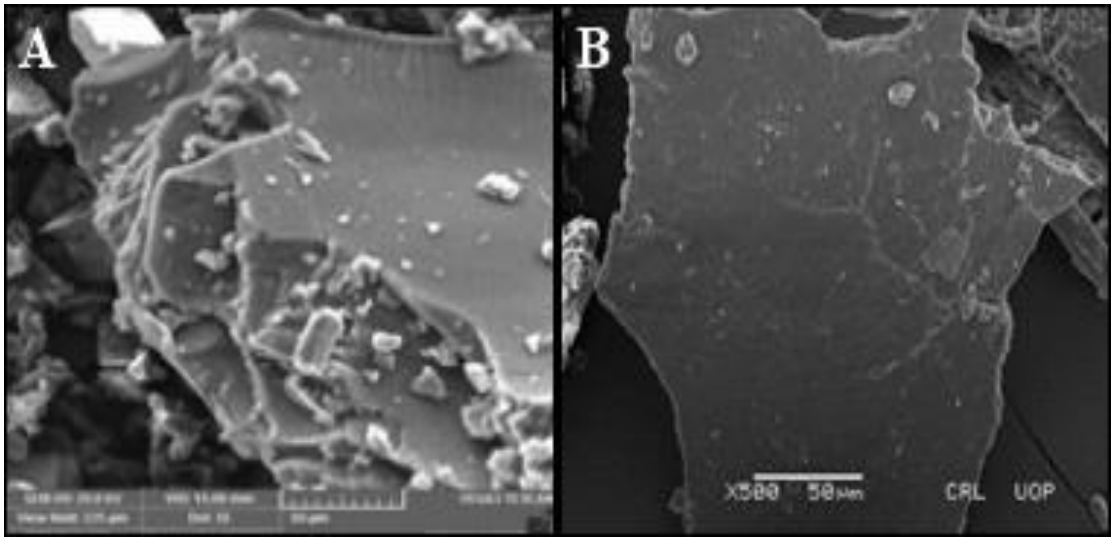


Figure 5.6: Scanning electron micrograph of EM-RS III; A) before digestion and B) after 6hr enzymatic digestion with pancreatic-α-amylase

5.4.11 Preparation and Characterization of EM-RS III Supplemented Cookies

Cookies formulation is mainly associated with water, milk and oil absorption capacities of starch[51]. Presence of hydrophobic proteins, differences in the ratio of amylose/amylopectin and the chain lengths of starch play an essential role in oil absorption and in the development of a product with desirable properties [52]. Compared to native, EM-RS III had a compact structure due to debranching and ordered conformation of granules which could have resulted in low porosity that restricted oil uptake. Milk absorption capacity is another desired property and depends on the amylose content of starch. Amylose and milk proteins interact to form a complex and hence greater the amylose content of starch greater is the ability of starch to absorb milk[53].

Low oil uptake and high milk absorption are the desired properties of RS III in bakery products but there is very little information available on the use of RS III prepared by enzymatic hydrolysis (EM-RS III) from maize flour in cookies making. Therefore, the inclusion of resistant starch (RS) ingredients in cookies formulas is gaining prominence, especially with the current positive health outcomes attributed to RS and the apparition of novel RS ingredients in the market[54].

Various cookies with different proportions of flour have been baked during the present study. The chroma (color intensity) of the cookies was dependent on the type of flour utilized. EM-RSIII based functional ingredient has improved the characteristics of cookies. Baked cookies are shown in Fig. 5.7.

Figure 5.7: Baked Cookies with different flour composition A) CBF (cookie baking flour), B) Native maize flour (NM) containing cookies C) EM-RSIII containing cookies, D) CBF+EM- RS III (1:1), E) NM+EM-RS III (1:1)

5.4.11.1 Sensory Evaluation of Resistant Starch Supplemented Cookies

The attributes of cookies based on questionnaire were assessed from the taste, aroma, texture, appearance and its overall acceptability. Fifteen panellists consisting of students and members of the laboratory were involved in the assessment. Each cookie sample was shown, and the observer's remarks were recorded to evaluate appearance, taste, texture, aroma and overall acceptability which are designated in the Table 5.All the cookies were considered acceptable but EM-RS III supplemented cookies either pure or in blends with maize flour was preferred due to its inimitable alluring aroma.

The dark brown colour of EM-RS III cookies may be due high amylose content and results in maillard reaction, which leads to the darker colour on baking. Native maize has sweet taste due to the presence of high polysaccharide content. Fewer cracks were also observed in EM-RS III cookies, which could be due to compact alignment of amylose granules to form a compact structure provides less crankiness.

Table 5.5: Sensory Evaluation of Resistant Starch supplemented cookies

Cookies	Parameters				
	Appearance/ colour	Aroma	Taste	Texture	Overall acceptability
Maize	4.01 ± 0.041 d	4.14 ± 0.04 d	4.35 ± 0.045 a	3.96 ± 0.039 e	4.37 ± 0.042 d
Maize+ EM-RS III	4.125 ± 0.044 a	4.31 ± 0.042 b	4.32 ± 0.042 b	4.15 ± 0.04 b	4.65 ± 0.044 b
EM-RS III	4.14 ± .041 b	4.53 ± 0.047 a	4.25 ± 0.043 d	4.25 ± 0.043 a	4.7 ± 0.048 a
CBF	3.92 ± 0.39 e	3.98 ± 0.038 e	4.17 ± 0.04 e	3.99 ± 0.038 d	4.21 ± 0.043 e
CBF+EM-RS III	4.09 ± 0.04 c	4.19 ± 0.043 c	4.29 ± 0.044 c	4.12 ± 0.041 c	4.54 ± 0.046 c

5.4.11.2 Resistant Starch Content of Cookies after Baking

To observe the effect of baking and processing conditions on the retention of resistant starch contents the RS content of baked cookies were determined. The EM-RS III content of the powder before cookies formation was compared to the content retained after baking in cookies formed and is represented in the Fig 8**.**Highest RS content was found in EM-RS III supplemented cookie (23.2%) ($p<0.05$) as compared to the least RS content (3.2%) of cookie containing baking flour only. Cookies formed of blends of EM-RS III with CBF or Native maize showed intermediate RS contents (Fig.5.8). During the processing of food items, it must be noted that not all ingredients containing RS preserve the RS content during baking. Any loss of RS content during processing has negative effect on product properties and consumers health[55]but EMRS-III supplemented cookies retained RS content after baking assumed as a best option to be used as functional ingredient in many bakery products.

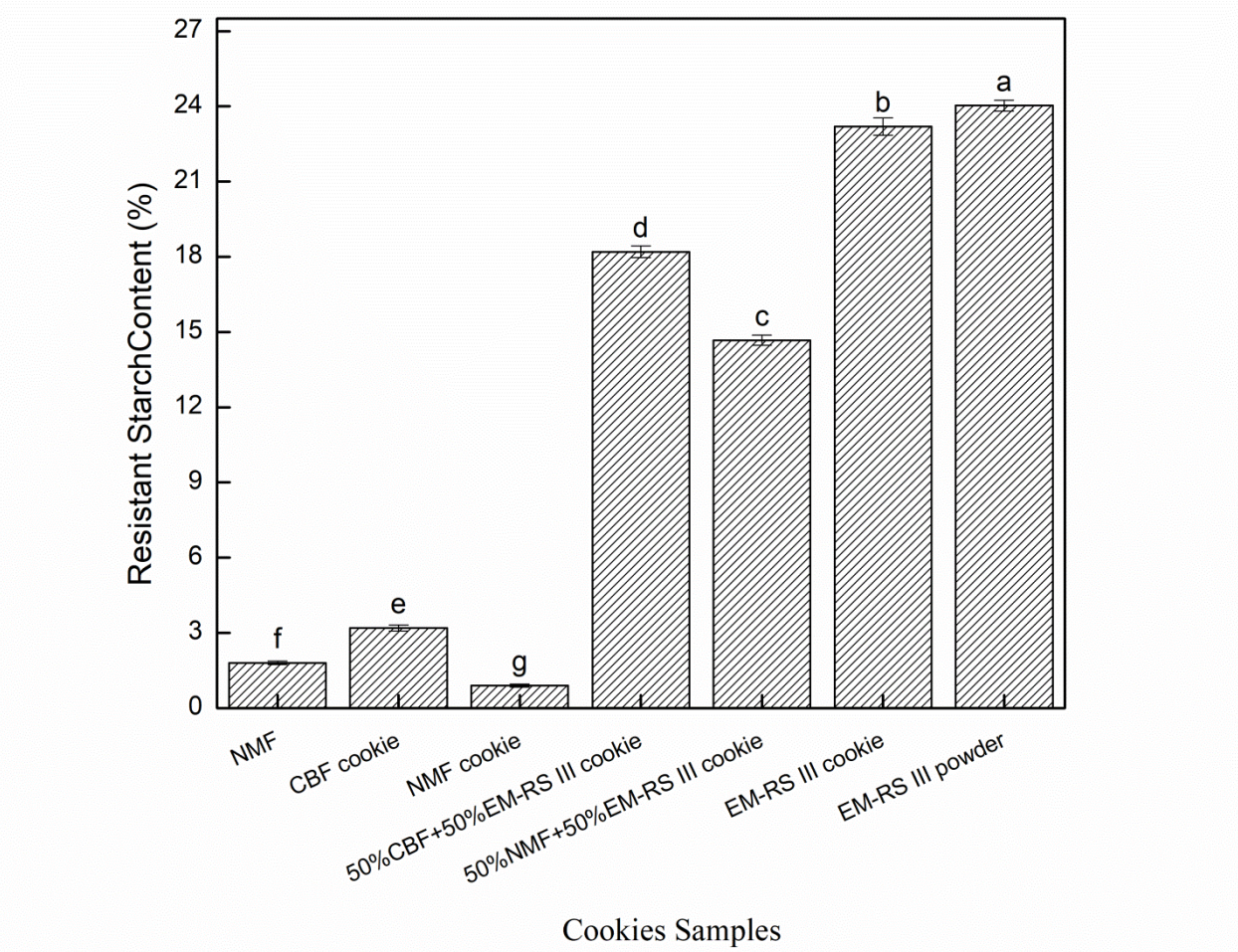


Figure 5.8: Description of resistant starch content (%) of cookies after baking

5.4.11.3 Colour Analysis before and after Baking of Cookies

Colour values of baked cookies differing in composition are represented in Table 5.6**.** EM-RS III containing cookie showed greater variation in colour values in comparison to the values of cookies made from pure as well as combination of cookie baking flour (CBF), and combination of untreated native maize (NMF) and EM-RS III. A considerable reduction in the L* values (lightness) of EM-RS III supplemented cookie was observed as compared to values of CBF and cookie from native maize flour which were prepared with the blends of EM-RS III with native maize or CBF showed intermediate values. Similarly, a* (redness), b* (yellowness) values of treated starch samples also showed minor increased with treatment. The variations in L* value showed similarities with the results of annealed yam starches[56] (Falade and Ayetigbo, 2015). Gelatinization alone and with enzymatic treatment lead to brown color of the starch samples, the changes in color can be due to maillard reaction.

Table 5.6: Colour analysis of Resistant Starch supplemented cookies

Cookie samples	Colour values		
	L*	a*	b*
CBF Cookie	64.6 ± 0.62 a	7.43 ± 0.08 e	35.45 ± 0.031 a
Native Maize Cookie	62.78 ± 0.67 b	7.66 ± 0.07 d	34.35 ± 0.032 c
EM-RS III Cookie	43.7 ± 0.49 e	13.41 ± 0.16 a	32.45 ± 0.038 d
50% CBF+50% EM-RS III Cookie	55.98 ± 0.51 d	10.47 ± 0.11 b	34.67 ± 0.032 b
50% N+50% EM-RS III Cookie	59.3 ± 0.54 c	8.17 ± 0.09 c	32.25 ± 0.034 e

5.4.12 CONCLUSION

Starch hydrolyzing enzymes play essential roles in the preparation of resistant starch, the most important are α-amylase and pullulanase, as revealed by the current study. The desired properties, such as low water binding ability, swelling power, low oil and water absorption capacity and increased in stability and milk absorption capacity of enzymatically prepared maize flour, provide better handling in processing leading to crispiness and crunchiness of the products (cookies).Resistant Starch supplemented cookies had better sensory properties and retained the resistant starch content even after baking and processing so could be successfully used in food industry as a new product.

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Chapter 6: *In-vivo* prebiotic potential of Resistant Starch

Paper 4:

Title: Physiological benefits and prebiotic potential of enzymatically prepared resistant starch type III on rats

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6.1 Abstract

The upsurge in consumer exigency for piercing quality food stuffs has led to an increase in the utilization of new ingredients and technologies. Resistant starch (RS) is recently recognized source of fibre and has potential functional properties and health advantages**.** However, knowledge about the fate of RS in modulating complex intestinal communities, the microbial members involved in its degradation, enhancement of microbial metabolites and its functional role on body physiology is still limited.

For this purpose, current study was designed to ratify physiological and functional health benefits of enzymatically formulated resistant starch (EM-RSIII) by using maize flour. To, approve the beneficial health effects as prebiotic; EM-RS III was supplemented in rats' diet. After 21 days of experiment EM-RSIII fed rats showed significant reduction in body weight gain, fecal pH, glycemic response, serum lipid profile, insulin level and modulation in gut microbiota and their metabolites compared to control were analyzed. The numbers of starch using and butyrate fabricating bacteria, such as *enterococcus, lactobacillus,* and *pediococcus genus*, in rat's gut upsurge after the processing of medium and high doses of EM-RSIII, whereas those of *E.coli* completely suppressed. The microbial metabolites such as short chain fatty acids precisely increased in feces of EM-RS III feed rats. Correlation analysis demonstrated the effect of butyrate on functional and physiological alteration on the body. This study demonstrated the tremendous metabolic health promoting benefits for body weight management along with modulation of gut microorganisms by utilizing EM-RS III.

Keywords: Resistant starch Gut microbiota, Obesity, Insulin sensitivity, Colonal health, SCFA

6.2 Introduction:

Fibre utilisation has been significantly lessened in western [1]. The main is modification in in life style, which reduce the fruit, legume, and vegetables consumption[2]. One example of such fiber rich food is resistant starch which has potential to be ruled as preventing agent for health-related problems. Resistant starch (RS) has pronounced prebiotic potential for promoting probiotic proliferation and having unique physicochemical characteristics to control satiety [3]. Resistant starch (RSIII) has ability to resist digestion in the small intestine, which then reached to the large intestine where fermentation is performed by bacteria, which results in the release of SCFAs, reduced fecal pH and increased fecal volume. RS III is also associated with other additional imperative physiological health benefits which include reducing blood glucose and insulin level, plasma lipid and cholesterol level and has positive effect on colonocytes proliferation[4]. Functional features of RS III involve the capability to: resistance to hydrolysis by pancreatic α-amylases, resistance to the absorption in the upper gastrointestinal tract, resistance to stomach low pH, the capability to be fermented by intestinal microbiome and provoke the activity and/or growth of intestinal bacteria related to the host health [5].

Diet rich in rapidly digestible starch results in elevation of insulinemic and glycemic responses, associated to poor satiety and enhanced energy intake [6, 7]. Therefore, changes in the quality and type of carbohydrate consumed have great impact on human health. Therefore, incorporation of RSIII in diet preparations is an excellent approach to diminish the food glycemic index [8]. Moreover, a negative correlation has been found between high density lipoprotein cholesterol and dietary glycemic index. Therefore, low glycemic index of food having a significant consideration, intended to upgrade the metabolic control of hyperlipidemia, specifically in the diet formulation for the diabetics [9]. To overcome and prevent the side effects of food with high glycemic responses, the prebiotic rich food could be the best strategy [10]. Most essential microbial metabolites such as SCFAs are mainly fabricated through microbial fermentation from dietary products in the gut. SCFAs includes butyrate, propionate, and acetate which are generated by specified genera of microorganisms. Major contribution of SCFAs is to control host's appetite and metabolism for body weight management. Activation of gut hormone receptors stimulate propionate production (FFAR2, Free fatty acid receptor 2; FFAR3, Free fatty acid receptor 3), which in turn reduces appetite. Propionate along with butyrate regulate appetite-

regulation hormone through expression of neuropeptide Y(NPY) in hypothalamus through stimulation of peptide YY (PYY) and *glucagon-like peptide 1 (*GLP-1) hormones in the colon. Butyrate helped to regulate metabolism energy through enhancing lipolysis and the repression of leptin as appetite suppressing hormone from adipose tissues. Therefore, SCFAs have pivotal mediating and regulating role leading to beneficial effect of EM-RSIII. The traditional prebiotics including fructo-oligosaccharides, inulin and glactooligosacchharide still provide the evidence of beneficial health effects [11] due to their fermentation and assessable literature. However, many other categories of food ingredients may equally or even more effective than the traditional prebiotics need to be explored for its physiological and functional benefits.

Therefore, current study was undertaken to introduce new food ingredient as a potential prebiotic prepared through enzymatic hydrolysis [12] as key player for the first time to explore the interlink between prebiotic intake, establishing the impacts of EM-RSIII on gut microbiota re-structure and SCFAs manufacturing and its effect on blood profile and body physiology. The purpose of latest work was to report the effect of different percentage of newly enzymatically prepared resistant starch (EM-RSIII) present in the standard control diet on the rat's body weight, fasting and postprandial blood glucose level, fecal pH, its effects on serum lipid profile, serum insulin level, modulation in gut microbiota, their metabolites and cecum health of control and EM-RS III fed rats to declare it as safe prebiotic. Furthermore, the correlation analysis of these effects was also achieved to explore the facilitating protagonists of gut microbiome and SCFAs in EM-RS III fed rats.

