Synthesis and Characterization of Enzymatically Produced Resistant starch coated Drug Microspheres and their Antimicrobial Potential

By

(Amna Bibi)

Department of Microbiology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad
2019

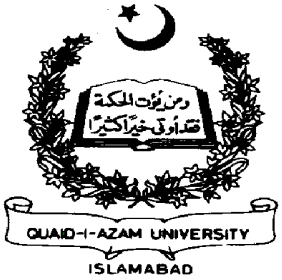
Synthesis and Characterization of Enzymatically Produced Resistant starch coated Drug Microspheres and their Antimicrobial Potential

A thesis submitted in partial fulfillment of the requirements for the Degree of

Master of Philosophy

In

Microbiology



By

Amna Bibi

Department of Microbiology

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad

2019

“IN THE NAME OF ALLAH THE MOST BENEFICENT, THE MOST MERCIFUL”

Dedication

I dedicate this humble effort of mine to my dearest *Abu*, *Ami* and Brothers.

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Amna Bibi

Certificate

This thesis submitted by ***Amna Bibi*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

Supervisor: _____

(Dr. Samiullah Khan)

External Examiner: _____

(Name)

Chairman: _____

(Dr. Rani Faryal)

Dated: _____

Amna Bibi

Contents

List of Figures

List of Tables

List of Figures

List of Abbreviations

RS	Resistant starch
Cipro	Ciprofloxacin
MPs	Micro particles
SEM	Scanning electron microscopy
XRD	X-ray Diffraction
FTIR	Fourier transform infrared spectroscopy
DLS	Dynamic light scattering
pul	Pullulanase
pH	power of hydrogen ion con concentration
mM	Milli molar
gm.	gram
RDS	Rapidly digesting starch
SDS	Slowly digestible starch
GOPOD	Glucose oxidase peroxidase reagent
M	Molar solution
Con	Concentration
KOH	potassium hydroxide
SCFA	Short chain fatty acids
mL	Milli liter
IR	Industrial resistant starch
OD	Optical density
Hrs.	hours
i.e.	That is
mg	Mili gram
℃	Degree Celsius/Centigrade
AMG	Amyloglucosidase
Min	Minute
%	Percentage

Acknowledgments

All praises to ***Almighty Allah,*** *The Light of Heavens and Earths, The One Who put good thoughts in one's mind, turn them into determinations and then makes the way towards their fulfillments showering all His Blessings throughout the journey.*

Best of the praises and Peace be upon all the Sacred Messengers and especially for the Last of them ***Hazrat Muhammad (SAWW)*** *who are the minarets of knowledge for all the mankind, who is forever a torch of guidance for the knowledge seekers and humanity as a whole.*

It is my privilege and honor to work under the supervision of ***Assistant Professor Dr. Samiullah Khan****, Department of Microbiology, Quaid-i-Azam University (QAU), Islamabad, Pakistan. I wish to express my deepest gratitude for his expert guidance, appreciation, sincere advice, and support during the period of my research work. I am also thankful to* ***entire Faculty*** *and* ***Chair person Dr Rani Faryal*** *her endless encouragement and familiar deeds have been the major driving force throughout my research work.*

I feel a deep sense of gratitude to Miss. ***Anum Khan****, Ph.D. student for help, useful suggestions, enabled me to complete my work successfully.*

I appreciate the support and encouragement of all my friends and colleagues including Dr.Shakirullah,Mr.irfan,Mr.Hazrat noor,Mr Rifaq,Mr Asaf,Mr Asad,,MrTalmeez,Mr Habib,,Mr Habibullah,Ayeisha,Mahnoor,Faryal,Huma,Safia,Hira and Asifa during my research.

I also express my sincere gratitude to Dr. Asim.,Dr Fakhar, and Miamooma Qindeel from Department of Pharmacy, for their help, and cooperation in my research work. I am very thankful to my ***parents and family*** *for their useful guidance and support during my study.*

Amna Bibi

Abstract

The aim of the present research was to synthesize and characterize enzymatically produced resistant starch coated drug microspheres,for the colon targeted delivery of Ciprofloxacin (Cipro).Resistant starch (RSIII) was produced by the debranching of maize flour using microbial amylase and pullulanase enzymes isolated and purified from *Bacillus licheniformis*.The single emulsion evaporation method was used for the synthesis of the microspheres,by the dropping of organic phase containing RS(III) and drug in dimethyl sulfoxide (DMSO) into aqueous solution containing 0.5% tween 80 as stabilizer under stirring.The encapsulation efficiency of 86.73% was found.The size and polydispersity index (PDI), was measured and analyzed by Dynamic Light Scattering (DLS**)**,X-ray Diffraction (XRD) analysis indicated that crystalline form of Ciprofloxacin is completely transformed into amorphous form and drug is encapsulated in resistant starch. The light microscopy and Scanning Electron Microscopy (SEM) showed that the different flat, compact block like structures of Resistant Starch were converted into the spherical microparticles, while Fourier Transform Infrared Spectroscopy (FTIR) results showed chemical and electrostatic interactions between the RS(III) and Ciprofloxacin used for the preparation of microspheres.Antimicrobial potential was measured by agar well diffusion method against *E.coli* and *S.aureus.*For *in vitro* drug release studies, RS(III) coated drug microspheres were incubated in simulated gastric fluid, intestinal fluid and colonic fluid, respectively.RS(III) remain stable and retarded drug release in stomach and small intestine,about 5% of drug was released in(stomach)acidic medium while at pH 7.8 drug release was about 52%.Release profile of drug from microspheres was evaluated by using various kinetic models these models showed concentration dependent sustained drug release.Drug release studies have shown that the enzymatically produced resistant starch could deliver the drug to the colon.These results indicate that the resistant starch prepared by bacterial enzymes can be used as a potential drug carrier in colon-targeted drug transport system.