6.3 Materials and Methods:

6.3.1 Rats and feeding regiments

Eight female rats of two months age and standard diet were provided by Department of Zoology, in the facility of Quaid-i-Azam University Islamabad, Pakistan. Rats were randomly divided into four groups in separate cages and were placed in an air-conditioned room provided with food and water for 21 days. The rats were maintained in specific pathogen free environment. Rats were sacrificed after anesthesia and all endeavours were attained to reduce their suffering. According to the National Institute of Health guidance for use and care of laboratory animals and the

treatment and care protocol were approved by the Institutional Animal care and use committee at the Quaid-i-Azam University Islamabad.

6.3.2 Experimental design for EM-RSIII feeding

The rats were randomly divided into four groups: control group did not receive EM-RS III in standard diet, and the other groups were given low-EM-RS III (2 g/kg body weight), medium-EM-RS III (4 g/kg body weight) and high-EM-RS III (8 g/kg body weight), mixed in the standard control diet and each group retained in wire cages in a atmosphere controlled environment with free approach to water and feed (Table 6.1). The temperature was sustained at 21°C ± 1°C with swapping 12 hours periods of darkness and light.

Table 6.1: Composition of experimental diet (g/100g diet)

Ingredient	Control	Low-EM-RS III	Medium-EM-RS III	High-EM-RS III
EM-RS III	0	2	4	8
Corn	50	48	46	42
Casein	10	10	10	10
Soybean oil	10	10	10	10
Cellulose	10	10	10	10
Vitamin mixture	5	5	5	5
Mineral mixture	15	15	15	15

6.3.3 Analytical Methods for measuring the effect of EM-RS III fed on rats Physiology:

6.3.3.1 Body weight Measurement

Each rat body weight was calculated every third day of the week by using weighing balance.

6.3.3.2 Measurement of fecal pH of rats

Fecal pH of rats from each group was recorded by preparing a suspension of one gram freshly collected feces in 10 mL of distilled water by using digital pH meter.

6.3.3.3 Measurement of blood glucose level of rats

Blood samples from the tail vein of rats were taken on the start of experiment at fasting, 40 min and 120 mins after consuming the feed to calculate serum glucose level and then on last three days of experiment at fasting, 40 min and 120 min after consuming the feed using glucometer.

6.3.3.4 Measurement of cecum length and weight of rats

Cecum from all groups was excised after dissection, their length, and weight were measured. Cecum content was gathered in separate tube and kept at -20 °C for further analysis.

6.3.3.5 Measurement of serum lipid profile of rats

After rat's dissection by cardiac puncture, 2 mL of blood from heart of each group of rats were collected in separate gel tubes by syringe and immediately centrifuged for 5 min at 800 rpm. Serum was collected in separate serum cups which were further processed for lipid profile in microlab 300.

6.3.3.6 Measurement of Cholesterol and triglycerides level of rats

Serum cholesterol and triglycerides were determined using10 µL serum sample with 1 mL cholesterol reagent and 1 mL triglycerides reagent from kit (cholesterol and triglycerides liquiColor kit) separately and incubated for 5 min at 20-25°C and absorbance values were spectrophotometrically recorded at 546 nm. HDL and LDL levels were determined according to the following formulas:

HDL determination:

HDL= Cholesterol/5

LDL determination:

LDL= Triglyceride/5+HDL-Cholesterol

6.3.3.7 Measurement of serum insulin level of rats

After rat's dissection, blood samples were collected from rats of each group separately in gel tubes as in case of serum lipid profile and immediately centrifugation was performed at 800 rpm for 5 mins. Serum was gathered in serum cups and analyzed for insulin level using ARCHITECT C400.

6.3.3.8 Histopathology of rat's colon

After 21 days of experiment, rats were anesthetized and killed by cardiac puncture. Colon of each group rats were removed, cleaned with normal saline and stored in 70% formalin. Segments were fixed in 10 % formalin for 48 hours, dehydrated with ethanol and embedded in paraplast medium. Cross sections with thickness of 7 µm

were stained with hematoxylin and eosin (Thermo, Shandon finesse 325, UK). Histological sections were examined under microscope at 40X resolution.

6.3.3.9 Microbial analysis from rat's cecum and fecal samples

Microbiological analysis was performed from luminal contents; samples were weighed, homogenized, and serially diluted in sterile peptone water. Serial 10-fold dilutions of homogenates were plated on specific media De Man, Rogosa and Sharpe *agar* (MRSA) and Rogosa agar, modified Columbia and Modified Yeast extract, Casitone and Fatty acid **(**YCFA) media used for the enumeration of probiotic bacteria. The samples were also checked for *E.coli* by spreading on eosin methylene blue agar (EMB). All the media plates were anaerobically incubated in sealed jar for 48 hours at 37°C. After incubation, the colonies on different selective media was recorded and catalogued, and chosen colonies were purified for identification. The final colonies counts were stated as colony forming units (CFU) from each sample. Probiotic strains were isolated and identified based on colony morphology, biochemical analysis, and by 16S rRNA sequencing.

6.3.3.10 Quantification of SCFAs levels in fecal samples of experimental rat's

The investigation of SCFAs in fecal samples were performed by gas chromatography fortified with a DB-FFAP capillary column and flame ionization detector. The standard of SCFAs (isobutyrate, I103524; isovalerate, I108280; acetate, A116173; butyrate, B11se0438; propionate, P110445;valerate, V108269;) purchased from Aladdin Bio-Chem Technology Co., LTD (Shanghai, China) were used for Quantification. For SCFAs mensuration, 200 mg fecal sample was mix with 1 mL of distilled H_2O; then, 0.15mL of dichloromethane (w/w) and 1.6 ml of ethyl acetate was appended; then samples were wobbled at 4℃ for 10 min and centrifuged at 10,000 rpm for 10 min; The organic phase was amassed, filtered and analyzed by gas chromatograph by the following the process as: carrier gas was hydrogen and used at 45 ml/min, injection of samples was made in split mode with an injection volume of 1µL, the injection was washed properly with methanol after injecting each sample. The oven initial temperature was 120°C and end temperature was 200°C, rate of change was 10 °C/min, total run time was 10 min, back inlet was set at 200°C, 25.5 psi pressure and 24.4mL/min flow rate. GC was equipped with Flame ionization detector with ´ON´ thermal zone, temperature 300 °C, makeup flow 15mL/min, H_2 flow 45ml/min and 149.9 ml/min air flow.

6.3.3.11 Statistical analysis:

The experiment was performed using a completely random design. The animals were taken as the experimental units. ANOVA was performed along with Duncan test for significance of treatments. Spearman correlation analysis in R software was used to evaluate the correlations among the butyrate and other biochemical indicators, comprising body weight, blood glucose, serum insulin, triglycerides and cholesterol level, and relative richness of intestinal microorganism. Data are depicted in the text as mean±SE. Different lowercase letters specify the significant differences among various groups considered significant at $p< 0{\cdot}05$.

6.4 Results

This work is continuation of our previous research work where resistant starch type III was prepared enzymatically and named as EM-RS III[12].The diet was prepared by supplementing the EM-RS III to standard control diet in specific ratio to prepare diet with no (control), Low, medium and high EM-RSIII in order to exploit the prebiotic nature of EM-RSIII. This diet was fed to rats for 21 days. The effects of EM-RS III diet on rat's physiology on body weight, blood glucose level, fecal pH, serum insulin, cholesterol level and colon microbiota of rats were observed during the current study.

6.4.1 Body weight of rats

The administration of EM-RS III supplemented diet and control diet to rats did not demonstrate any sign of presence of diarrhea or toxicity. However, all EM-RS III fed rats displayed a decreased weight gain compare to control group, as obvious after 2 weeks of receiving EM-RS III diet. This effect was linked to minimum food consumption as seen in all low, medium and high EM-RS III fed rats in comparison with the control group. Rats were generally in good health throughout the experiment. The average body weight of all the rats was 170 g initially and after 21 days of experiment average weight gain of high EM-RS III fed rats (208 g) was significantly lower than that of control (250 g) as shown (Table 1).The same trend was followed in case of low and medium EM-RSIII rats compared to control. The average weight gains of rats were in the order of control > low-fed RS III >medium-fed RS III > high-fed RS III as demonstrated in Table 6.2.

Table 6.2: Average body weight gain of rats at start and end of experiment.

Groups	Body weight(g) initial	Body weight(g) final
Control	170±2	250±2
Low RS III	170±1	243±4
Medium RS III	170±3	230±3
High RS III	170±1	208±4

6.4.2 Blood glucose level of rats

The blood glucose level examined throughout the experiment indicated the diet specific response towards starch molecules supplemented in food formulations. The blood glucose level of high EM-RS III fed rats was significantly decreased, when measured at the end of the experiment as compared to control (Table 6.3). The average blood glucose level values can be articulated as control > low EM-RS III fed rats > medium EM-RS III fed rats > high EM-RS III fed rats. At start of experiment blood glucose level for all the groups were analyzed at fasting, 40 min and 120 min after consuming EM-RSIII containing feed and blood glucose level was observed for all rats i.e., on fasting have 117, 113, 106 and 104mg/dL for control, low, medium and high EM-RS III fed rats respectively and after 40min of consuming feed the blood glucose level become 125, 121,113 and 111mg/dL respectively and then after 120 minutes the blood glucose level become 132,125,118 and 116mg/dL for control, low, medium and high EM-RS III respectively indicated that at the start of experiment the blood glucose level were high even from the normal blood glucose level i.e. (70-110 mg/dL) of all experimental rats [13].But after EM-RSIII feed the increase in blood glucose level of rats seems to be inversely proportional to the EM-RSIII concentration in rat's diet with alternate time period as shown in table 6.3. Results indicate that there was decrease in the blood glucose level of EM-RS III fed rats specially the rats with high amount of EM-RS III feed attributed the diet specific response of blood glucose due to difference in type of starch molecules, arrangement and specific structure.

Table 6.3: Average blood glucose level mg/dL of rats at different time interval i.e. fasting, 40 min and 120 min at the start and end of experiment.

Groups	Time	Initial Blood glucose level mg/dL	Final Blood glucose level mg/dL
Control	0 mint	117 ±0.19	116±0.05
	40 mint	125±0.07	125±0.13
	120 mint	132±0.04	134±0.14
Low RS	0 mint	113±0.02	113±0.09
	40 mint	121±0.10	118±0.69
	120 mint	125±0.0.5	123±0.18
Medium RS	0 mint	106±0.06	97±0.019
	40 mint	113±0.08	110±0.03
	120 mint	118±0.12	116±0.05
High RS	0 mint	104±0.11	91±0.06
	40 mint	111±0.09	104±0.11
	120 mint	116±0.13	108±0.07

6.4.3 Fecal pH of rats

Administration of EM-RS III containing diet affected the pH values of intestinal contents of rats, significantly. Reduction in pH of caecum and colon contents was observed as compared to control fed rats. The average fecal pH at first three days of the experiment was 8.0 but after 21 days, the pH of high EM-RS III fed rats was significantly dropped to 6.5 as compare to initial pH of experimental days as seen in table 6.4. Similar fashion of decreased pH in fecal samples related to experimental diet was observed in low and medium EM-RSIII fed rats samples while in case of control pH remains the same (pH 8) from the start till the final days of experiment. The pyramid of pH values can be expressed as control > low RS III fed >medium RS III fed > high RS III fed rats.

Table 6.4: Average fecal pH at start and end of the experiment

Groups	Fecal pH(initial)	Fecal pH (final)
Control	8±0.1	8.075±0.015
Low RS	8±0.2	7.58±0.015
Medium RS	8±0.3	6.8±0.1
High RS	8±0.4	6.5±0.15

6.4.4 Cecum length and weight of rats

The average length and weight of cecum was measured after rat's dissection. Significant increase in weight and length of EM-RS III fed rats were observed with the high EM-RSIII fed group showing the highest cecum length (6.35cm) and weight (1.42g) as compared to cecum length (5.8cm) and cecum weight (1.25g) of control group rats. Low and medium EM-RS III fed rats cecum sample also showed the healthy, lengthier and weighed cecum compared to control fed rats as shown in table 4. The average cecum length and weight was in the order of control < low RS III fed rats < medium RS III fed rats < high RS III fed rats.

6.4.5 Serum lipid profile of rats

After dissection, rats blood samples were analyzed and results indicated a considerable decrease in serum triglyceride, cholesterol and LDL values in the low and medium EM-RSIII fed groups with significant decrease observed in high EM-RSIII fed rats (33 mg/dL) as compared to control (45 mg/dL). On contrary, an increase in serum HDL values were recorded in EM-RS III fed rats as compared to control (16 mg/dL) with significantly increased HDL values in high EM-RS III fed rats (22 mg/dL)(Table 6.3). The average cholesterol, triglyceride and LDL values changed positively in the following order: control > low EM-RS III fed rats > medium EM-RS III fed rats > high EM-RS III fed rats respectively while the average HDL values were control < low EM-RS III fed rats < medium EM-RS III fed rats < high EM-RS III fed rats.

6.4.6 Serum insulin level of rats

Serum insulin level was determined in blood samples, which revealed significant decrease in the serum insulin levels of rats after supplementation with maximum decrease observed for high EM-RS III fed rats as compared to control. The average insulin level of control was 0.8 while that of high EM-RS III fed rats was 0.344 mU/mL. The decreasing trend was also observed in case of low and medium EM-RSIII fed rats samples compared to control rats samples demonstrating the diet specific response of insulin sensitivity. The drift of average insulin level was in the order of; control > low RS III fed rats' > medium RS III fed rats' > high RS III fed rats as demonstrated (Table 6.5).

Table 6.5. Average cecum length and weight after dissecting the rats and average serum cholesterol, triglyceride, LDL, HDL and insulin level from rat's blood samples.

Parameters studied	Control	Low RS	Medium RS	High RS
Cecum length(cm)	5.8±0.1	6±0.1	6.305±0.005	6.35±0.15
Cecum weight(g)	1.25±0.05	1.235±0.005	1.39±0.01	1.42±0.01
Serum cholesterol level (mg/dL)	87±1	84±1	80±1	77±1
Serum Triglycerides level(mg/dL)	288±2	154±2	119±1	107±1
Serum LDL level(mg/dL)	45±1	43±2	39±1	33±1
Serum HDL level(mg/dL)	16±2	16.5±0.5	17±1	22±2
Serum insulin level(mU/mL)	0.8±0.1	0.4±0.1	0.36±0.14	0.345±0.145

6.4.7 Colon tissue histopathology of rats

The colonic tissue of control group showed ulcer symptoms and mild ulcer was also seen in the colon tissue of low and medium EM-RS III fed rats. Differences in mucosal linings were also observed in rats' groups, thin mucosal lining was observed in control in contrast to the healthy thick and intact mucosa of high EM-RS III fed rats with prominent lymphocytic aggregates as shown in figure 6.1. The damage order of the tissue was control> low EM-RS III fed rats > medium EM-RS III fed rats > high EM-RS III fed rats.