Key words; Resistant starch, Ciprofloxacin, Microspheres, In vitro release, Antibacterial potential.

Introduction

One of the broad research field in pharmaceutical sciences is the drug delivery, for which novel material appropriate for the controlling of drug release based on therapeutic needs to be developed(Wang, Hu et al. 2010, Carbinatto, de Castro et al. 2012, Prezotti, Meneguin et al. 2012) Modified starch that contains 70% of amylose have improved properties for controlled drug delivery.(Abbas and Baeten 2016;Soares, de Castro et al. 2013)As the bio-based RSIII has public acceptance hence its potential use for food industry is potentially favored. The RSIII has high resistance making it resistant to enzymatic digestion, hence using it as a coating material for vitamin, probiotics and controlled drug delivery into colon.

The RSIII has resisted enzymatic digestion even after 120 min (Ashwar, Gani et al. 2016) and granules water compartments that entrape molecule and affects action of delivery.Hence the polysaccharides serve for and effective drug delivery purposes via gastrointestinal tract (Singh, Dhiman et al. 2016).Release of drug into GIT and its spatial control represents a crucial research topic in field of drug delivery as particular targeting to tissue or organ can expand the bioavailability of numerous drugs specifically those that are stable, soluble or have permeability problems in drastic conditions of the upper part of GIT i.e. stomach.But to find an appropriate and efficacious way to overcome the problem. Colon provides conditions like slow transit time, less proteolytic activity and pH close to neutral that makes the environment favorable for drug delivery mainly peptides and proteins.

For drug targeting, coating of solid dosage forms with polymeric material that is degraded by the colonic microbiota or whose solubility is dependent on pH is quite a relevant approach.Additionally, this polysaccharide could escape the digestion and it is fermented under enzymatic action of the colonic bacteria (Mura, Cirri et al. 2016). Though starch is non-toxic but native starch is modified physically and chemically to enhance its properties (da Rosa Zavareze and Dias 2011). So the starch derivatives are being used in making of micoparticles, tablets and coating films in order to achieve a required drug release rate (Marinich, Ferrero et al. 2012: Teacă, Bodîrlău et al. 2013, Meneguin, Cury et al. 2014). The RSIII resists the enzymatic digestion in duodenum,

stomach but ultimately degraded by the microbiota in colon (Htoon, Uthayakumaran et al. 2010). Properties of resistant starch make it a favored candidate for designing the colon-based delivery of drug system. The RS is categorized into various subtypes, one such is retrograde starch (RSIII) which is prominent because of its low solubility, hardness, birefringence analysis, rheological and thermal stability hence making it appropriate for the colon based drug delivery after testing its digestion against the pancreatic enzymes.

The antibiotics administrated orally have very limited bioavailability. Some portion of dose is metabolized or even expelled out of body before biological materials could overcome it. So in order to maintain the therapeutic drug concentration at the site of infection more enhanced and frequent dose is required. But unfortunately such dosage may cause high level of toxicity and cause side effects that is inconvenient for patients. Though some antibiotics that are delivered intravenously they have 100% bioavailability, but still fail to reach some body parts like bone. Additionally, another obstacle is formation of bacterial biofilm responsible for infection in patients with medical devices like urinary catheters or implants. The conventional antibiotic therapy is sometimes not effective much due to enhanced bacterial resistance inside biofilms. The increased resistance may also be due to lack of proper use or using of broadspectrum antibiotics. All such problems have paved the way for development of effective infection therapies, novel drug delivery systems(DDSs) way that can help in making treatment more effective.

Use of polymers for purpose of drug delivery opens up new possibilities for antibiotics delivery to the particular tissues, cells etc. and provides an effective way for drugs to reach at infection sites. Various other advantages involve drug modifications and its pharmacokinetics (controlled release, protection against elimination or fast degradation) (Chen, Li et al. 2018). So drug dose must be reduced and administrated less frequently to improve the patient amenability. Active compounds delivery to the colon can prove to be quite effective for numerous applications like reduce administrated dose, avoiding unnecessary digestion, improved bioavailability of drugs for the systemic absorption, side effects of anti-inflammatory drugs for local treatment etc (Afifi, Mandour et al. 2015). Also RSIII retrograded starch was also reported to be

used as film coating material with a high affinity for the colon-based drug delivery merely by physically modifying it (Chen, Yu et al. 2007) Coating of drug by adding various material could improve the mechanical strength that would prevent unnecessary early release (Yang, Salas et al**.** 2016). Nano drug delivery systems in patient treatment, and its safety is of great concern. Smaller size nanoparticles show highest toxicity due to their increased surface to volume ratio (Dunphy Guzman, Taylor et al. 2006). Nanoparticles synthesized from metals such as copper, cobalt, titanium and silicon and of their respective oxides have inflammatory and severe toxic effects on cells (Dunphy Guzman, Taylor et al. 2006)). An innovative oral colon based delivery of drug has been developed by using resistant starch (Chen, Pu et al. 2011) The present research focuses on the development of a colon-specific sustained release system in the form micro particles by a single emulsion evaporation method. The approach is based on two main parts; the first part is the preparation of RSIII. It is prepared in form of paste by the high pressure/temperature (HTP) followed by the enzymatic debranching and retro gradation. Efforts were also made to determine the RSIII resistance to acidic pH (gastric), intestinal enzymes present in upper gastrointestinal tract by observing its resistance to digestion and various characteristics (Chen, Li et al. 2018). The second part deals with preparation of drug loaded microspheres and their characterization t. An active compound, Ciprofloxacin was selected as a model drug because it was easily available and its surveillance was comparatively easy, to study the behavior during the release from microspheres in the *in vitro* experiments in the conditions of simulated gastrointestinal fluids (Chen, Li et al. 2018). The drug chosen was encapsulated in the resistant starch microspheres. It belongs to the second drug generation i.e. antibacterial fluroquinolones extensively used to deal with numerous infectious diseases like urinary tract infections, osteomyelitis, respiratory, gonococcus and enteric infections.

The purpose of current study was to synthesize resistant corn starch crusted drug loaded microspheres and their characterization. The influence of solvent extraction and enzymatic debranching on granular configuration and morphology of starch samples were analyzed by the FTIR, XRD and SEM (Chouhan, Bajpai et al. 2010). Parameters like chemical composition, size of microspheres, encapsulation efficiency morphology release behavior, antimicrobial affinity of enzymatically formed RS-based

Ciprofloxacin loaded microspheres were examined analytically.Further it was studied on basis of particle size value, drug loading efficiency, antimicrobial study, X-ray diffraction,FTIR analysis, morphology, in vitro discharge comportment of ciprofloxacin. The FTIR studies showed an interaction between RSIII and drug. Sample formulation was studied to be stable with desirable drug entrapment efficiency (86.73%), its size ranged in micrometers 964 nm about 1µm. *In vitro* studies indicated a cumulative drug release (> 40%) for up to 8 h was observed.

Challenge in field of functional foods for the aim of disease deterrence and health elevation is to know about the incorporation of bioactive compounds in food to maintain its stability in food processing, for storage and in GIT system. It is necessary to enable the availability of such compounds by the controlled release at the target site in GIT (Chen, Liang et al.2016).

Aim and Objectives

Aim:

Aim of the current study was to utilize the natural, biodegradable and biocompatible enzymatically produced resistant starch for the synthesis of drug loaded microspheres in colon targeted drug delivery.

Objectives:

1. Preparation of Drug loaded microspheres through Single emulsion evaporation technique and optimization of process parameters.

2. Evaluation of microspheres and drug loading through different characterization techniques.

3. Evaluation of sustained drug release from microspheres in terms of *in vitro* release studies.

4. Determination of antimicrobial potential of microspheres by using agar well diffusion method.

Review of Literature

2.1 Enzymes:

Enzymes are natural catalytic bodies which are responsible for catalysis of biochemical reactions in a living cell. They speed up conversion of substrate into product by lowering down activation energy(Mollania, Khajeh et al. 2010).As they can accelerate the reaction rate up to 10^{12} times, so they are considered as highly effective biocatalysts. In their absence, many metabolic reactions would be too slow for a normal metabolism.

Enzymes have the potential to specifically catalyse their respective substrates in a chemical reaction. Substrate specificity makes enzyme more beneficial for industrial use (Li, Yang et al. 2012).For catalytic activities enzymes are more preferred than chemicals because of many reasons. Some of them are; regiospecificity, ease of product formation, economic feasibility, optimization and modification etc., Enzyme catalysis results in production of a product in pure state. Making modification and optimization of product easily. Which further lowers down the overall downstream problems and cost of purification.

All enzymes are protein in nature. They are biomolecules composed of long linear amino acid chains that are folded into a 3D structure. Catalytic site (a small portion of enzyme consists of 2-4 amino acids) perform substrate (Gurung, Ray et al. 2013). Enzyme catalysed processes have attained more interest because they are non-toxic, cost effective, eco-friendly and required less processing time. Today, enzyme is considered as a best substitute for the previously used toxic chemical catalysts. Thus their use on industrial scale is more beneficial. Microbial enzymes have been used from old Greek times up till now for different applications such as baking, alcohol, cheese production, brewing and in pharmaceutical and chemical industries (Dhar, Banerjee et al. 2012) The global industrial enzyme market is estimated to exceed 7.1 billion dollars by 2018 that would have about 8.2% CAGR (Dhar, Banerjee et al. 2012). In the global enzyme market, carbohydrases have the maximum share of approximately 37% and have been mainly utilized in bakery. Different hydrolytic enzymes are used in industries for processing of starch. Where starch containing biomass such as, wheat, potato, sago,

banana etc. are utilized as substrates for production of energy, pharmaceutical products, chemicals and biofuels.

Both enzymes and chemicals are exploited for breaking of polymeric starch into monomers. Previously, chemicals and acids were used for starch hydrolysis but they are now replaced by enzymatic hydrolysis, which is more efficient and beneficial. Enzyme specificity and hydrolytic properties results in a syrup of sugar with less side reactions and undesirable changes (browning).

2.1.1 Starch Hydrolysing Enzymes:

A starch usually contains amylose and amylopectin. Starch hydrolysed into low molecular weight monomeric compounds by group of enzymes.

Generally, Starch hydrolysing enzymes can be divide into the following four groups: (Van Der Maarel, Van der Veen et al. 2002)

1. Endoamylases

2. Exoamylases

3. Debranching enzymes

4. Transferases (Van der Veen et al. 2002)

2.1.2Debranching Enzymes:

Hydrolysis of α-1, 6-glycosidic bonds OF starch molecule is carried by these enzymes. and convert starch into low molecular weight particles. Pullulanase and Isoamylase are categorized in this group.(Van Der Maarel, Van der Veen et al. 2002)

2.2 Starch:

Starch is the well-known polysaccharide found on the earth. It is considered as one of the most vital component of life because of its usage as energy source specifically as an animal and humans feed across the whole world (Cherubini and management 2010)(Streb and Zeeman 2012)It is 98-99% composed of two D-glucose

homopolysaccharides namely amylopectin and amylose. Starch obtained from most plants consists of amylopectin and amylose.These two alpha-glucans are further made up of number of glucose units connected with glycosidic bonds (Wandee, Uttapap et al.
2017).