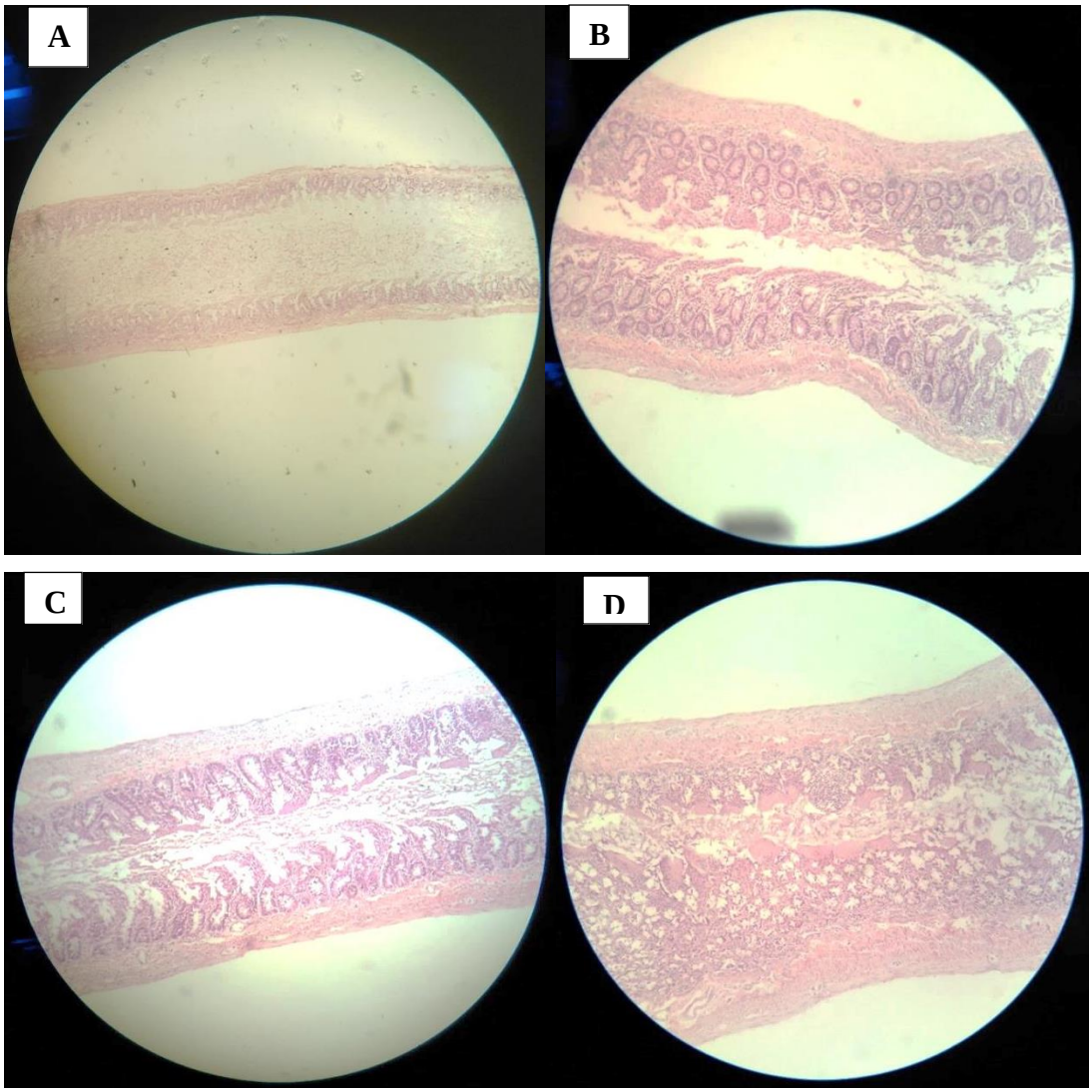

Figure 6.1: Colon histology of cecal rat's samples **A**: Colon histology of control group **B**: Colon histology of low EM-RS III fed group **C**: Colon histology of medium EM-RS III group **D:** Colon histology of high EM-RS III group

6.4.8 Microbial analysis of fecal and cecum content of rats

The microbiological analysis of fecal and cecum rat's samples revealed that administering EM-RS III diet leads to modification of intestinal and fecal microbiota of rats as compared to control group. The analysis of fecal and cecum contents showed the high count of *lactobacilli* in high EM-RS III fed rat samples as compared to low EM-RS III, medium EM-RSIII and control fed rat samples. Morphological characteristics of colonies were observed on all the specific media containing agar plates. Morphologically different types of colonies appeared on all specific media containing agar plates including white creamy smooth, opaque or transparent and some are small and large colonies also with smooth or irregular margins (Appendix S1-S4). Biochemical analysis revealed that all the strains are Gram positive,

anaerobes, oxidase and catalase negative. Twenty-four strains were sequenced from rats fecal and cecum samples of control and EM-RS III fed rats. Most of them were *lactobacillus, enterococcus* and *pediococcus* genus. Different *lactobacilli* strains found were *Lactobacillus reuteri, Lactobacillus planterum, Lactobacillus gasseri, Lactobacillus johnsoni, Lactobacillus curvatus. Enterococcus faecium* and *pediococcus pentosaceus* were also identified. Most of the *lactobacillus* species were identified from high RS III fed rats' samples while in control samples only *enterococcus faecium* and *lactobacillus curvatus* were detected. The rat's samples were also checked for *E.coli* by spreading on eosin methylene blue agar (EMB), the CFU/g of *E.coli* was higher in control as compared to low, medium and high EM-RS III fed rat's samples. *E.coli* was identified by their metallic sheen color on EMB media. The CFU/g calculated was in order of control> low EM-RS III fed rat sample > medium EM-RS III fed rat sample > high EM-RS III fed rat sample pattern (table 6.6).

Table 6.6: Use of different culture media for quantitative isolation of microbes identified based on 16 sRNA.

Culture media	**Main organisms counted**
Anaerobic culture	
MRS agar	*Lactobacillus reuteri, Lactobacillus gasseri, Lactobacillus johnsoni, pediococcus pentosaceus, pediococcus sacidilactici, enterococcus faecium, enterococcus durans,*
Rogosa agar	*Lactobacillus plantarum, Lactobacillus curvatus and lactobacillus reuteri*
Modified Columbia agar	*Lactobacillus fermentum, Pediococcus acidilactici*
Modified YCFA	*Enterococcus faecium, pediococcus spentosaceus*
Aerobic culture	
Eosin methylene blue agar	*E.coli*

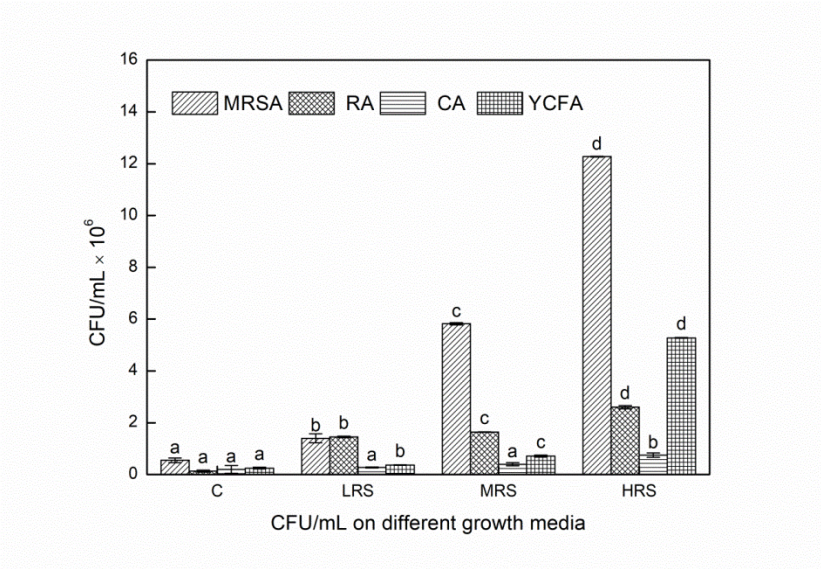

Figure 6.2: CFU/mL counted on the probiotic media used. (MRSA; De Man, Rogosa and Sharpe *agar*, RA ; Rogosa agar, CA; modified Columbia Agar and YCFA media used for the enumeration of probiotic bacteria.)

6.4.9 RS feed improved the generation of SCFAs

SCFAs were analyzed in fecal samples by using gas chromatography (Appendix S5-S8). Compared to the control group, RS feed significantly enhanced the butyrate levels in feces of rats. RS feed also significantly improved propionate and acetate in feces of experimental group as compared to control group rats. Moreover, it was also noted that the profusion of butyrate producing microorganisms including *lactobacillus*, *enterococcus* and *pediococcus genus*, were as significantly improved in high RS feed rats. These verdicts showed that RS feed improved butyrate production in EM-RS III fed experimental rats.

6.4.10 Correlation analysis for the serum/fecal butyrate levels and other biochemical catalogue

The Spearman's correlation analysis was executed to elucidate the correlation among the biochemical index, microbiota, and microbial metabolites (Fig 6.3). For example, the fecal butyrate levels, augmented by high RS feed, showed significantly negative correlation with body weight increase (r=-0.84), blood glucose level (r=-0.78) serum insulin (r=-0.62), fecal pH(r=,-0.82) and positively correlated with cecum length (r=0.77) and cecum weight (r=0.7).

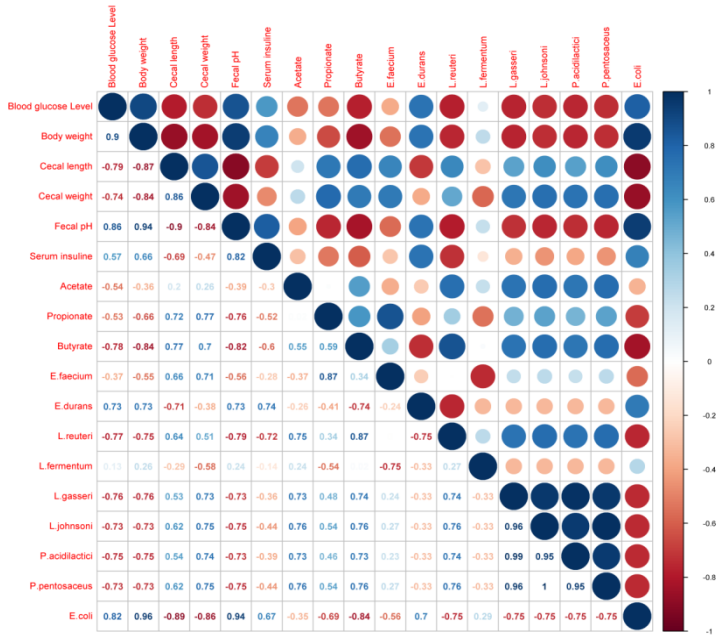

Figure 6.3 Correlation analyses between the biochemical indexes and serum/fecal butyrate levels. In the upper right side of the matrix, the color and size of the circles explain the level of the correlation index, and the relevant numeric value for correlation index are available on the right side.

6.5 Discussion:

Dietary fiber is not a "nutrient", it is on the other hand a key player of our diet to modulate human health. Dietary fiber is in direct relation with "diabesity" which show the interdependent association between diabetes and obesity. Obesity is observed in about 80% of the diabetic patients. [14]. RS III has enthused the researchers because of its health benefits[12]. RS III does not digest in small intestine, it is subjected to fermentation in the large intestine. Current study aims to summarize the present information relevant to the dietary RS III on prevention of obesity and related outcomes. This study provides valuable findings relevant to directly assessing enzymatically reformed resistant starch (EM-RSIII) by using maize flour as prebiotic on manipulation of gut microbiota and on metabolic and physiological behavior of rats. The data revealed that RSIII induced substrate specific shifts in the microbial community of fecal and colon samples that were tightly associated with the consumption of food between experimental and control group. The low weight gain in the resistant starch fed rats particularly in the high RS III fed rats were observed as compared to control, medium and low EM-RS III fed rats. There were many

attributes of resistant starch as compared to normal starch which could promote body weight loss/weight maintenance includes reduce postprandial insulinemia and increase level of gut satiety peptides, lower fat storage in adipocytes, increase fat oxidation and preservation of lean body mass and most importantly period of satiety[15]. Fermentable carbohydrate such as RS III as diet supplementation has been shown to enhance the discharge of gut hormones having roles in appetite regulation [16, 17]and ultimately controlling body weight gain in high EM-RS III-fed rats.

As demonstrated in current study that lower blood glucose level and serum insulin level accredited to the RS III fed especially observed in rats fed with high EM-RS III diet as compared to control group. This decrease in glucose and insulin level is mainly attributed to the release of glucagon-like peptide-1 (GLP-1) in the gut. GLP-1 excreted by intestinal endocrine cells is mainly responsible for maintaining glucose homeostasis by improving insulin secretion, lowering plasma glucose concentration, and maintaining beta cell functions of pancreas. The secretion of GLP-1 is associated with stimulation by short chain fatty acid specially butyrate and acetate which are the main product of resistant starch digestion by colonic bacteria is majorly the main reason for reduced glucose and insulin level in high EM-RS III fed rats in the current study[18, 19].

The outcome of reducing of glycemic response by RS III is in similar to the preceding studies[20]. A study conducted by Aziz *et al* concluded that a low glycemic retort was provoked in obese rats by consuming elevated amylose-resistant corn starch. Whereas, in current study during correlation analysis the negative correlation were observed in case of blood glucose (r=-0.78) and serum insulin level (r=-0.62)with that of butyrate representing role of SCFA in maintaining blood glucose and serum insulin level. Compared with control, low, medium and high EM-RS III fed rats reduced the serum triglyceride and cholesterol level after the experimental rat's blood samples analysis. Similar observations have been reported by the trials on feeding RS containing food like Tebou bean starch (TS) and Adzuki bean starch (AS) to rats showed that the high RS diet have more effect on lowering serum cholesterol due to increase the levels of hepatic SR-B1 (scavenger receptor class B1) and cholesterol 7alpha hydroxylase mRNA[21]. SR-B1 is a cell surface HDL receptor that mediates HDL-cholesteryl ester uptake in liver where it provides cholesterol for bile acid synthesis. Cholesterol 7 alpha- hydroxylase is important for oxidation and regulation of cholesterol level in serum. Another study conducted on hamsters fed with cassava

starch extruded with 9.9% oat fiber and cassava starch extruded with 9.7% RS, results showed that food containing RS lowers serum cholesterol level and used as preventing agent for cardiovascular disease[22].

To get better understanding the effect of EM-RSIII on rat's gastrointestinal health, histopathological examination using colon tissue samples were conducted. The description of mucosal pattern and histological observations exhibited low level insinuation of the colon in control, low and medium EM-RS III fed rat as compared to high EM-RS III fed rat's samples. The reason for the contradictions with the very low or medium EM-RS III fed is that the active ingredients concentration may be not enough to overcome the oxidative stress in the colon. While in case of high EM-RS III fed rat's samples the colon showed thick mucosal lining with no sign of infiltration or inflammation related to its unique antioxidant activity compared to native maize flour[12]. The crypts had a nearly normal architecture and goblet cell component, accompanied by mucin replenishment of high EM-RS III fed rats, compared with control. In addition to this the cecum wall length and weight was also examined in control and EM-RS III fed rats and results depicted the increase in cecum length and cecum wall weight specially in the rats fed with medium and high EM-RS III diet. The possible explanation for this is production of SCFAs as a result of RS III fermentation by colonic microorganisms, which provides energy to the colonocytes and the cells grow and renew, which affect the cecum length and weight. A study conducted on rats, RS III diet with 25% raw potatoes raised the cecum size and cecum pool of SCFAs, and also elevated the absorption of SCFAs and lowers the plasma triglyceride and cholesterol level[23]. Along with metabolic or physiological effects the diet specific microbial shift in fecal and intestinal content was examined during the study. For example, we did not examine the impact of these prebiotics in any of these disease phenotype. The increase in the *Lactobacilli* in the intestinal and fecal samples of high fed EM-RS III rats was observed which attributed to the stabilization and stimulation of the beneficial microflora in the colon. *Lactobacillus* are accounted as beneficial bacteria and recommended as probiotic. They may enhance endurance to disease by decreasing the growth of putrefactive and pathogenic bacteria by lessening pH, yielding inhibitor molecules, exhilarating the whole immune system, and contesting directly for mucosal attachment locations and substrate. Our findings also display that prebiotic treatment increased *Lactobacilli*, *Prevotella*, in rats fecal microbiome, further suggesting that utilization of EM-RSIII may increase the butyrate

producers abundance. Overall, enhanced SCFAs levels and *Bacteroidetes* , reduced *Firmicutes*, and increased microbial diversity evince that these probiotic *Enterococcus* and *Lactobacillus* strains may be useful in bettering gut microbiota dysbiosis in numerous human diseases such as IBDs, cancer, type 2 diabetes, and obesity. Several studies have shown the beneficial physiological function of *lactobacilli* in the animal colon [24]. These verdicts confirm that little change in starch can bring modification in intestinal microbiota. In spite of the evidence the administration of RS III that the gut microflora composition, it is hard to distinguish the specific microorganisms accountable for these fluctuations. It appears that numerous RS III vitiating bacteria are in the gut performing their metabolic activities. These organisms cause hydrolysis of RS III which lead to the deposit of intermediates, for example malt oligosaccharides. Our study implies that EM-RS III stimulated the occurrence of probiotic bacteria and completely suppressing the growth of *E.coli* in high EM-RS III fed rats. The *L.reuteri*was isolated from high EM-RS III fed rat at somewhat higher frequencies than low and medium EM-RS III fed rats but was not found in the control

6.6 Conclusion:

In current study, we developed a concept to consume novel prebiotic EM-RSIII with a ability to important member of uropathogenic *Enterobacteriaceae* group. Low and high RS supplemented diet reshaped the gut microbiota and increased the production of SCFAs in rat's feces. Conclusively, current study provides the evidence regarding utilization of EM-RSIII as biotherapeutic regimens for treating human ailments related to gut health. Our data could be beneficial for future studies aimed at investigating the influence of prebiotics on human microbiome, metabolism and associated diseases.

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Chapter 7: Application of Enzymatically Prepared Resistant Starch

Paper: 5

Title: Development of Prebiotic Resistant Starch Film-coated Microparticles for an Oral Colon-Specific Drug Delivery

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7.1 Abstract

In this study, novel enzymatically modified resistant starch type III (EM-RSIII) derived drug loaded microspheres were developed, for oral colon-specific delivery of Ciprofloxacin HCl, used as a model drug. EM-RS III was enzymatically prepared using starch hydrolyzing enzymes amylase and pullulanase. The Prebiotic effect of EM-RSIII was successfully scrutinized for promoting the growth of probiotic bacteria e.g. *Lactobacillus reuteri.* Drug loaded microspheres were synthesized from the EM-RSIII through single emulsion evaporation method. The pH sensitive drug loaded microspheres were characterized through scanning electron microscopy, XRD and FTIR. Release studies confirmed the stability of drug loaded microspheres at acidic pH while sustained release was observed at pH 7.8 demonstrating a site-specific delivery of drug to colon. *Invitro* release mechanism of drug was evaluated using various kinetic models. The study also revealed that EM-RSIII derived microspheres were biodegradable and were degraded by the natural microflora of the colon, which was indicated by stimulating growth of probiotic bacteria. The microspheres were exploited as drug carriers for the colon specific release of Ciprofloxacin HCl and its encapsulation was further confirmed from the antibacterial activity.