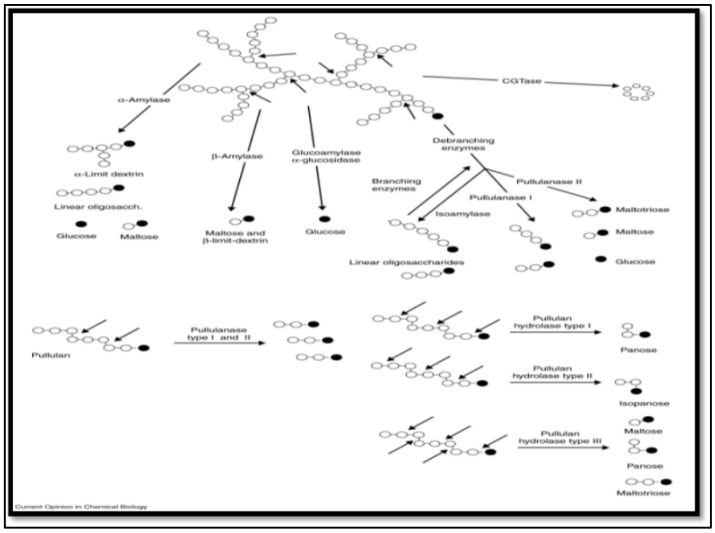


Figure 2.1: Action pattern of starch degrading enzymes (Yang, El-Ensashy et al. 2013)

2.2.1 Amylose:

Amylose is one of the major constituent that made up polymeric starch.,Mostly occurs in a liner configuration and is connected by alpha-1-4 glycosidic linkages (Parada and Aguilera 2009).Its degree of polymerization is studied to be 6000. mylose have an average molar mass of nearly 105-106g/mol. Most commonly, it is found in a right handed helical or spiral structure (Karmakar, Ban et al. 2014, Kong, Zhu et al. 2015).

2.2.2 Amylopectin:

The second major component of polymeric network of starch is generally known as amylopectin. Which is extensively branched and contained 1-6 glycosidic linkages. This homopolymer is abundant in glucose units which are arranged in a linear configuration (Karmakar, Pahari et al. 2014)Figure 2.2.2 shows amylopectin`s structure.(Kalinga, Bertoft et al. 2014)

2.3 Classification of starch:

Several enzymes, convert starch into its monomer i.e. glucose. For example in digestive system, starch is first encountered by α-amylase of oral cavity. Then its digestion is further conducted by pancreatic enzymes in small intestine(Syahariza, Sar et al. 2013)For the purpose of nutrition, starch is classified into different fractions. Such classification is mainly based of degree of digestibility of starch.

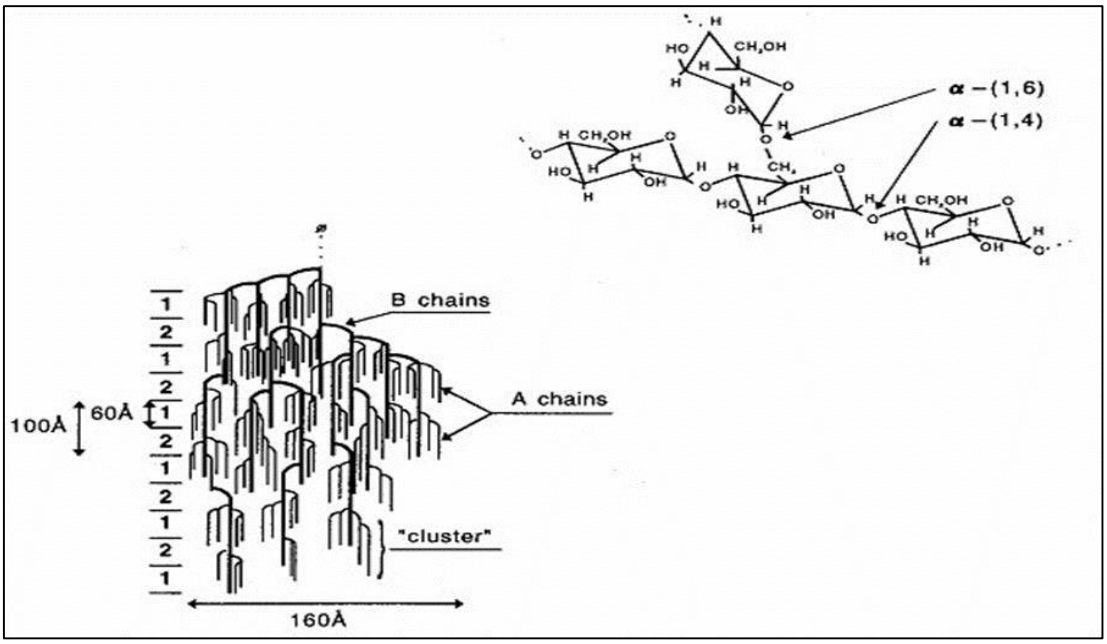


Figure 2.2 Amylopectin cluster model indicating A, B and C chains (Whistler and BeMiller 1997)

2.3.1 Rapidly digestible starch (RDS):

Is a fraction of starch which is digested by enzymes rapidly, when ingested, in just 20 minutes. Such fraction is naturally in amorphous form. When consumed, RDS is quickly hydrolysed into monomers (glucose), by the action of small intestine enzymes. This rapid release of glucose units into the bloodstream cause increased sugar and insulin level in blood. Therefore, considered a potential cause of diabetes and cardiovascular diseases.

2.3.2 Slowly digestible starch (SDS):

Starch which gets digested in a time interval of 20-120mins is called SDS. Invitro digestion models of SDS proposed that SDS after the first 20 mins is first hydrolysed into glucose by α-amylase of intestine. While SDS complete digestion is occur within 120 mins. This causes a slow release of glucose in the blood. (Lehmann, Robin et al. 2007)

2.3.3 Resistant Starch (RS)

RS is the content of starch that is resistant to enzymatic digestion by pullulanase and αamylase treatment in vitro. It is considered as an important part of total dietary fibre (TDF) and resist to digestion by enzymes of small intestine (Lehmann, Robin et al. 2007). RS resists the action of α-amylase and Pullulanase for more than two hours and is not converted into glucose/dextrin. In its intact form, RS escapes from small intestine into large intestine in a healthy individual. There, it is degraded by the colonic microbiota. (Lockyer and Nugent 2017). RS content is directly proportional to the content of amylose the starch.have been proposed a positive correlation between amylose contents of maize starch and RS content. Resistance starch fermentation in large intestine by colonic microbiota results in the reduction of pH of intestine, phenols, acids and ammonia. Which further promotes the production of shot chain fatty acids (SCFAs) especially butyrate. SCFAs are anti-inflammatory and anti-carcinogenic maintaining colonic health(Zhang, Tang et al. 2012)

2.3.4 Status of research interest in the field of resistant starch:

One of the important energy source for humans is resistant starch. Starch normally have two physical forms i.e. granular and amorphous starch. The former resists rapid digestion while the later form gets readily hydrolysed leading to increased blood glucose level which can cause serious health issues (Juntunen, Niskanen et al. 2002). (Sekirov, Russell et al. 2010)

RS has several health benefits by producing short chain fatty acids. Short chain fatty acids produced with the colonic fermentation of RS, helps to control diabetes, obesity and reduces the risk of cardiovascular diseases(Fuentes-Zaragoza, Riquelme-Navarrete

et al. 2010) (Paterson, Wendel et al. 2012)Moreover, RS has numerous physiochemical properties making it a suitable food ingredient.(Simsek and El 2012)

Both RS and SDS as compared to RDS, are more health beneficial as they reduce the risk of; diabetes incidence by prolonged blood glucose release, harmful metabolic disorders and colonic cancer. Because of such potentials, the interest of nutritionals have been increased in RS(Reddy, Suriya et al. 2013)

2.4 Classification of resistant starch:

On the basis of conformational and structural changes and addition of RS to the polymer, RS can be categorized to five types i.e. RS1-RS5 (Haralampu 2000)

2.4.1 Resistant starch type I:

RSI is a type of starch that is difficult to digest due to the presence of external hard coating surrounding the grain. It is also known as physically inaccessible starch entrapped inside partial or whole milled seeds and grains.(Singh, Dartois et al. 2010)

When grains are meshed and milled, the protecting matrix finally broken down exposing the starch on the interior of matrix to the action of amylase (Singh, Dartois et al. 2010, Alsaffar and technology 2011)

2.4.2 Resistant Starch type II:

RSII is a kind of resistant starch whom resistance to digestion is because of configuration and organized structure of the raw native granules. (Lehmann, Robin et al. 2007)On contrary, RSII upon heating converted to an easily digestible amorphous structure which is more susceptible to enzymatic hydrolysis(Jyothsna, Hymavathi et al. 2017) Both RS1 and RS2 can lose their resistivity and rigidity to digestion, during the process of cooking.

2.4.3 Resistant starch type III:

RSIII is also known as retrograded starch because when it is heated at high temperatures its amorphous polymers gelatinized completely. Through which it exhibits a robust

crystalline configuration that is recalcitrant to action of enzyme in the small intestine. This property also causes the starch to be high heat stable, which is more beneficial when using this starch in food and other edible products. This kind of starch can sustain a heat of about 120℃.(Yuan, Xu et al. 2011)

2.4.4 Resistant starch type IV:

RS IV is also referred as chemically modified starch because it is synthesized through chemical modification and its acceptance is low because of consumer's choice. In which different functional groups including esters, ethers or phosphate groups are added to starch for making it resistant to hydrolytic action of various enzymes(Zięba, Szumny et al. 2011)The resistivity of starch can be further enhanced by adding resistant content i.e. corsslinkers.

2.4.5 Resistant starch type V:

RS V is a complex synthesized from the combination of linear amylose and lipids. The starch used here is high in amylose that at high temperatures quickly gelatinizes and is quite susceptible to retro gradation. (Ng, Fleming et al. 2014).

2.5 Important Characteristics of Resistant starch type III:

There are many methods for the synthesis of RS but synthesis through enzyme is mostly preferred. Because enzymatic synthesis is safe, cost effective, thermal stable, ecofriendly, quick and effectively improve the percentage of RS contents(Raturi 2016).

2.5.1 Physical Modifications:

RSIII physical modification can be carried out by exposing resistant starch to high thermal treatment and gelatinization at high-pressure and then finally storing at low temperature (Zhou, Baik et al. 2010)While other methods include annealing, pressure treatments, freezing and hydrothermal treatments.Starch of treated rice showed more RS content (30.31-38.65%) than starch of untreated rice (4.42-10.94%) (Ashwar, Gani et al. 2016)Longer the cooling greater will be the percentage of retrograded starch with higher RS content(Hidayat, Muslihudin et al. 2018).

2.5.2 Chemical Modifications:

Chemical modifications lead to increase in the content of amylose. This method includes alcohols and acids treatment which hydrolyse the amorphous regions of starch especially amylopectin portion. Treatment with acids and alcohols are included in chemical modifications which cause hydrolysis of starch amorphous regions particularly the amylopectin branches producing linear residues(Rafiq, Singh et al. 2016)

2.5.3 Enzymatic debranching:

Enzymatic treatments are also done to modify the starch making it more resistant to digestion till small intestine while its digestion is by colonic bacteria. The increase in RS is primarily because debranching enzymes which increases the amylose content. This causes an increase in its retro gradation which results in tightly packed, organized crystals of amylose units contributing ultimately to enhanced crystallinity(Ma, Shen et al. 2011). Pullulanase and Isoamylase are the most commonly used debranching enzymes for modification of starch.

2.5.3.1 Reaction pattern of pullulanase:

Starch is composed of amylopectin,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 hydrolysing pullulan, amylopectin and all other saccharides containing α-(1,6)-glycosidic linkages.