Keywords; Probiotic, Oral colon-specific delivery, Resistant starch, Ciprofloxacin HCl delivery, Controlled release

7.2 Introduction

Polymer based microparticles for site-specific delivery of a drug to colon is becoming a hot spot in applied research. Utilizing specialized polysaccharide as a colon specific drug carrier employ tremendous benefits including its biocompatibility, inexpensiveness, non-toxicity and resistivity to the digestion in upper GIT tract and targeted delivery to colon. Moreover, its biodegradation resulted in bundles of benefits including stimulation of probiotic growth in colon with minimal loss of loaded compounds during the early stage of digestion [1]. In this respect, use of unique and specialized polysaccharides harboring such characteristics like resistant starch as drug carriers is a sustainable and novel approach [2].

Resistant starch, a natural polysaccharide, is a renewable and biodegradable resource with the advantages of being indigestible in upper GIT tract and fermented by colonic bacteria [3]. Native starches are not appropriate for direct use due to limitation of self-assembly and sensitivity to gastric acid and pancreatic enzymes, which can easily hydrolyze them as they have loose and long chain structures [4]. To make them resistant to digestion, various strategies are used like, physical modifications (e.g. pre-gelatinization) [5], chemical modifications (e.g. oxidation, etherification) [6]and/or enzymatic modifications to improve its physicochemical properties [7].The current study focused on novel enzymatically modified resistant starch (EM-RSIII) from maize flour, with prominent characteristics including high resistance against enzymatic digestion, its low solubility, hardness, birefringence analysis, rheological, pH and thermal stability. These properties make it appropriate for targeted delivery of drugs to colon. EM-RSIII derived microparticles with small particle size and slow digestibility rate play a key role in pharmaceutical industry. These microspheres have dual functionality i.e. as colon specific drug carrier and additionally act as prebiotic for stimulating the growth of probiotic bacteria in colon. Additionally, they are stable at acidic pH and resist digestion to pancreatic enzymes. Moreover, biobased EM-RSIII has a public acceptance due to its potential use in food and pharmaceutical industries [8]. The use of EM-RSIII derived microspheres from maize flour opens new possibilities for delivery of drugs to tissues and cells. It can provide an effective way for drugs to reach the specific sites like local treatment of colon ailments, reduced administered dose by avoiding unnecessary degradation and digestion, improved bioavailability of drugs and most importantly in remediating the patient compliance for drug administration [2]. The EM-RS III derived

microspheres are composed of natural polymer with no side effects for human use. Previously, chemically synthesized resistant starch (RS-IV) has been used as drug coating material. Chemically synthesized resistant starch (RS-IV) is stable under intestinal conditions, but the problem associated with it, is the strong interaction and complexation of drug with coating material due to formation of chemical bonds. This type of coating materials could be a problem for dose dependent release of drug. In addition to this there is some ethical concern with the involvement of chemicals in the preparation process of RS-IV. Therefore, enzymatically prepared EM-RSIII containing coating material can be the most preferred approach for the site-specific delivery of drugs and other active materials compared to the chemically synthesized resistant starch (RS IV). The EM-RSIII derived microspheres is exploited for the first time in the current study.

The current study aimed at synthesis of colon-specific EM-RSIII derived microspheres for sustained release of drugs. The study is further based on; validating the prebiotic potential of EM-RSIII for promoting growth of *Lactobacillus reuteri* and the use of EM-RSIII derived microspheres for coating ciprofloxacin HCl, a fluoroquinolone antibiotic used as a model drug for colon delivery. Characterization of microspheres was carried out with respect to their size, encapsulation efficiency, morphology, chemical and electrostatic interactions and crystallinity of ingredients used in preparation processes. Finally, the *in vitro* release of ciprofloxacin HCl and its antimicrobial assays were performed.

7.3 Materials and Methods:

7.3.1 Materials:

Ciprofloxacin HCl powder was kindly gifted by Global Pharmaceuticals, Islamabad (Pakistan), Acetone, Methanol, Monobasic Potassium Phosphate, Dimethyl sulfoxide (DMSO), Tween 80and Sodium Hydroxide was purchased from Merck (Germany). All the chemicals and solvents used in the study were of analytical grade. Resistant starch as potential prebiotic was produced from enzymatically modified maize flour (EM-RSIII) using microbial α-amylase and pullulanase purified from indigenously isolated *Bacillus licheniformis*-C1 strain [9]. Standard diet with addition of EM-RSIII was provided by the Department of Animal Sciences, primate facility Quaid-i-Azam University Islamabad, Pakistan. With the guide for the care and use of laboratory animals, as communicated by the National Institute of Health were approved by the Institutional Animal Care and

Ethical Committee at Quaid-i-Azam University Islamabad (No. BEC-FBS-QAU2017-10).

7.3.2 Preparation of Enzymatically Modified Maize Flour (EM-RSIII):

Resistant Starch was synthesized from maize flour. Five grams of maize flour in 0.5 M citrate buffer (pH 5) was dispersed, and the mixture was pregelatinized by autoclaving (121°C for 20 min). The gelatinized mixture was cooled to 37°C and protease (200U/ mL: 250 µL) was added and incubated for 40 minutes. The enzymatic reaction was terminated by boiling the mixture in a water bath at 100°C for 5 minutes to denature the protease. After cooling the starch gelatinized slurry amylase and pullulanase were added to the protease treated maize flour, and was incubated at 50°C, 100 rpm in shaking incubator for 16 hours. Enzymes' inactivation was achieved at 99°C in water bath for 10 minutes. Then the mixture was autoclaved (121°C, 15 psi) for 1 hour, and the paste was cooled at -4°Cfor 24 hours. A negative control without any enzymatic treatment was run in parallel. Dried resistant starch was grinded into fine powder of approximately less than 0.1 mm and stored in the airtight container for further analysis.

Resistant starch content of native and enzyme treated maize flour was determined by Megazyme Resistant Starch Assay Kit and the sample codes used for native maize flour were NMF and enzymes treated maize flour as EM-RSIII during characterization.

7.3.4 Prebiotic Potential of Enzymatically Produced Resistant Starch

The prebiotic potential of enzymatically prepared maize four was evaluated compared to that of native maize flour in order to utilize it as a safe drug carrier with dual functionality i.e. as a drug carrier and additionally act as a prebiotic for stimulating the growth of probiotic bacteria in the colon.

7.3.4.1 Isolation and Identification of Probiotic Strain

Probiotic strain was obtained from the colon content of the rats feed with EM-RSIII after dissection. The strain was isolated on specific medium (De Man, Rogosa and Sharpe agar; MRSA) sequenced via 16S ribosomal RNA gene sequence and identified as *Lactobacillus reuteri*. The medium used in the current study was De Man, Rogosa and Sharpe agar broth for growth and inoculum preparation of probiotic bacteria and adjusted to pH 7.

7.3.4.2 Inoculum Preparation of Probiotic Strain

The probiotic strain i.e. *Lactobacillus reuteri* was cultured on selective agar of De Man Rogosa and Sharpe (MRSA). All plates were incubated at 37 °C under anaerobic conditions for 48 hours. A single selected colony of the *L. reuteri* from the respective

agar was then transferred into 50 mL of MRSB and incubated for 24 hours at 37 °C to be used as inoculum.

7.3.4.3 Growth of *Lactobacillus reuteri* on EM-RSIII Containing Media

After 24 hours of incubation, the inoculum (5 %) was transferred to 50 mL of three fermentation media that contained different carbon sources. Fermentations were carried out in 50 mL Schott bottles for 48 hours. The prebiotic potential of enzymatically modified resistant starch from maize flour in comparison to the untreated native maize flour was assessed. Three fermentation media were prepared, one containing untreated native maize starch as a carbon source, the 2nd was supplemented with enzymatically modified resistant starch from maize flour as a carbon source while the third one was kept as negative control medium containing no carbon source. The probiotic strain was then anaerobically inoculated into the different starch supplemented fermentation media separately. After that bottles were placed in a jar containing Gas Pak envelopes (Becton Dickinson, New Jersey, United States) at 37°C and incubated for 48 hours to observe effects on growth and pH of fermentation media. Samples were withdrawn after every 12 hours and their pH as well as absorbance values at 600 nm were measured and compared.

7.3.5 Preparation of Drug Loaded Microspheres

The aim of the following experiment was to prepare and evaluate the ciprofloxacin HCl loaded sustained release microspheres by single emulsion evaporation technique. Emulsifying agent e.g. Tween 80 was used in the preparation of drug-loaded microspheres. It has the properties of adsorbing to the interface and stabilizing the emulsion droplets by preventing drug and polymer aggregation[10]. Tween 80 (0.5%) as stabilizer/emulsifier was taken in a beaker with distilled water and was stirred for 10 minutes. The formulation mixture containing EM-RSIII and ciprofloxacin HCl (10 mg) in DMSO was poured drop wise with varying injection rates into the aqueous phase (0.5% tween 80)and placed on a hot plate magnetic stirrer. The mixture was then continuously stirred for a specified time and speed, resulting in the formation of microspheres. Afterwards, the dispersion was centrifuged at 12,500 rpm for 20 minutes (Hermlelabortechnik, Z-206A). The supernatant was discarded, and microspheres were collected at the bottom, washed twice with distilled water to remove un-entrapped material, and frozen before being lyophilized.

7.3.5.1 Experimental Design for Optimization of Process Parameters

Various parameters were optimized to get the desired size and entrapment efficiency of the microspheres. Formulations with different phase ratios from aqueous to organic (1:2,

and 1:4), various stirring speeds (200, 400, 600, 800, and 1000 rpm) for different time periods (1, 2, 3, 4 and 5 hours) and different injection rates of organic phase (0.5 mL/min, 1mL/min and 1.5 mL/min) were prepared (Table 1). The size of spheres and encapsulation efficiency were checked for each formulation. The formulation having the lowest size and highest encapsulation efficiency was selected as the optimized one.

7.3.5.2 Encapsulation Efficiency of Microspheres

The drug encapsulation efficiency is important variable for assessing the drug loading capacity of microspheres. This parameter is dependent on the process of preparation, physicochemical properties of the drug, and formulation variables. The objective of this experiment was to encapsulate Ciprofloxacin HCl within enzymatically prepared resistant starch EM-RSIII derived microparticles with high encapsulation efficiencies with high theoretical drug loadings by single emulsion evaporation method. The encapsulation efficiency was measured by centrifugation of micro suspension for 30 minutes at 12,500 rpm. Both microspheres and supernatant were collected separately. Standard calibration curve of ciprofloxacin HCl with different concentrations were prepared with regression value (R^2) of 0.999. The collected supernatant was analyzed by measuring the absorbance values on UV-Vis spectrophotometer at 271 nm. The concentration of free drug in the supernatant was determined through the regression equation obtained from standard calibration curve of ciprofloxacin HCl. Encapsulation efficiency was measured through the following formulae [11].

$$\%\ Encapsulation\quad Efficiency\qquad (EE) = \frac{w_1 - w_2}{w1} \times 100$$

w1= total Ciprofloxacin HCl added

w2= free Ciprofloxacin HCl in supernatant

As mentioned above, the formulation with lowest size and highest encapsulation efficiency was selected as the optimized one.

7.3.6 Characterization of Optimized Drug loaded Microspheres

The optimized microspheres were characterized for the shape and surface properties through various techniques as mentioned below.

7.3.6.1 Average Particle Size Distribution

Size distribution plays a very important role in determining the release characteristics of the microsphere. Particle size of prepared formulations and poly dispersity index (PDI)

was analyzed through Dynamic Light Scattering (DLS Brookhaven Instrument Corporation, BI-200SM, New York USA).

7.3.6.2 Morphology of Optimized Microspheres

Shape and surface properties of optimized microspheres were studied through scanning electron microscopy (VEGA-3 by TESCAN Czech Republic with oxford EDS detector, Oxford Instrument). Samples were dried at 100°C for 12 hours to prevent the interference of water vapors. Then, these dried samples were coated with a thin layer of gold and fixed on an aluminum stub using adhesive tape.

7.3.6.3 Percentage yield of Drug Loaded Microspheres

Percentage yield (W/W) was measured through the weight of dried drug loaded optimized microspheres per quantities of drug and EM-RSIII initially used in the preparation of the microspheres during the experiment. Following formula was used for this purpose.

$$Percentage \quad yield = \frac{\text{Weight of dried MPs}}{\text{weight of polymer} + \text{drug}} * 100$$

7.3.7 Fourier Transform Infrared Spectroscopy

Interactions between the ingredients of formulation were studied through FTIR analysis. Ciprofloxacin HCl, EM-RSIII, and dried optimized microspheres were fixed with KBr disc and their spectra were assessed using FTIR spectrophotometer (100 L160000A, Perkin Elmer, USA).

7.3.8 X-ray Diffraction

XRD (Theta, STOE German) analysis was performed to check crystallinity of ingredients used in the preparation of microspheres i.e. Ciprofloxacin HCl, EM-RSIII, their physical mixture and the drug loaded microspheres. Philips X-Pert Pro diffractometer of CuKα was used to produce X-ray Diffraction patterns of all the samples at a wavelength of 1.54 Å with intensity in the range of 2º-80º.

7.3.9 *In vitro* Drug Release Studies from Microspheres

The purpose of the experiment was to check the release profiles of formulations with negligible burst effect and slow release profile. The formulations that showed little burst effect at higher drug-to-polymer ratio, rectifying better sustained release. For this purpose, *in vitro* drug release studies were performed by placing the formulation in a dialysis membrane placed in a shaking water bath. In this experiment, 3 mg of dried microspheres were taken in the dialysis membrane containing5 mL of respective release medium. The sample was taken and treated with α-amylase (3 U/mL) in 5 mL of

potassium phosphate buffer (5 mL, pH 7), followed by incubation at 37°C for approximately 2 min in a shaking water bath to mimic salivary amylase. To mimic the stomach conditions, sample was mixed with 0.1 N HCl solutions, and the pH was adjusted to 1.2 to mimic the gastric ph. To mimic intestinal conditions the sample was mixed with pancreatic α-amylase (300 U/mL; phosphate buffer) in the dialysis bag with a molecular weight cut of 14 kDa. The dialysis membrane was then placed in a beaker containing 50 mL of release medium and the beaker was then placed in a shaking water bath at 37°**C**. A sample of 1 mL was taken from release medium after 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 12, and 24 hours and equal volume (1 mL) was replaced with respective release medium after each sampling to maintain the sink conditions. Calibration curves were constructed using the buffers with respective pH values. All the samples from simulated stomach and intestinal fluid were analyzed at 271 nm. The absorbance values collected were incorporated into regression equation (R^2 value0.999) to determine the percent drug release at that specified time interval. All readings were taken in triplicate.

7.3.10 Drug Release Kinetics:

Various kinetics models including first-order [12], zero-order [13], Hixon-Crowel [14], Higuchi [15] and Koresmeyer Peppas [16] were applied on *in vitro* release data to check the mechanism of drug release from the enzymatically modified resistant starch derived microspheres.

7.3.11 *In-vitro* Antibacterial Assay

Drug loaded microspheres were dissolved in DMSO and their antimicrobial activities were determined by agar well diffusion method to further confirm the drug encapsulation in the microspheres [17]. Bacterial lawn of different strains (*Escherichia coli* and *Staphylococcus aureus*) was prepared on the whole surface of Mueller Hinton Agar (MHA) containing plates. A hole of 8mm was punched with a sterile borer and sealed the bottom with agar to prevent diffusion. A volume (100 µL) of sample solution was introduced into the well. Ciprofloxacin HCl was taken as positive control while water and DMSO were taken as negative controls. The MHA plates were incubated at 37°**C** for 24 hours. Zones of inhibition were examined onwards.

7.4 Results

7.4.1 Preparation of Enzymatically Modified Resistant Starch from Maize Flour:

The RS content of untreated native maize as well as enzymatically treated flour and gelatinized maize control was quantified according to the standardized protocol through Megazyme Assay Kit (Ireland). The Resistant starch content of enzymatically produced starch was found to be 24.4% (Fig. 7.1). The RS content in the sample corresponds to the amount of amylose, which is produced as a result of enzymatic hydrolysis. The amylose produced recrystallized into a more tightly packed resistant structure represents the RSIII content.

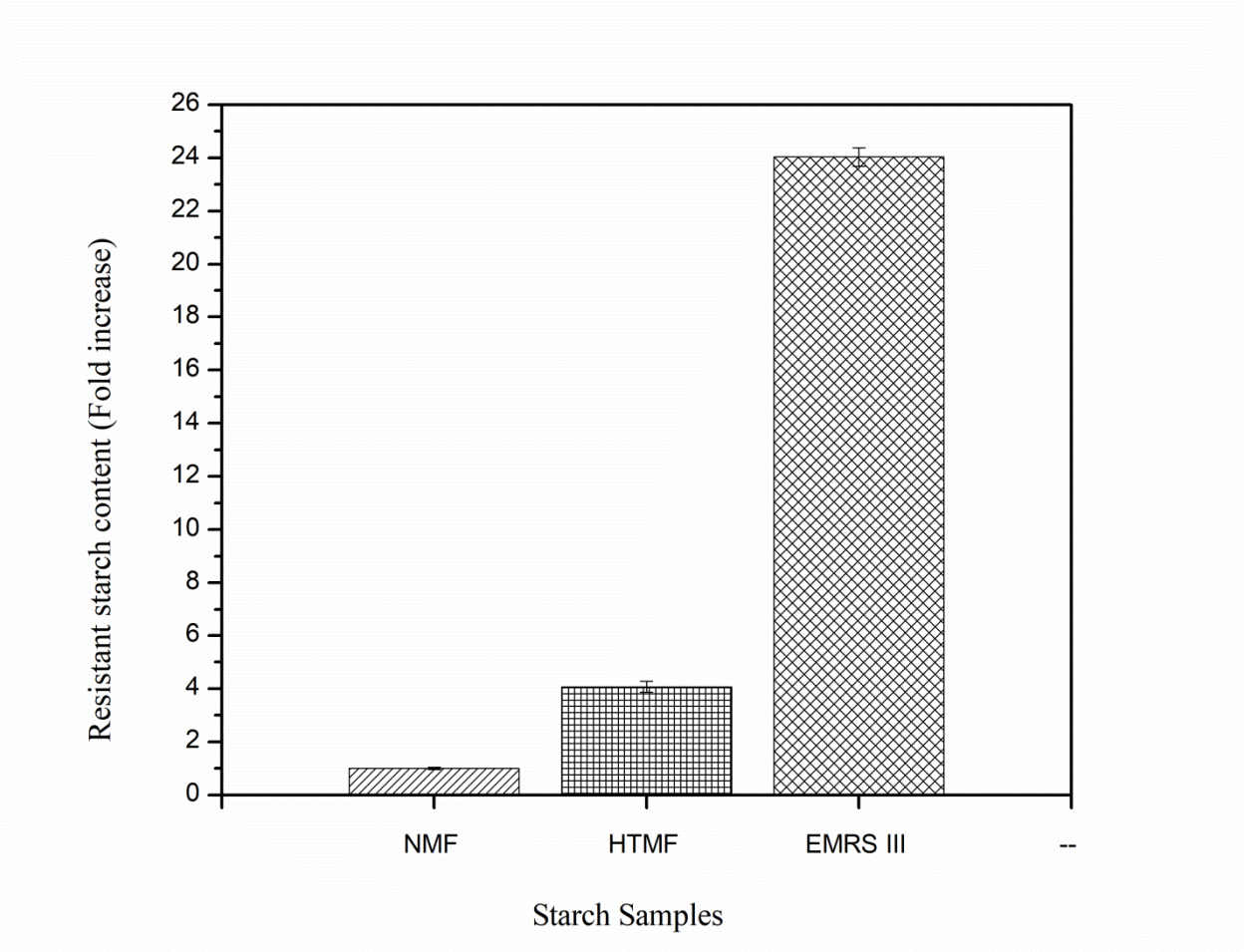

Figure 7.1: Enzymatic preparation of resistant starch from maize flour

7.4.2 Effect of Different Starches on Proliferation of *L. reuteri*

The growth rate of *L. reuteri* strain in different starch supplemented fermentation media over a period of 48 hours was examined as described in Fig. 7.2. Maximum growth (absorbance) was observed in the case of EM-RSIII from maize flour supplemented medium with a continuous increase in the absorbance values (2.57) after 48 hours as compared with the untreated native starch with final absorbance of 2.0. The higher

absorbance values($p<0.05$) indicating the greater prebiotic potential of EM-RSIII as compared to untreated native maize flour. Initially the growth pattern of untreated native maize flour was higher (1.32) as compared with EM-RSIII (1.25) because readily accessible starch was available for growth in the untreated native maize flour fermentation medium, however, after 24 hours the growth pattern was observed to be slow due to vindication of utilization of readily accessible starch. In case of EM-RSIII initially the growth pattern was slow due to its compact hard surface not easily accessible to the bacteria but after adhering of probiotic bacteria to starch molecules, the growth rate was harmonized from 1.25 to 2.57 in 48 hours. The fermentation medium with no carbon source supplementation (NCS) showed the lowest absorbance value of 1.26 after 48 hours indicating that carbon source is necessary for flourishing the growth of *L.reuteri*.

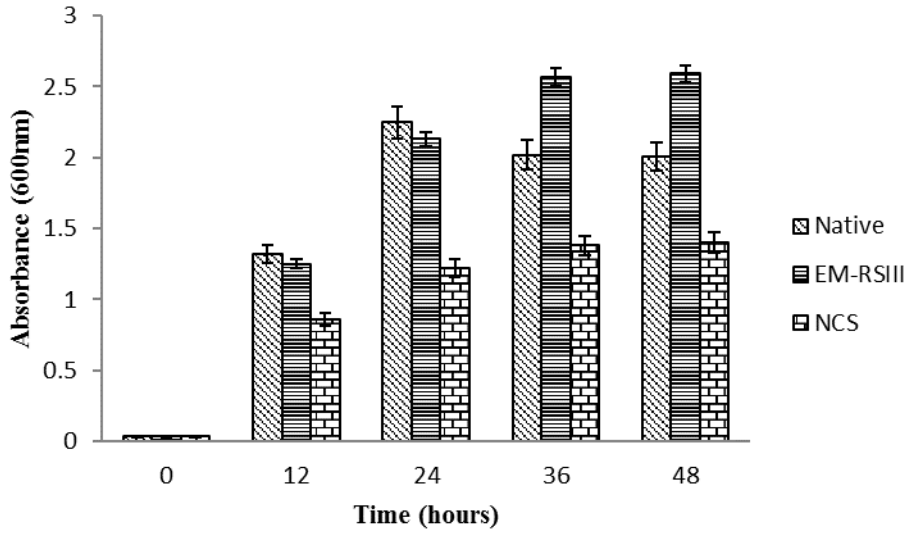

Figure 7.2: Effect of different starches as carbon source on proliferation of *L. reuteri* at various time intervals

7.4.3 Effect on pH of Fermentation Medium

The decline in pH of different starch supplemented fermentation media by inoculating *L.reuteri* over a period of 48 hours was also observed as shown in Fig. 7.3. Maximum decline in pH (5.07) was observed after 48 hours in case of EM-RSIII supplemented fermentation medium as compared with the pH (5.98) of the untreated native maize supplemented media. This indicated a greater acid production by *L. reuteri* in EM-RSIII from maize flour as compared with the untreated native maize flour. No carbon source supplemented fermentation medium (NCS) showed the lowest decline in pH (6.5) after 48 hours.

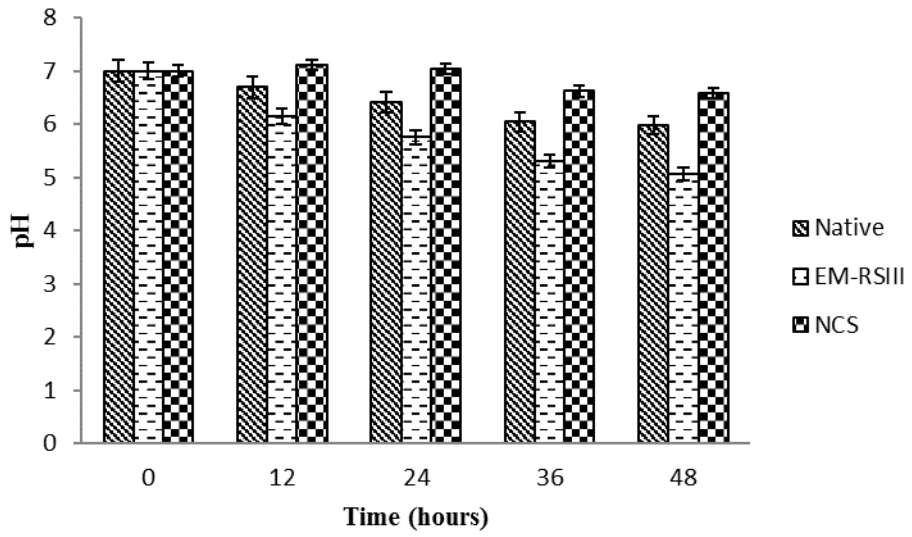

Figure 7.3: Effect on pH of fermentation medium by inoculating *L. reuteri* in the presence of EM-RSIII, native maize flour and media containing no carbon source

7.4.4 Preparation and Selection of Optimized Drug Loaded Microspheres

Drug loaded microspheres were synthesized and an optimized formulation was selected based on smallest size with maximum encapsulation efficiency for further characterization as shown in Table 7.1. Based on the above criteria formulation (Sr. 4) containing 100 mg EM-RSIII, 10 mg ciprofloxacin HCl and 0.5% tween 80 was considered as the optimized one as has the minimum particle size (964 nm) with maximum encapsulation efficiency (86.73 %).

7.4.5 Characterization of Drug Loaded Optimized Microspheres

The drug loaded microspheres were characterized for its size, morphology and drug release profile as below.

7.4.5.1 Size analysis of Drug Loaded Microspheres

Particle size and poly dispersity index (PDI) of optimized drug loaded microspheres was analyzed through Dynamic Light Scattering technique (DLS). Size measured was 964 nm (about 1µm) and PDI was 0.452 with mono dispersed system. Results are shown in Table 7.1 and Fig. 7.4.

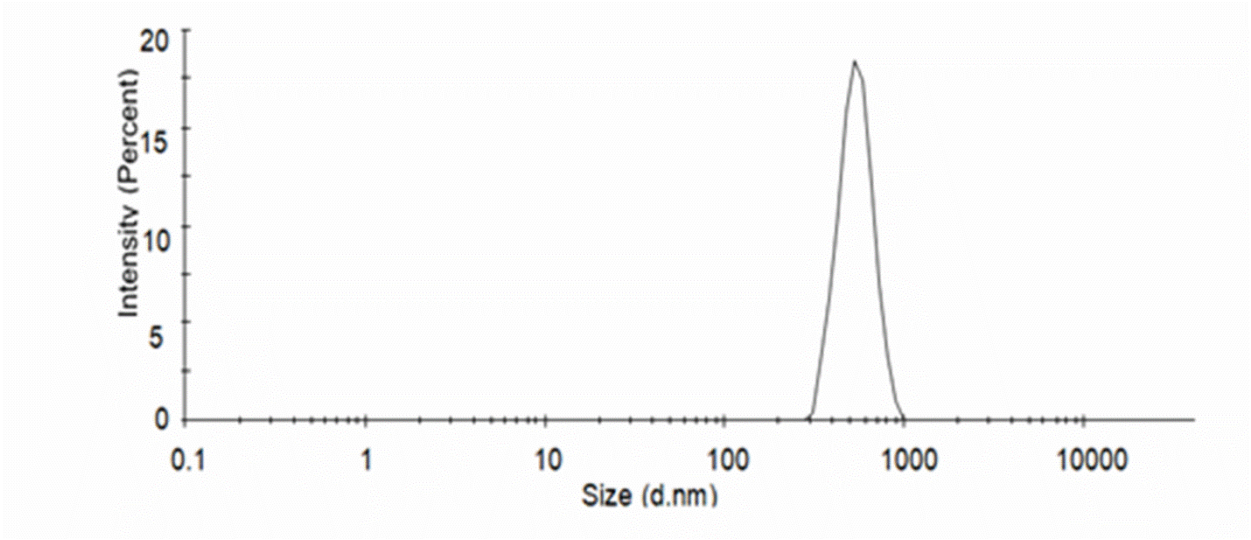

Figure 7.4: Size distribution of optimized Drug loaded Microspheres

Table 7.1: Optimization of process parameter for the desired size and encapsulation efficiency of drug loaded microspheres and selection of optimized drug loaded microspheres as indicated in Sr. No: 4

Sr no	Stirring speed (rpm)	Stirring time (Hrs.)	Injection rate (ml/min)	Organic to aqueous phase ratio	Resistant starch (mg)	Drug (mg)	Size (nm)	EE* (%)
1	200	1	2.5	1:2	20	10	4156	53.3
2	400	2	2	1:2	60	10	1548	58.6
3	600	3	1.5	1:4	80	10	1041	79.0
4	800	4	1	1:4	100	10	964	86.7
5	1000	5	0.5	1:4	120	10	743	65.5

EE= Encapsulation efficiency (%)

7.4.5.2 Morphology of Microspheres

The surface morphology of the microspheres was determined by observing them under scanning electron microscope. SEM analysis as shown in Fig. 7.5 (A) showed compact block like aggregates of EM-RSIII and drug loaded microspheres obtained from single emulsion evaporation method have spherical shape as shown in Fig. 7.5 (B).

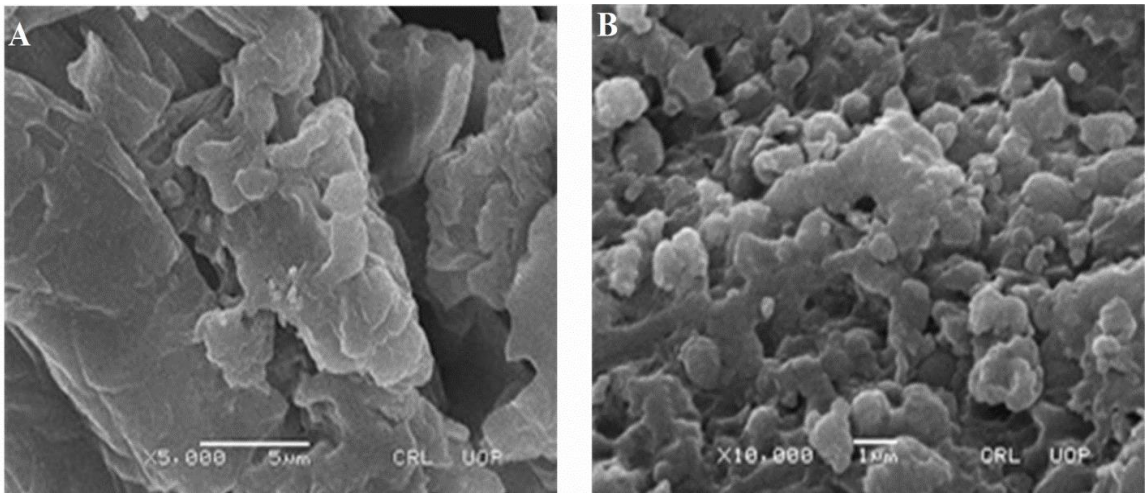

Figure 7.5: SEM Analysis of (A) Enzymatically modified resistant starch from maize flour (EM-RSIII) (B) Drug loaded Microspheres

7.4.5.3 Fourier Transforms Infrared Analysis

The FTIR spectra of the optimized drug loaded microspheres, Ciprofloxacin HCl and EM-RSIII is shown in Fig. 6.FTIR spectra of drug loaded microspheres showed prominent bands in the region of 3550 and 3500 cm^{-1}, which can be due to single bridge hydrogen bonding. While the bands in the range of 3450 to 3400 cm^{-1} was assigned to polymeric hydrogen bonds. The strong hydrogen bonding is represented by bands between 2650 and 2600 cm^{-1}. The bands from 1650 to 1600 cm^{-1} showed carbonyl stretching vibration. C-F groups are indicated by the peaks in the range of 1100 and 1000 cm^{-1}, while peaks at 800 cm^{-1} in the spectra is an indication of meta distribution of aromatic group. FTIR spectrum confirmed the presence of chemical and electrostatic interactions of the components used in the preparation of microspheres.

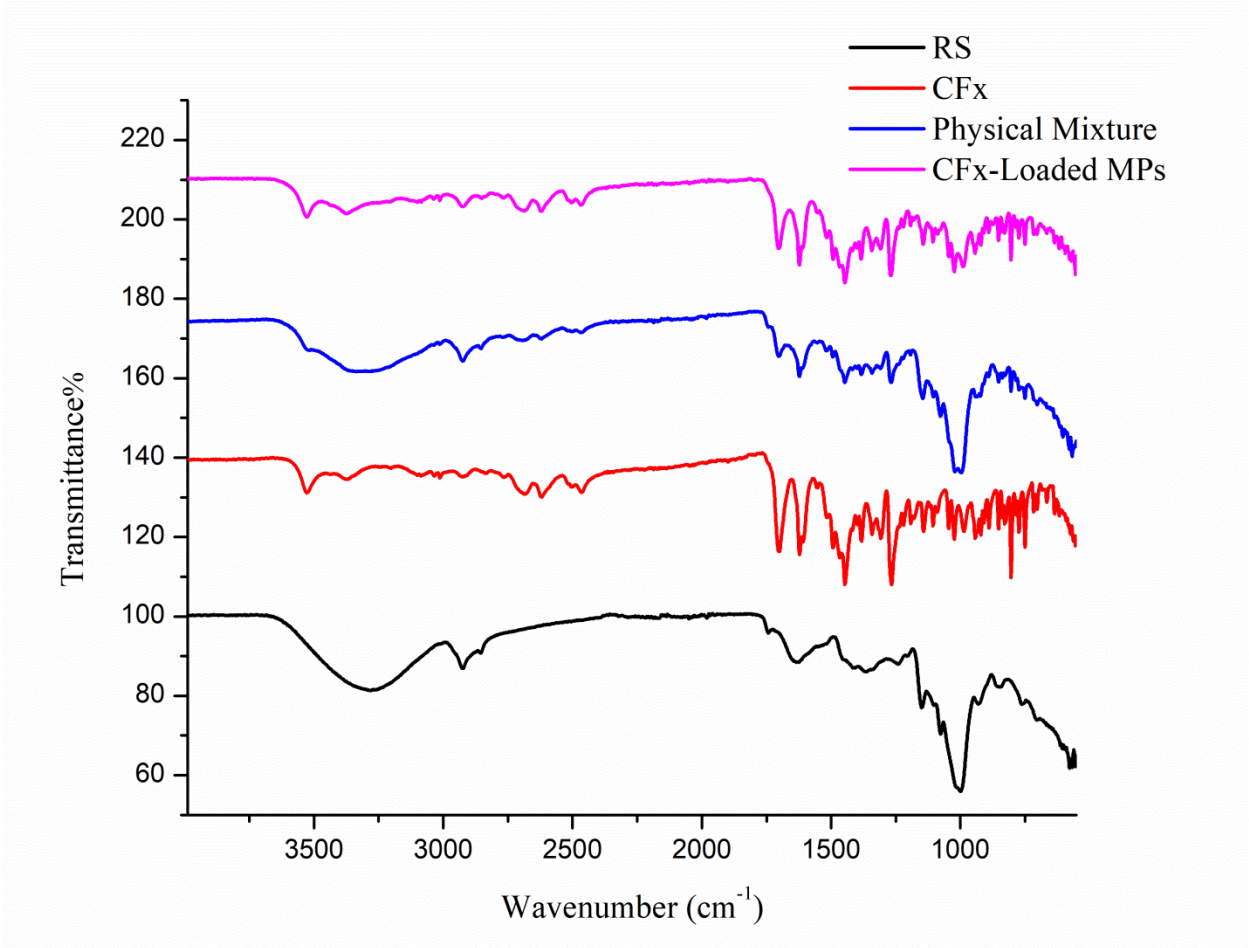

Figure 7.6: FTIR Spectrum of drug loaded microspheres, Ciprofloxacin HCl and EM-RSIII.