2.5.3.2 Reaction pattern of Alpha amylase:

Amylolytic enzymes is the class of all the enzymes which can cleave the glycosidic bonds present in the starch molecule. These enzymes are produced by all living organisms (Guo 2018).And α-amylases results in cleavage of the α-1, 4 glycosidic linkages liberating oligosachharides with alpha configuration in starch. For example, on comparison of alpha amylases produced from *Bacillus licheniformis, Pseudomonas fluorescens,* the later has the best catalytic action because of its ability to attack on

multiple chains of starch polymer producing low molecular weight products (Ooms, Vandromme et al. 2018).

2.6 Resistant Starch content:

RS contents in food can be determined through several methods. Berry method is the most commonly used method (Berry, 1986), it quantify RS in food after digestible starch removal from the food(Jeong, Han et al. 2018).(Shi, Sun et al. 2019) is most extensively use nowadays.Increase resistant starch content is due to increase amylose content as reported by(Jeong, Han et al. 2018). Linear chain of amylose is formed as a result of amylopectin debranching(Shah, Masoodi et al. 2018).

2.7 Physicochemical properties of Resistant Starch:

2.7.1 Amylose content:

The extent of digestibility can be reduced further by using amylose which form complexes with metals in comparison to carbohydrate alone(Ai and Jane 2018).Therefore enhancement of amylose and RS3 content is occurred by debranching of starch through pullulanase.

2.7.2 Water absorption capacity (WAC):

Starch water holding capacity is called water absorption that is an essential parameter for viscosity(Saito, Tamura et al. 2019).Maxing condition is represented by WAC, increased starch WAC represent increased solubility and leaching.

2.7.3 Iodine Staining Index (ISI):

It is one of the important property of any type of starch due to the iodine residue in starch. The complexing ability of starch can be indicated by this tool. Resistant starch sample must have this ability for its various application like an encapsulating agent, drug carrier, baking and cooking due to interaction of starch with other compounds. Amylase determination is also done by this procedure (Knutson 2000, Lei, Shao et al. 2016)

2.7.4 Fatty acid binding:

(Yotsawimonwat, Sriroth et al. 2008) method can be used for determination of starch sample fatty-acid binding capability with some modification and iodine staining index can be evaluated before fatty acid addition. The difference in starch-FA complex and free starch ISI values (Δ) represent the ability of fatty acid binding to starch in which the formal one is more stable. Butyric acid and palmitic acid is used to represent the short and long chain of starch.(Klaochanpong, Puttanlek et al. 2015)

2.7.5 Antioxidant potential of RS:

DPPH radical scavenging activity and reducing power:

This method is used for the antioxidant potential assessment of starch sample in which antioxidant molecule squinch DPPH purple colour and convert into colourless products detected by spectrophotometrically. Depending on the sample reducing power, various shades of green color are formed. Hydroxyl group present in starch polysaccharide are related directly to the extent of starch antioxidant potential.(Oliveira, Sousa et al. 2008)

Similarly, the free radical scavenging activity is due to hydroxyl group(Oliveira, Sousa et al. 2008, Sreeramulu and Raghunath 2010) Thereby reducing power methods and DPPH scavenging method show increase radical scavenging ability(Ashwar, Gani et al. 2016)

2.8 Digestion of starch:

When mechanical digestion such as chewing and mastication occur starch digestion starts. During this time salivary α-amylase also help in digestion through hydrolysis of the starch glycosidic bonds, α-1,4 glycosidic bonds are cut down randomly by αamylase and products like dextrin's, maltotriose and maltose occur. The process of digestion occurs in stomach where pepsin activation occurs by HCL. A protein, act as a barrier protection for starch is degraded by pepsin and assist in the hydrolysis of starch (Dhital, Warren et al. 2017)After this pancreatic enzyme undergo the resting process of digestion in duodenum (Maughan 2009).Pancreatic amylase also perform the function

as that of salivary amylase and oligosaccharide formation occur which on hydrolysis by the enzyme like sucrose, maltase, lactase form monosaccharide.

The portion of starch that remain undigested e.g.,short chain fatty acid are produced by the digestion of resistant starch in small intestine by bacteria.(Alsaffar and technology 2011)

2.9 Invitro digestibility of Resistant Starch:

In vitro elucidation of starch is being digested through various methods in laboratory (Singh, Dartois et al. 2010)Lipase, pepsin, amyloglucoidase, and pancreatic alpha amylase are used in this process. Gelatinization of starch is one of the method used for hydrolysis of starch enzymatically. After treatment with enzyme it is incubated for 30 minutes at 37°C in shaker water bath. Dinitrosallicylic acid DNS is used for quantification of the products.

Pancreatin, pepsin and salivary α-amylase are used for digesting starch samples sequentialy in the in vitro enzymatic digestion system (Sigma Chemicals Ltd.) This mimic the condition from mouth to small intestine correspondingly(Fässler, Arrigoni et al. 2006) (Pongjanta, Utaipattanaceep et al. 2009)resistant starch was lower in digestion as compared to low resistant starch of native flour containing lower resistant starch.(Giuberti, Gallo et al. 2015).

2.10 Starch derivatives in the emerging field of nanodrug delivery

2.10.1 Nanotechnology

With the help of nanotechnology different problems have been removed that are associated with many of drug molecules. When these particles are converted into micron size their properties like degradation, uptake, flow and physicochemical properties changed completely. This delivery system is very suitable which solved many conventional formulation limitations and related pharmacokinetic limitations (Cevc and Vierl 2010)Many different system related to nanoparticles like, polymeric nanoparticles (Han, Wang et al. 2017)dendrimers(González-Rubio and Liz-Marzán 2018) quantum dots (Joos, Ding et al. 2017)liposomes, have been prepared

successfully. Now a days considerable efforts have been made for biodegradable nanoparticles designing (Kumari, Pandey et al. 2017)Polymers have been utilized that can distribute drugs to specific targeted sites. The main objective of therapeutic system is to release the therapeutic agents continuously at an optimum concentration for a long time (Stebbins 2014).Polymeric nanoparticles have some advantages like, it improves drug molecules stability encapsulated within the polymers. Nanoparticles preparation from these biodegradable polymers occur through natural or synthetic sources. Due some of their improved properties like specific targeted distribution of drugs the biodegradable polymers are very important part of the present research work.(Uchegbu and Schatzlein 2006).

2.10.2 Barriers in Oral Route

Mostly drugs are delivered through oral route due to their great patient compliance and ease of administration while the most important thing is the cost effectiveness of this route(Baghel, Cathcart et al. 2016).Delivery through other routs generally have some barriers like gut wall permeation and GIT fluid dissolution which are overcome by this system (Vo et al., 2013). GIT dissolution is the major problem among these (Childs et al., 2013). But there are also some issues related to oral route like solubility problems. If the solubility does not occur properly in GIT, drug molecules will not be absorbed through this route.(Bou-Chacra, Melo et al. 2017)

The second problem is lumen gut enzymes and enzymes released by bacteria. Here the drug substances are metabolised by these enzymes which will prevent the drug fractions to reach the systematic circulation and bioavailability of these drug substances are diminish there. (de Sousa and Bernkop-Schnürch 2014)

Metabolism of some drugs occur completely in first phase either in liver or gut before reaching the systemic circulation which cause a major problem in oral route delivery (Wilkinson 2005).

2.10.3 Nanotechnology in Pharmaceutical Field

New opportunities are being provided by pharmaceutical nanotechnology which have many applications in management of disease and diagnostic field. Beside these nanoparticles are also

Used for drug delivery as a carrier agent for diagnostic and bioactive agents.(Farokhzad and Langer 2009).This delivery system offers some advantages like efficacy, tolerability of drugs with improved safety. Nanoparticles also have other applications in increasing the residence time of medicinal agents and improving the solubility which reduces the toxicity and expenses and the patient are benefited(Akerib, Araújo et al. 2016).Nanoparticles prevent quick degradation of drugs and drug are absorbed slowly in favoured environment. It also increases the bioavailability and tissue retention time. The preference of nanoformulations over other conventional formulation is its persistency and the long retention time in blood circulation(Surendiran, Sandhiya et al. 2009).

2.10.4 Different Nanosystems and their characteristics.

Table explains the variety of nanocarriers,and their role in drug delivery.

Table 2.10.4 Different nanosystems and their functions(Nahar, Dutta et al. 2006)

Types of nanosystems	**Size (nm)**	**Characteristics**	**Applications**
Carbon nanotubes	0.5-5 nm	Carbon mostly occur in allotropic form, carbon nanotubes is one of them which have two categories of single and multiple walled nanotubes.	Breakdown of nanotube bundle occur during functionalization which result in increased solubility and incursion to cytoplasm of the cell. Through these gens and

			peptides are delivered into the cell nucleus. Drug are also being delivered to the cancerous/tumour affected cells through these.

Dendrimers	<10 nm	Due to the distinctive three dimensional structure of dendrimers it has some superior chemical and physical properties like monodispersity molecular weight and well define structure over other.	Its utilization occur in targeted and controlled delivery drugs of bioactive to liver targeting and macrophages.
Liposomes	50-100 nm	Liposomes are present in small vesicalar shape. These vesicles are consisted of phospholipids and cholesterol. The entrapment efficiency of these vesicles are also good.	It has long and safe circulatory time. Peptides, protein, drugs and other agents are delivered active and passively.
Metallic nanoparticles	<100 nm	Silver, gold and iron oxide nanoparticles which are having large surface area to volume ratio and having dangling bonds in large	Binding of magnetic nanoparticles to biological molecules occur easily and drug and genes delivery

		number as well as low coordination sites. Also poses excess electron storage capacity.	occur in highly sensitive diagnostic assays.
Polymeric nanomicelles	10-200 nm	Self-assembly of block copolymers are used in polymeric micelles that are core shell type nanoparticles.	The use of these nanoparticles occur in passive and active targeting of drugs as well as for diagnostic purpose.
Polymeric nanoparticles	101000 nm	Drug is found entrapped in nanoparticles, or it could be dissolved or attached to the matrix highest entrapment efficiency has been found as compared to other drug delivery systems. In polymeric nanoparticles drugs are entrapped in solid colloidal particles in which drugs are dissolved. As compared to other system they are possessing high entrapment efficiency	Sustainable and controlled release of drug occur through these nanoparticles. Bioactive materials occur active and passively through these surface modified nanoparticles.

2.10.5 Polymeric Nanoparticles as Carriers for Drug Polymeric nanoparticles (PNPs) are in the form of colloidal particles in solid form in the size range of 10

100 nm while the carrier drugs are attached or dissolved to the matrix of nanoparticles (Jawahar, Meyyanathan et al. 2012).Currently there are present many innovative drug delivery systems with the help of advancement in new technology. PNPs are an interesting option for controlled and targeted drug delivery. In recent years PNPs are gaining more and has a very important role in different fields. The current market requirements are being fulfilled by the increased tendency of the PNP(Sumit and Sciences 2012). PNPs can also be used to deliver drugs to targeted tissues and to other site like for tissue engineering. Volatile drugs get stability when entrapped in these nanoparticles. Manufacturing of these nanoparticles occur through different methods(Jawahar, Meyyanathan et al. 2012).Polymers used for the preparation of certain nanoparticles should meet with certain requirements like its compatibility with body fluids and organs and should be economical. It should be nontoxic non-immunogenic should be characterised and manufactured easily and should be biodegradable (Dadwal, Solan et al. 2014)

2.10.6 Methods of preparation of Polymeric Nanoparticles

Polymeric nanoparticles can be prepared through numerous methods. Main categories are the following(Rao and Geckeler 2011).