7.4.5.4 X-Ray Diffraction Analysis of Microspheres

In the diffractogram of Ciprofloxacin HCl, crystalline nature was confirmed from the sharp peaks in the range of 20-40° as shown in Fig. 7 but when drug was encapsulated into EM-RSIII, no sharp peaks were observed in that range, thus confirming encapsulation or loading of drug into EM-RSIII derived microspheres. Crystallinity of drug was completely masked within the polymeric microspheres. In case of the physical mixture of ciprofloxacin HCl and EM-RSIII, diffractogram of drug remained the same, so it is proved that encapsulation of drug into EM-RSIII occurred in microspheres through proper preparation method.

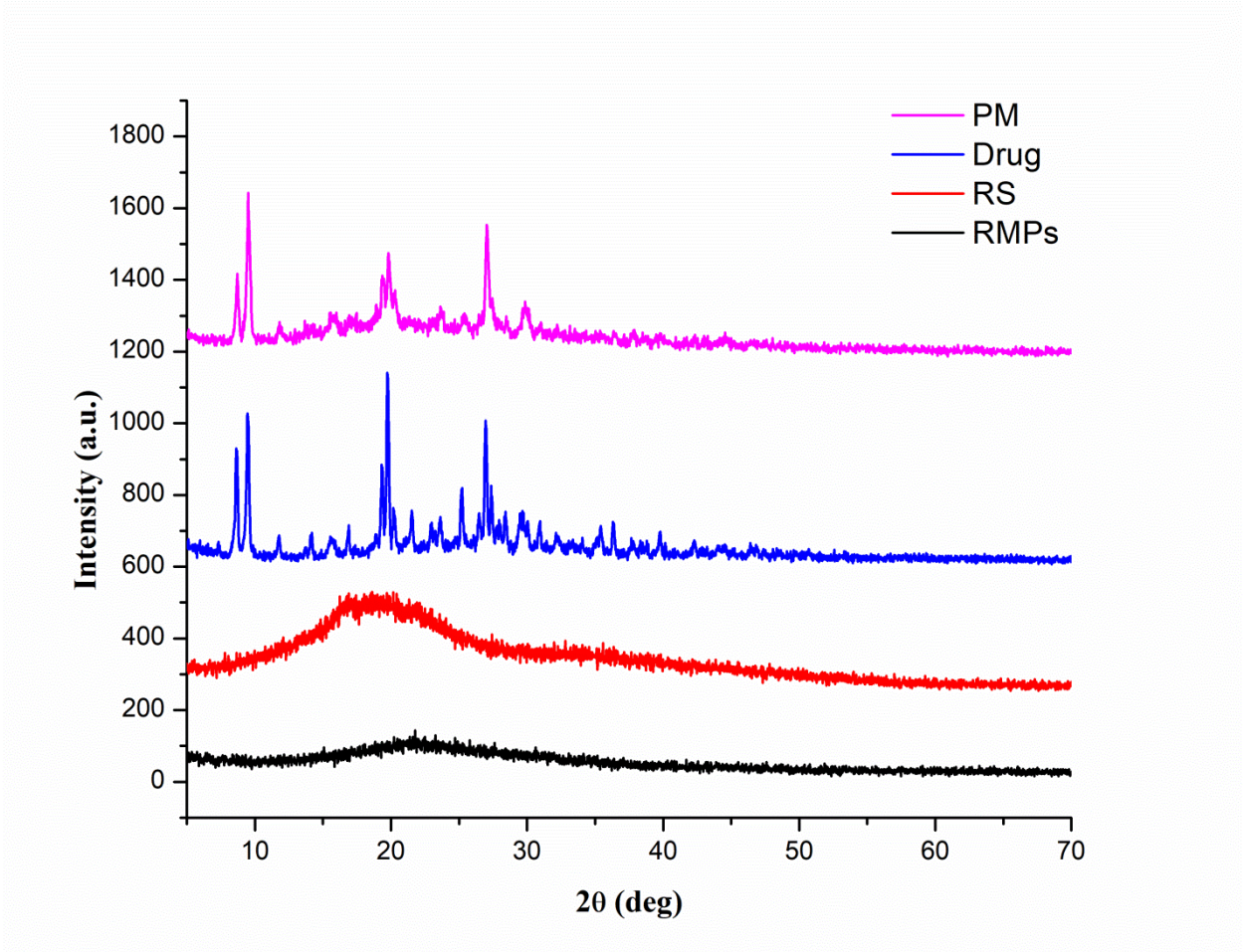

Figure 7.7: X-Ray diffraction (XRD) analysis of Microspheres, Ciprofloxacin HCl, EM-RSIII and their physical mixture.

7.4.5.5 Percent Yield of Optimized Formulation

Percentage yield of the optimized formulation was found to be 79.3%.

7.4.5.6 *In Vitro* Drug Release from Microspheres

The release profile of Ciprofloxacin HCl from enzymatically produced resistant starch derived microspheres was evaluated in simulated gastrointestinal fluids at 37 °C in shaking water bath. Drug release from microspheres was 38% within 6 hours and about 52% in 14 hours at pH 7.8, indicating a sustained release pattern of drug from the microspheres (Fig. 7.8). However, in case of simulated gastric fluid only 5% of the drug was released from microspheres($p<0.05$), confirming the successful encapsulation of drug into EM-RSIII derived microspheres, having the potential to deliver the drug to colon.

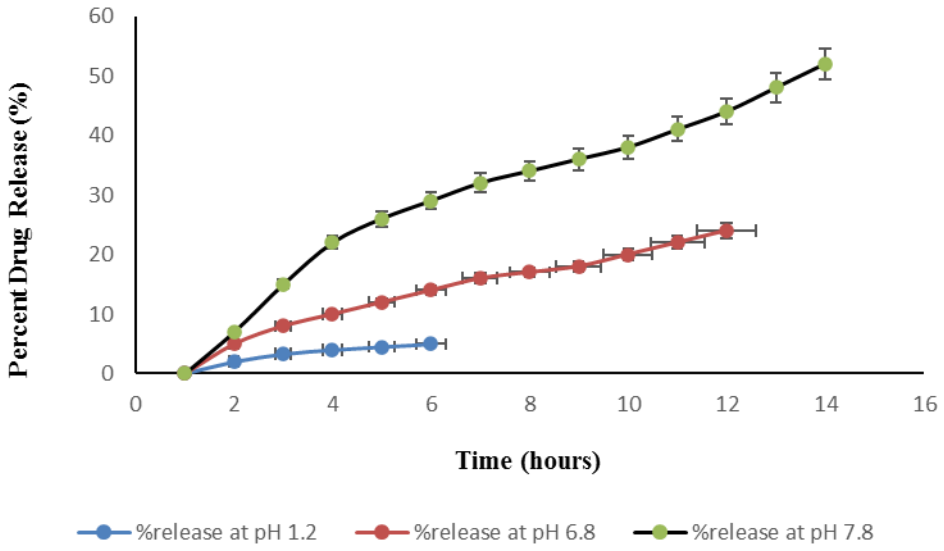

Figure 7.8: Cumulative drug release profile of Ciprofloxacin HCl from enzymatically produced resistant starch derived drug loaded microspheres at different Gastrointestinal conditions

7.4.6 Drug Release Kinetics from Microspheres

The drug release profile of microspheres was evaluated using different kinetic models such as zero-order, first order, Higuchi, Korsmeyer peppas and Hixon-Crowel models. These models were applied on the release data. The values obtained from the coefficient of regression (R^2)proposed that 1st order kinetics was followed in acidic medium while Higuchi release model was followed in simulated intestinal and colonic conditions as described in Table 7.2.

Table 7.2: Coefficient of regression (R^2) value of drug release kinetics

R^2 value of drug release	Zero Order	1st order	Korsmeyer peppas	Higuchi	Hixon-Crowel
Simulated Stomach conditions	0.900	0.9109	0.567	0.833	0.87
Simulated Intestinal conditions	0.909	0.913	0.616	0.953	0.844
Simulated Colonic conditions	0.630	0.658	0.214	0.901	0.801

7.4.7 *In Vitro* Antibacterial Potential of Drug loaded Microspheres

In order to confirm the encapsulation of drug into the microspheres, antimicrobial activities of the drug loaded microspheres were determined by agar well diffusion method [16] against *Escherichia coli* and *Staphylococcus aureus*. The drug loaded microspheres showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* with zone of inhibition (ZOI) 22 mm and 20 mm respectively ($p<0.05$) like that of pure Ciprofloxacin used as a positive control (Fig. 7.9) while EM-RSIII in water (control) and DMSO used as negative control did not show any antibacterial activity.

These results proved the drug was successfully encapsulated into EM-RSIII derived microspheres and showed antibacterial activity on dissolution in DMSO.

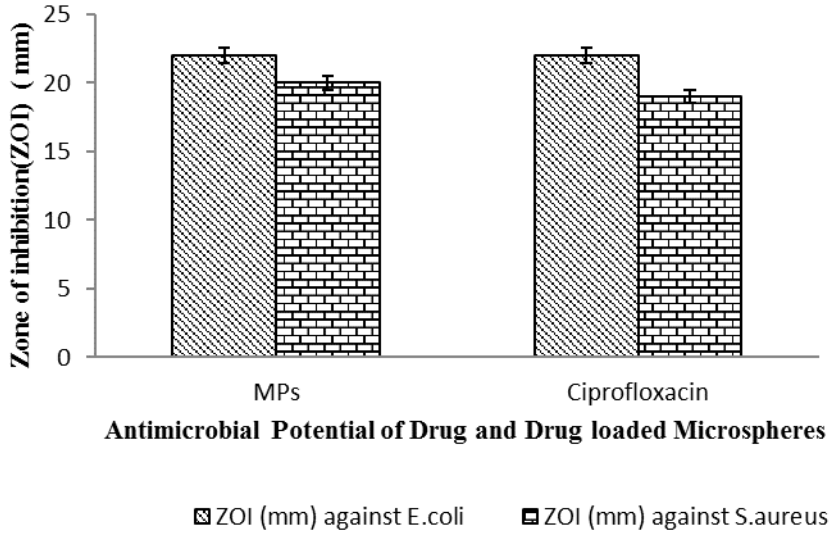

Figure 7.9 Antibacterial potential of EM-RSIII and Drug loaded microspheres against *S.aureus* and *E.coli*

7.5 Discussion

The current study explored the ability of enzymatically modified resistant starch (EM-RSIII) derived microspheres from maize flour as a potential drug carrier for colon targeted delivery of drugs. Enzymatically prepared resistant starch is preferred over chemically synthesized resistant starch as it is free of any toxic residues due to the high selectivity and specificity of enzymes for their substrate and the biodegradable nature of the enzymes [22].The enzymes used for preparation of resistant starch wereα-amylase and pullulanase that specifically cleavedα-1,4- and α-1,6-glycosidic linkages of amylose and amylopectin in polysaccharides respectively, resulted in the production of short, linear and branched oligosaccharides of varying length. The linear amylose recrystallized into a more tightly packed resistant structure called as resistant starch, which was found to be

24.4% (Fig.1).Prebiotic potential of resistant starch III (EM-RS III) was determined from the growth of *Lactobacillus reuteri*(Fig. 2) and from the drop in pH of the fermentation medium during the growth of *Lactobacillus reuteri*. The drop in pH was due to the production of short chain fatty acids (butyrate, acetate and propionate etc.). Among the acid's butyrate was of the most important acid produced by bacterial fermentation. It plays an important role in metabolic hemostasis and supplying energy to the epithelial cells in the large intestine[18].The production and quantification of the acid was further confirmed by gas chromatography and the result indicated the highest production of butyrate (unpublished data).Resistant starch derived microspheres were designed to deliver model drug i.e. Ciprofloxacin HCl to the colon via oral route for achieving the advantage of enhancing growth of probiotic bacteria on one side and to minimize toxicity on the other side. Ciprofloxacin HCl was selected as model drug as it was easy to determine the release profile and encapsulation efficiency with simple spectrophotometric analysis (by taking absorbance of Ciprofloxacin HCl at 271 nm). Moreover, Ciprofloxacin HCl is an effective drug for controlling inflammation in bowel diseases [19]. Hence targeted and dose dependent efficacy of Ciprofloxacin HCl can be further explored based on the current study. Due to the biodegradable and prebiotic nature of resistant starch, it is an important biopolymer, to be used as a drug carrier for colon specific drug delivery [17]. It has been studied that native starch is completely broken down by pancreatic enzymes after oral ingestion, with successive absorption from the small intestine into the systemic circulation and has limitation of provoking glycemic index [20]. Whereas, resistant starch has the potential to escape digestion in the small intestine because of its compact crystalline structure and undergoes fermentation by bacteria in the colon. To decrease the enzymatic degradation in the stomach and to allow acceptable amount of a therapeutic agent to be absorbed, starch in maize flour has been modified with enzymes α-amylase, which resulted in increasing content of resistant starch with compact crystalline [9].

Current study was designed to fabricate EM-RSIII derived drug loaded microspheres and their characterization. For the synthesis of drug loaded microspheres, single emulsion evaporation method was used. This method is simple, and no expensive technology is needed. Both polymer and model drug used in the present study were hydrophilic, which ultimately promoted the synthesis of microspheres [21]. Various formulations were prepared with different concentrations of tween 80 for optimum size of microsphere. With an increased concentration of tween 80, smaller particle size was obtained and vice

versa. The concentration of EM-RSIII and tween 80 has a great impact on encapsulation efficiency. It was observed that upon increasing the concentration of EM-RSIII, encapsulation efficiency was increased and vice versa. When an aqueous phase without Tween 80 was used as an internal phase, microspheres were aggregated. Increasing the concentration of emulsifying agent (tween 80) resulted in the reduction of microspheres size [23].The reason for improving encapsulation efficiency is the hydrophilic nature of both EM-RSIII and ciprofloxacin HCl and higher viscosity of oil phase, which promotes the escape of drug from the oil phase to the aqueous phase. Beside the optimization of polymer and emulsifier concentrations, other factors like stirring speed, stirring time, injection rate and aqueous to organic phase ratio and their effect on particle size and encapsulation efficiency was also evaluated. The particle size increased with increasing aqueous phase because the evaporation of organic phase become difficult with increasing the volume of aqueous phase, it results into the formation of aggregates hence producing large size microspheres. Rate at which organic phase is injected into the aqueous phase is very crucial factor that play a major role in the preparation of optimal size microspheres. It was observed that upon slow addition of organic phase, particle size got condensed. The credible reason is that higher injection rate of organic phase results in the improper mixing of two phases, while slow injection promotes the prolonged contact time of the organic phase with the aqueous phase that can result in smaller particle size [24]. High stirring speed is helpful for proper mixing and getting smaller particle size, because it allows rapid diffusion of the oil phase into the aqueous phase resulting in diffusion of more drug into the aqueous phase and ultimately affect the encapsulation efficiency [25].

SEM images showed that EM-RSIII has compact, block like structure, while the drug loaded EM-RSIII microspheres have spherical and uniform distribution and have a small size of about 1 micrometer. The FTIR peaks assigned to C-O and a C-O-C bond representing acrylates and esters confirm the esterification interactions between polymeric OH group and -COOH group of drugs (Ciprofloxacin HCl) as described in Fig. 6. C-F group showed stretching vibrations and remained almost unchanged. FTIR peaks between 3550 and 3500 cm^{-1}, 3450 and 3400 cm^{-1}, and 2650 and 2600 cm^{-1} are assigned to prominent intermolecular hydrogen bonding, indicate single bridge O-H…O, polymeric O-H.OH…O-H and strong hydrogen bonding. The bands from 1650 to 1600 cm^{-1} showed carbonyl stretching vibration. C-F groups are indicated by the peaks in

the range of 1100 and 1000 cm^{-1}, while peaks at 800 cm^{-1} in the spectra is an indication of meta distribution of aromatic group. FTIR peaks of Ciprofloxacin HCl in the range of 1750 to 1700 cm^{-1} were not detected in the final formulation most probably due to interaction with polymer (EM-RSIII). FTIR spectrum confirmed the presence of chemical and electrostatic interactions of the components used in the preparation of microspheres. FTIR bands are sharp in case of intramolecular hydrogen bonding while broad bands represent intermolecular hydrogen bonds, [26, 27]. XRD results as described in Fig. 7 illustrated that Ciprofloxacin HCl have sharp peaks in the range of 20-40° confirming the crystalline nature of drug. But, when it was loaded into EM-RSIII, there were no characteristic peaks in the range of 20-40°; however, the spectrum of EM-RSIII remains the same before and after loading of drug into polymer. Physical mixture of drug and EM-RSIII also confirmed that complete encapsulation of drug only occurs by single emulsion evaporation method. Therefore, the crystalline nature of pure drug was completely masked within the microspheres and the drug did not show any peak when combined with polymer by following proper encapsulating procedure while clear peaks of drug was observed in case of physical mixture. These results are in accordance with the results reported by Eesfandiar pour Boroujeni, as the drug changed its nature from crystalline to amorphous form when encapsulated [28].

Percent yield of microspheres is critically important from industrial and economic point of view. Higher the percent yield of microparticles more will be the economic benefit of selected method used for preparation. Percent yield of microspheres was measured to be approximately 79.3 %, which proved that the number of reactants lost during the preparation method was much less than product produced. Hence, the preparation method is highly beneficial. High encapsulation efficiency is the most important feature for delivering high dose of drug to the targeted site. Encapsulation efficiency measured was 86.73±0.014% for the optimized formulation. Higher encapsulation efficiency was achieved as compared to the previous studies where encapsulation efficiency was found to be 67 % ± 8% in case of pH sensitive curcumin loaded NPs [29].

In vitro release studies of the drug encapsulated microspheres revealed a fast and high release of drug at pH 7.8 (simulated colonic fluid) and almost 52% of drug was released from microspheres in 24 hours. The coating of EM-RSIII derived microspheres is resistant to gastric conditions but sensitive to higher pH and fermented by colonic bacteria, facilitating release of drug in colon. In the absences of colonic bacteria, the outer coating of EM-RSIII imparted additional stability to the microspheres. Although

even in simulated colonic fluid (phosphate buffer of pH 7.8) the release of drug from microspheres was only 52% in 24 hours, but the colonic bacteria can digest the polymer and can enhance the release of drug in the colon [30]. In case of simulated gastric and intestinal fluids, only a small percentage of drug was released from microspheres. Similar results were also reported where, pH sensitive nanoparticles showed significant difference in release profile at neutral to acidic pH [31]. The *in vitro* release of Ciprofloxacin HCl from the microspheres as described in Fig. 8 at pH 7.8 was observed to be in a constant manner. The reason for this constant release is due to the functional groups of polymers (EM-RSIII) that gets ionized and resulting into structural changes like swelling of polymer, which ultimately tailor the slow release of drug from network of microsphere [32]. To investigate release mechanism of drug from the microspheres, various kinetic models were applied on the release data [33]. As R^2 value for microspheres in acidic media was highest for 1^{st} order kinetics, hence a concentration dependent release of drug was shown. While in case of intestinal and colonic simulated fluids Higuchi model was followed as has the highest R^2values, which explains diffusion-based drug release [34].

Antibacterial assay for the drug loaded microspheres was carried out to have strong evidence that drug was encapsulated into EM-RSIII as described in Fig. 9. The antibacterial effect seen in the case of drug loaded microspheres was the confirmation of encapsulation ability of EM-RSIII derived microspheres.

7.6 Conclusion

It was concluded from the current study that EM-RSIII is a potential drug carrier in the colon targeted oral delivery of drugs. EM-RSIII has prebiotic potential and therefore, provoked the growth of *L. reuteri* known as probiotic bacteria. Microspheres were successfully prepared by single emulsion evaporation method. Characterization studies including ingredients compatibility, particle size(964 nm), polydispersity index(0.452), morphology, encapsulation efficiency (86.7 %) and antibacterial assay of the drug loaded microspheres explains their compatibility for targeted delivery of drug. *In vitro* drug release study explained the pH dependent release profile of the drug from the microspheres. In case of mimicking colonal conditions substantial amount of drug was released at pH 7.8 and negligible amount of drug was released in acidic medium.

Acknowledgements

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Conflict of Interest

We declare no conflict of interest.

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Conclusions

Starch hydrolyzing enzymes play essential roles in the preparation of resistant starch, the most important of which is α-amylase and pullulanase from *Bacillus licheniformis*, as revealed by the current study (**Papers I, II, and III**).α-Amylase and pullulanase from *Bacillus licheniformis* successfully cloned into *pET* 24a and *pGEX-2T*respectively and finally expressed and purified from *E.coli* BL21 (DE3). The purified enzymes were utilized individually and in combination for the preparation of resistant starch. Combination of enzymes (amylase and pullulanase) resulted in more resistant starch content (24 %) as compared to only amylase (16%) or only pullulanase (20 %) in the preparation process of resistant starch. The enzymatic treatment of starch prior to retrogradation aligns the short amylose chains for the formation of double helices, ultimately enhancing the degree of retrogradation. In the current study, a transformation of starch from amorphous to crystalline structures contributed to an increased resistance of α-amylase and pullulanase-treated maize flour (EM-RS III). The desired properties, such as low water binding ability, swelling power, oil and water absorption capacity and increased milk absorption capacity of EM-RS III, provide better handling in processing leading to crispiness and crunchiness of the products formed, usually in cookies, crusts, breakfast cereals and bakery products. EM-RS IIIsupplemented cookies had better sensory properties and retained the resistant starch content even after baking and processing so could be successfully used in food industry as a new product(**Paper III**). EM-RS III showed lower *in-vitro* digestibility compared to untreated native maize flour. We herein develop a concept to utilize novel prebiotic EM-RSIII during *in-vivo* experiment with a potential to inhibit the growth of *E.coli*. Feeding of selected prebiotic to experimental rats also modulates the fecal microbiome and enhances the production of SCFAs in rat's gut and feces (**Paper IV**). This work provides evidence that such EM-RSIII prebiotics could be exploited as biotherapeutic regimens for human diseases associated with gastrointestinal track. In addition, the increased complexing ability of EM-RS III with iodine as well as with fatty acid compared to that of native maize flour makes enzyme-treated maize flour suitable for encapsulating material for site-specific drug delivery (**Paper V**). *In-vitro* drug release study explained the pH dependent release profile of drug from microspheres. In case of mimicking colonal conditions substantial amount of drug was released at pH 7.8 and insignificant amount

of drug was released in acidic medium. Therefore, prepared EM-RSIII have potential to be used for targeted and site-specific delivery of drugs.

Future Prospects

Future prospects to continue this study in future:

- Synergistic studies of Resistant Starch with other dietary fibers could be carried out to determine the most effective composition with maximum prebiotic potential can be performed.
- Role of Resistant Starch in prevention of colorectal cancer at molecular level could be explored
- Investigations can also be done for the development of symbiotic containing probiotic bacteria complexed with Resistant Starch as a prebiotic for enhanced health benefits.
- Use of resistant starch as an encapsulating material for site-specific delivery of drugs to colon for anti-inflammatory drugs in IBD, anticancer, flavonoids and antioxidants.
- Studies on positive influence of RS on satiety and short-term food intake can be conducted along with role of RS in lipid metabolism and in reducing serum cholesterol.
- Different sources of starch can be employed for the enzymatic synthesis of resistant starch with effective physicochemical properties and enhanced RS content.
- RS can be used as a functional ingredient for the synthesis of innovative foods in future with greater health benefits and can be used for the treatment of obesity and for weight loss.
- There are gaps in the clinical and experimental studies despite of the extensive research on analyzing *In-vivo* prebiotic effect. Most of the studies were carried out on healthy subjects for short time period; it is very important conduct well-designed long-term studies on diabetic and obese human subjects.
- Certain efforts were made by researchers to investigate the mechanism of action of RS on obesity and diabetes. Production of SCFAs as result of colonic fermentation and modification in the expression level of certain genes, providing evidence for the action of RS. But still there is need of more research to know the exact biochemical and molecular mechanism of the action of RS.

- *In-vivo* study should be conducted for further permeation of drug loaded microspheres for control and target delivery.

Appendix 1: Additional file 1

Table S1: Water absorption capacity, water binding capacity Swelling power, at different temperatures and light transmittance at different time of native maize flour (NMF) and amylase treated maize flour (ATMF)

Parameters	**Samples**	
	NMF	**ATMF**
Water absorption capacity (WAC) %		
30°C	180	166
60°C	240	199
90°C	387	245
Water binding capacity (WBC) %		
30°C	80	73.3
60°C	140	87
90°C	287	147
Swelling power (SP) %		
30°C	268	244
60°C	285	265
90°C	414	284
Light transmittance (%)		
24h	0.089	0.129
48h	0.073	0.09
72h	0.053	0.082
96h	0.029	0.08
120h	0.019	0.069

Table S2: Iodine binding capacity of native maize flour (NMF) and amylase treated maize flour (ATMF) at 460 nm and 570 nm

Samples	Wavelength(nm)					
	Starch Concentrations %w/v (460 nm)			Starch Concentrations %w/v (570 nm)		
	1%	3%	5%	1%	3%	5%
NMF	0.13	0.31	0.35	0.12	0.15	0.25
ATMF	0.15	0.33	0.36	0.14	0.28	0.33

Table S3: Complexation of native maize flour (NMF) and amylase treated maize flour (ATMF) with fatty acids and butyric acid

Starch Samples	NMF	ATMF
Without FA complexation		
ISI -460nm	0.048	0.116
ISI-570nm	0.071	0.103
With butyric acid complexation		
ISI-460	0.011	0.092
Δ460	0.037	0.024
ISI-570	0.015	0.057
Δ570	0.056	0.046

Table S4: Digestion rate of native maize flour (NMF) and amylase treated maize flour (ATMF) at different Gastrointestinal track compartments

Type of Digestion (Compartment of GI tract)	**Samples**	
	NMF Reducing sugar released (mg)	**ATMF Reducing sugar released (mg)**
Digestion in Mouth by α-amylase		
30 sec	0.016	0.012
60 sec	0.023	0.018
90 sec	0.033	0.027
120 sec	0.049	0.041
Digestion in Stomach (Gastric pH stability)		
	0.015	0.012
40min	0.025	0.013
80min	0.031	0.015
120min		
Digestion in small intestine by pancreatic-α-amylase		
	0.085	0.062
1h	0.163	0.151
2h	0.206	0.192
3h	0.216	0.202
4h	0.229	0.218
5h	0.237	0.221
6h		

Table S5: Solubility of Resistant Starch (RS) in different solvents:

Resistant starch in DMSO.	Resistant starch in ethanol	Resistant starch in methanol	Resistant starch in Dichloromethane	Resistant starch in Acetone	Resistant starch in water
1.2606	0.090	0.234	0.093	0.108	0.206

Table S6: Solubility of Ciprofloxacin HCl in different solvents:

Ciprofloxacin in DMSO	Ciprofloxacin in ethanol	Ciprofloxacin in methanol	Ciprofloxacin in Dichloromethane	Ciprofloxacin in Acetone	Ciprofloxacin in water
0.291	0.292	0.250	0.023	0.04	0.380

Table S7: Iodine staining index of RS at 460 and 570 nm:

Starch concentration (%w/v)	Absorbance at 460 nm	Absorbance at 570 nm
Native starch	0.007	0.0016
Resistant starch	0.06	0.05

Table S8: Calibration curve of ciprofloxacin HCl in distilled water

Sr NO.	Concentration in mg/mL	Absorbance at 271 nm
1	0.1	0.020
2	0.2	0.041
3	0.3	0.062
4	0.4	0.081
5	0.5	0.110
6	0.6	0.120
7	0.7	0.140
8	0.8	0.161
9	0.9	0.18

Table S9: Calibration curve of ciprofloxacin in HCL buffer of (pH 1.2)

Sr number	Concentration in mg/ml	Absorbance at 271 nm
1	0	0
2	0.1	0.124
3	0.2	0.21
4	0.3	0.31
5	0.4	0.392
6	0.5	0.481
7	0.6	0.582
8	0.7	0.681
9	0.8	0.782
10	0.9	0.883

Table S10: Concentration versus absorbance of ciprofloxacin in phosphate buffer of (pH 6.8)

Sr number	Concentration mg/mL	Absorbance at 271 nm
1	0	0
2	0.1	0.12
3	0.2	0.2
4	0.3	0.28
5	0.4	0.36
6	0.5	0.44
7	0.6	0.52
8	0.7	0.6
9	0.8	0.68
10	0.9	0.76
11	1	0.84

Table S11: Calibration curve of ciprofloxacin in phosphate buffer of (pH 7.8)

Sr number	Concentration mg/Ml	Absorbance at 271 nm
1	0	0
2	0.1	0.11
3	0.2	0.18
4	0.3	0.26
5	0.4	0.31
6	0.5	0.37
7	0.6	0.42
8	0.7	0.48
9	0.8	0.54
10	0.9	0.61
11	1	0.67

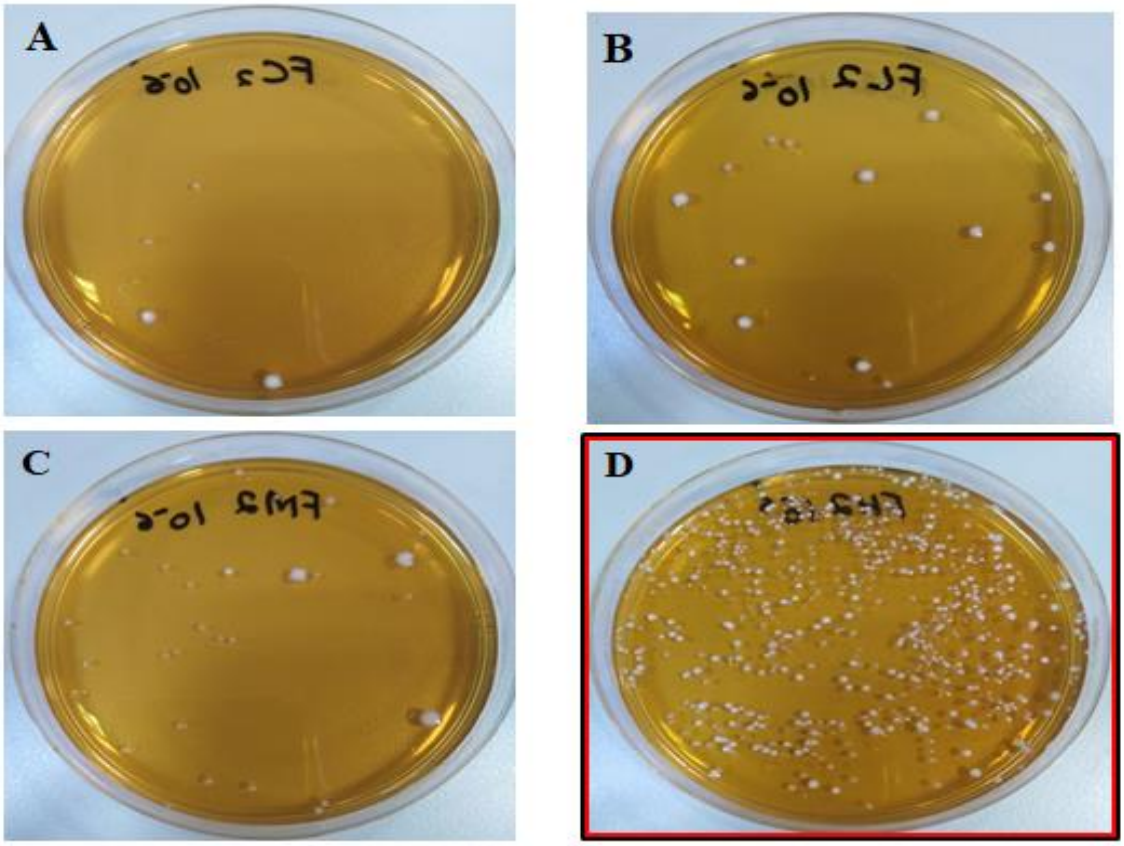

Figure S1: Isolation of Faecal Probiotic bacteria on de Man, Rogosa and Sharpe agar **A**: Control Fed Diet, **B:** Low EM-RSIII Fed Diet C: Medium EM-RSIII Fed Diet, **D: High** EM-RSIII Fed Diet

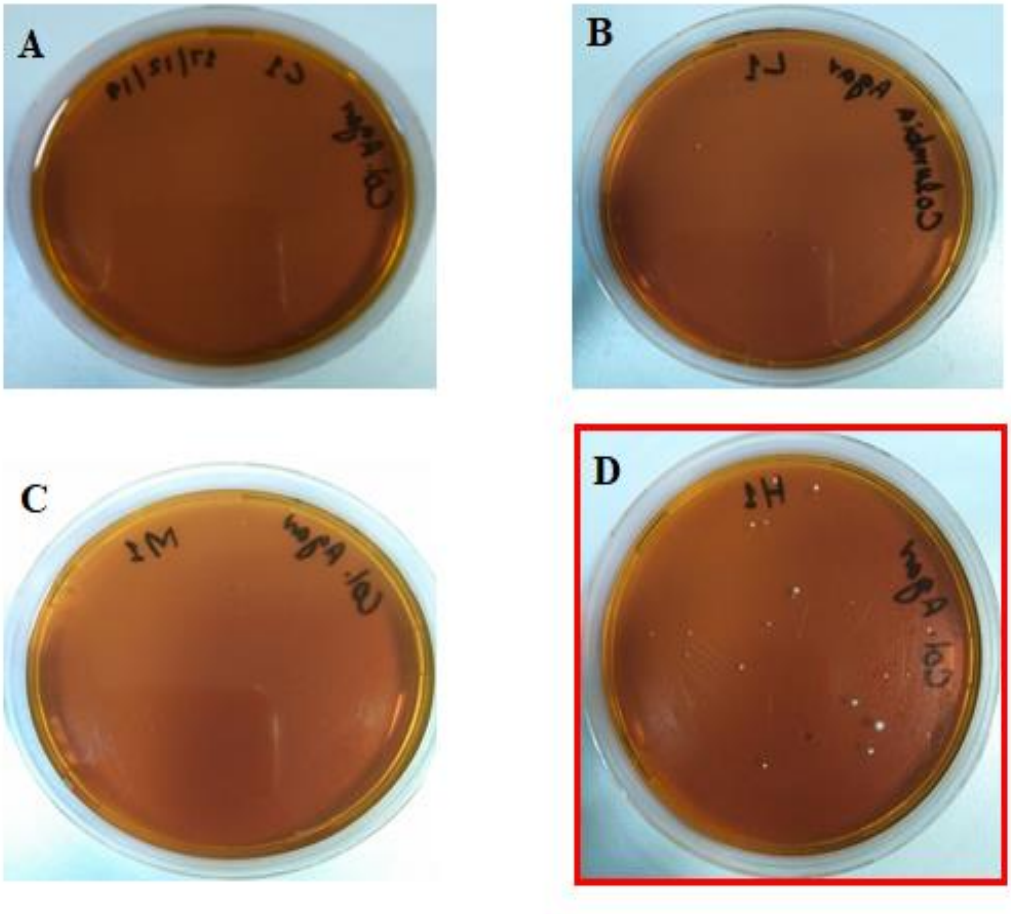

Figure S2: Isolation of Faecal Probiotic bacteria on Modified Columbia agar plates **A**: Control Fed Diet, **B:** Low EM-RSIII Fed Diet, **C**: Medium EM-RSIII Fed Diet, **D: High** EM-RSIII Fed Diet

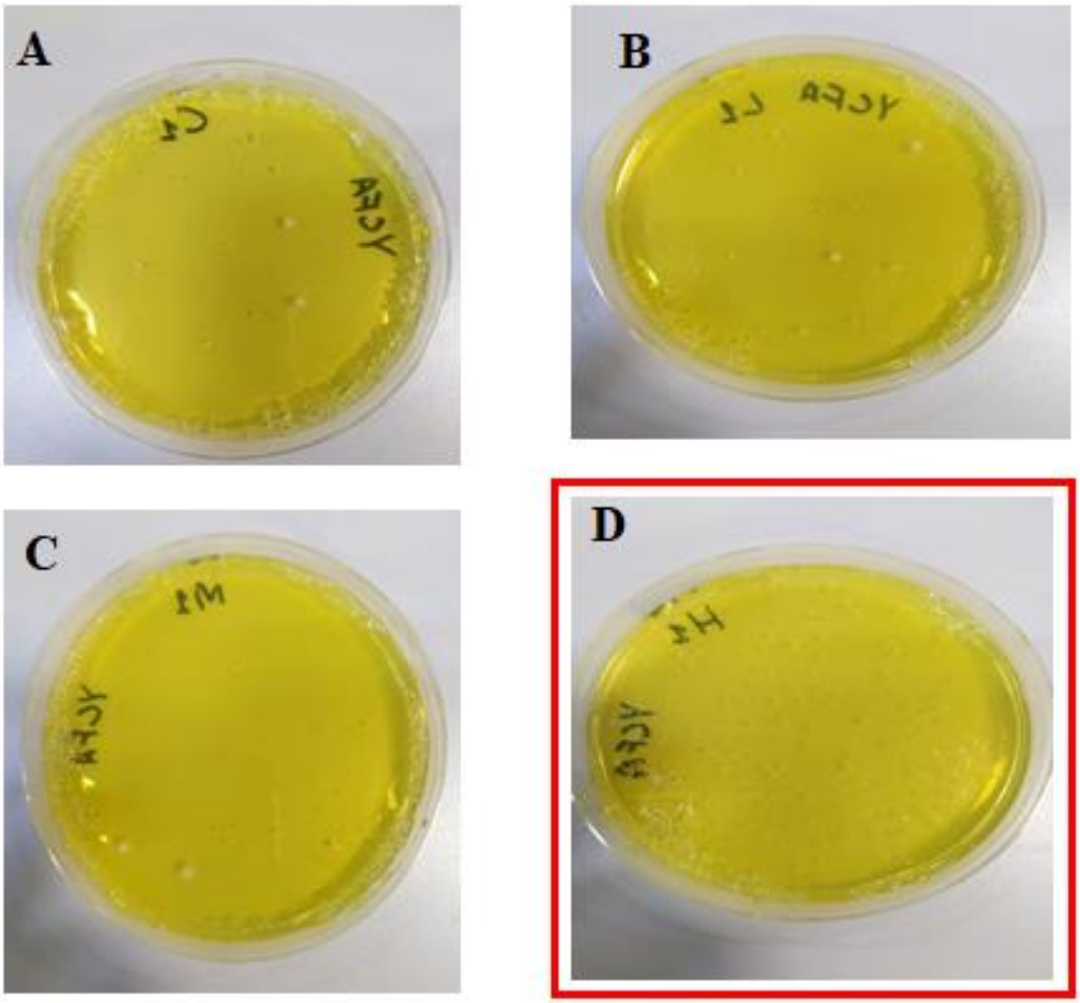

Figure S3: Isolation of Cecal Probiotic bacteria on Modified YCF agar plates, **A**: Control Fed Diet, **B:** Low EM-RSIII Fed Diet **C**: Medium EM-RSIII Fed Diet, **D: High** EM-RSIII Fed Diet

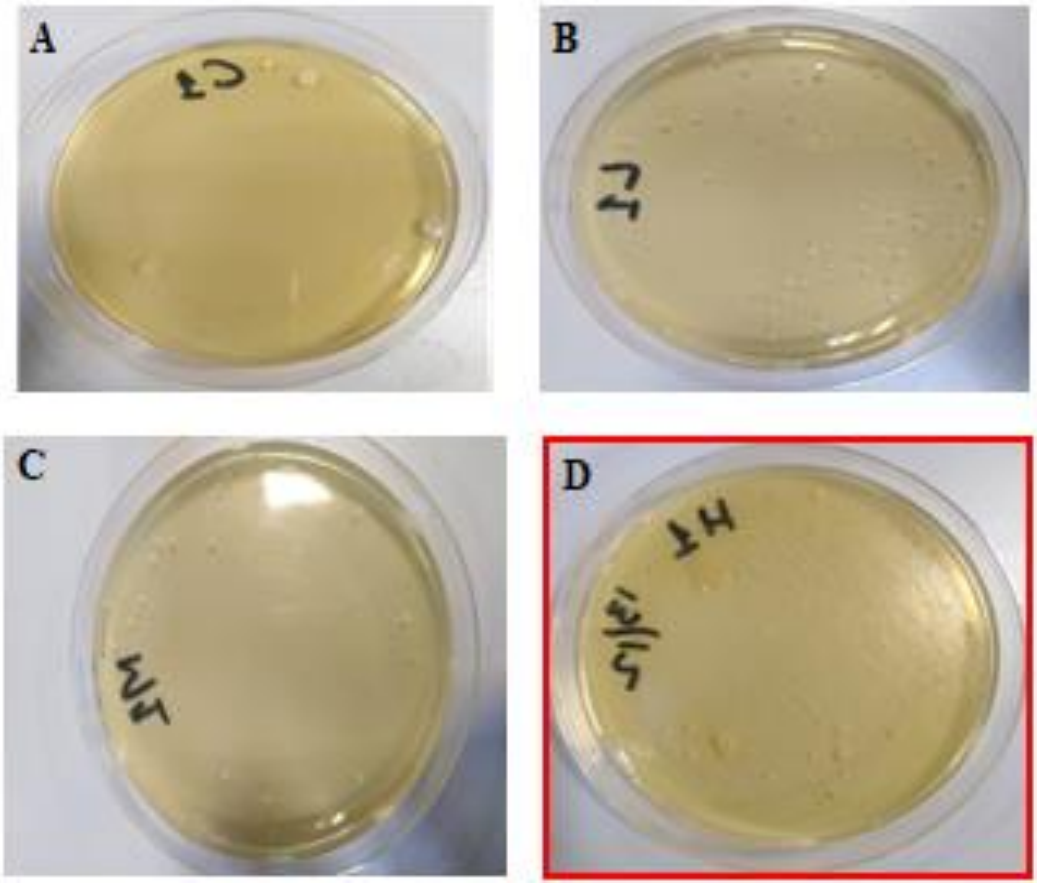

Figure S4: Isolation of Cecal Probiotic bacteria on Rogosa agar plates, **A**: Control Fed Diet, **B:** Low EM-RSIII Fed Diet C: Medium EM-RSIII Fed Diet, **D: High** EM-RSIII Fed Diet

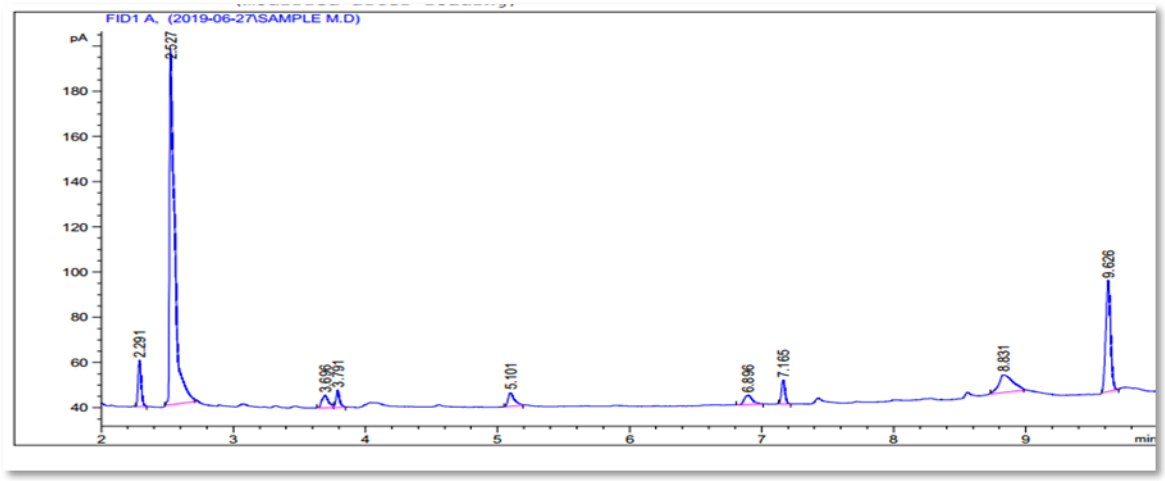

Figure S5: GC-spectra of Fecal sample of control Fed rat's sample

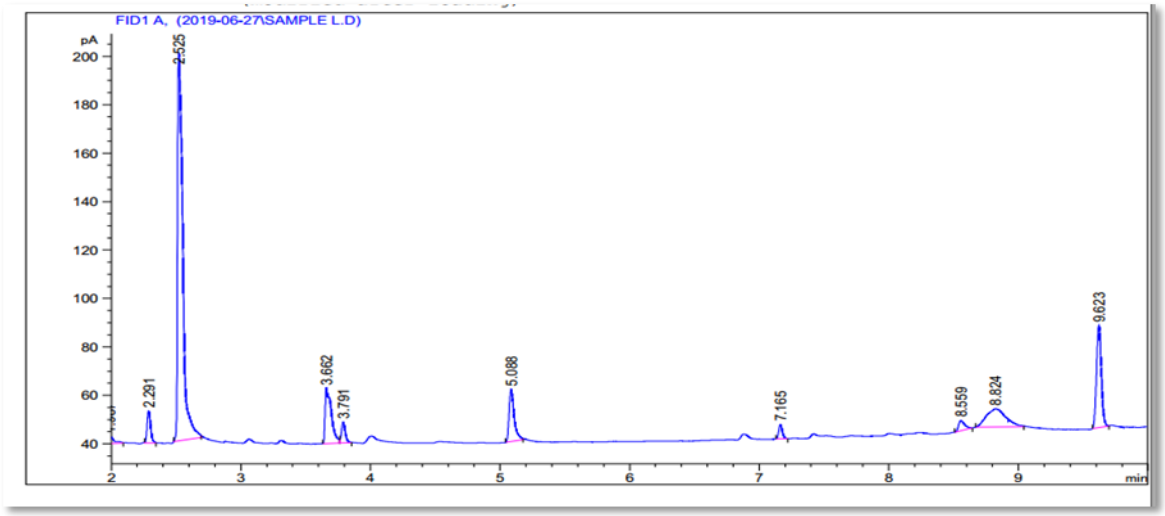

Figure S6: GC-spectra of Fecal sample of Low RS III Fed rat's sample

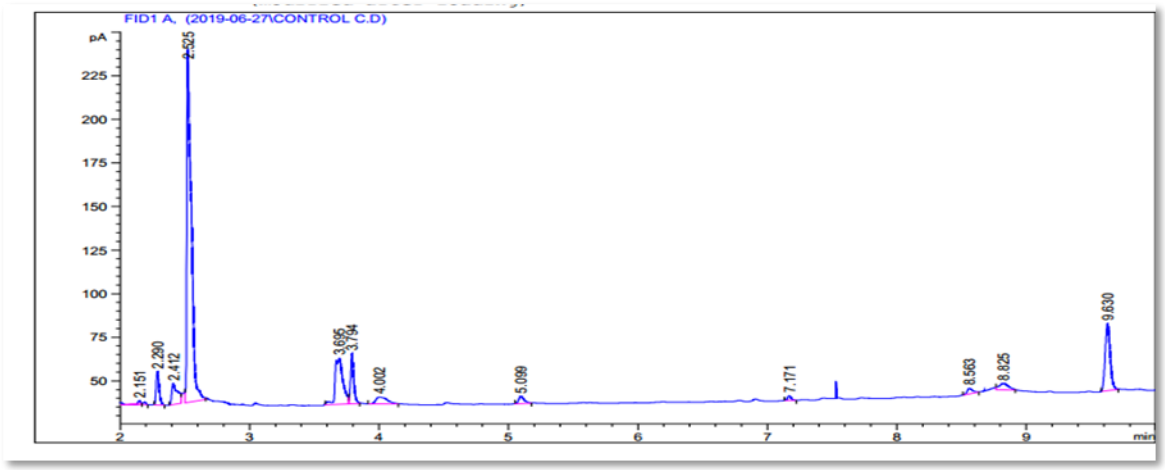

Figure S7: GC-spectra of Fecal sample of Medium RS III Fed rat's sample

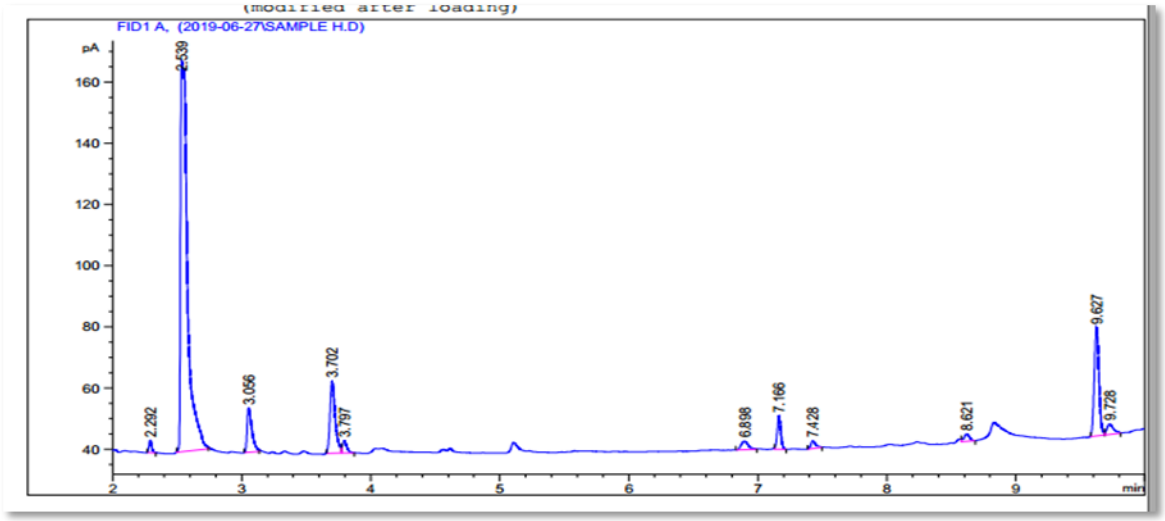

Figure S8: GC-spectra of Fecal sample of High RS III Fed rat's sample