- Through the polymerization of monomers
- Ionic gelation method
- from dispersion of preformed polymer
- Dialysis
- Nanoprecipitation

2.10.6.1 PNPs through the polymerization of monomers

Preparation of particular size nanoparticles require suitable polymers which are designed and prepared by monomer's polymerization. These polymerization methods include interfacial polymerization, (Mahdavian, Ashjari et al. 2007)micro and miniemulsion polymerization(Landfester 2009),(Landfester 2009).

2.10.6.2 Ionic gelation

In ionic gelation PNPs preparation through hydrophilic biodegradable polymers, gelatine and chitason. There are two aqueous phases in ionic gelation method one containing sodium tripolyphosphate and the other containing chitosan. Cationic group of chitosan interact electrostatically with tripolyphosphate which is an anionic group and form coacervates.

2.10.6.3 PNPs from dispersion of preformed polymers

Dispersion of performed polymers include emulsification/solvent diffusion (GalindoRodriguez, Allemann et al. 2004, Hu, Hong et al. 2004) supercritical fluid technology (SCFT) and solvent evaporation (Desgouilles, Vauthier et al. 2003).Superficial fluids are used for designing non-toxic method for PNPs preparation.(Banik, Fattahi et al. 2016) (Sane and Limtrakul 2009)

2.10.6.4 Dialysis

Dialysis is a valuable and easy method to prepare PNPs. Utilization of a variety of copolymers with polymers are used to prepare PNPs in this method.(Guo, Liu et al. 2014)

2.10.7 Nanoprecipitation

Nanoprecipitation or single emulsion evaporation method is cost effective and easy method. In this technique organic phase is drop wise poured into aqueous phase.In Current work polymeric microspheres are prepared through nanoprecipitation method due to its ease and simplicity and cost-effectiveness. There are numerous process which are describe in detailed in this method and can affect particle formation. (Miladi, Sfar et al. 2016).This process was developed by Fessi and his co-workers which result in instant formation of nanoparticles. A solution of polymers is made in organic or non-organic polar solvent that are water soluble which are then injected into the surfactant present in the organic phase.(Maaz, Abdelwahed et al. 2014)

2.10.8 Mechanism of particle formation by nanoprecipitation

Aggregation, growth and nucleation are the major three mechanisms for particle formation (Budhian, Siegel et al. 2007)Supersaturation is the driving force for all phases it is very important for nucleation (Maaz *et al*., 2014).Surface tension difference is important factors for nanoparticles formation McManamey finding described this (McManamey and Woollen 1973). Aqueous phase has more surface tension as compared to organic phase therefore the molecule of aqueous phase are pulled more strongly in aqueous phase. This difference lead to thermal variation and interfacial turbulence which cause solvent twisters in both phases.(Mora-Huertas, Fessi et al. 2010)

2.10.9 Components of methodology

Following components are required in the preparation of PNPs through nanoprecipitation technique.

A Drugs

Hydrophilic drug molecules were found better as compared to nanoprecipitation techniques in lipophilic drug molecule incorporation. PCL was used in aqueous medium as polymer in NPs synthesis by Bilensory et al (Bilensoy, Sarisozen et al. 2009). The same techniques has been used by Govender and his colleagues in PLGA NPs synthesis (Govender, Stolnik et al. 1999).

B Organic phase

Hexane, Methylene chloride and Methanol Ethanol, water soluble organic solvents are present in oil phase. Other compounds include vegetable oil, triglycerides, hydrophobic surfactants etc. when these substances are added as a result the formation of Nano capsules instead of nanospheres (Bukhari, Idris et al. 2014)

C Aqueous phase

In this phase same hydrophilic surfactants which are either natural or synthetic are present in water for particles aggregation like tween 80 and PVA(Bukhari, Idris et al.

2014). Beside these some other substances like drug molecules and polymers are present in aqueous phase.

D Polymers

PNPs preparation utilize a number of polymers most common of which are PCL, PLA, and PLGA which are resistant type-3 polysaccharide polymers with suitable performance(Noor, Shah et al. 2018)

2.10.10 Influence of operating conditions

Three steps are involved in nanoprecipitation in which first step involved organic and aqueous phase preparation in second phase injection of organic phase occur into aqueous phase under continuous stirring condition and finally organic phase of the solution is evaporated either through rotary evaporator or at ambient temperature There are a number of parameter that influence nanocarriers characteristics (Dong *et al*., 2015).

a. Amount of polymer

Nanoparticles characteristics with reference to concentration of polymers have been checked through various studies. Increase occur in encapsulation efficacy and particle size as we increase concentration of polymers.

b. Molecular weight of the polymer

Particle properties are greatly influenced the parameter that is very critical. Lince and co-workers associated.

c. Surfactant concentration

A number of studies shows the effect of concentration of surfactant on particles characteristics. This study shows that larger particles are formed in presence of high polyvinyl alcohol (PVA) concentration (5-10%)(Arica, Lamprecht et al. 2005)While decrease in surfactant concentration give smaller sized nanoparticles(Allémann, Gurny et al. 1992)

d. **Organic to aqueous phase ratio**

In another study Fonseca et al. shows that reduction occur in 1; 2 oil to water phase (Fonseca, Goya et al. 2002).

e. Stirring rate

Magnetic stirring is frequently used for stirring in nanoprecipitation in which particle size get reduce if we increase the stirring rate.For the sake of gaining excellent characteristics of the particles all these parameters including should be control.

The importance of this method is its simplicity and importance and NPs precipitate faster. Relatively low concentration of surfactants and polymers is required for this method. Achieving small and controlled size particles is easy in method (Miladi *et al*., 2016). Figure 2.10 represent the flow diagram for drug loaded nanoparticles preparation through nanoprecipitation techniques.

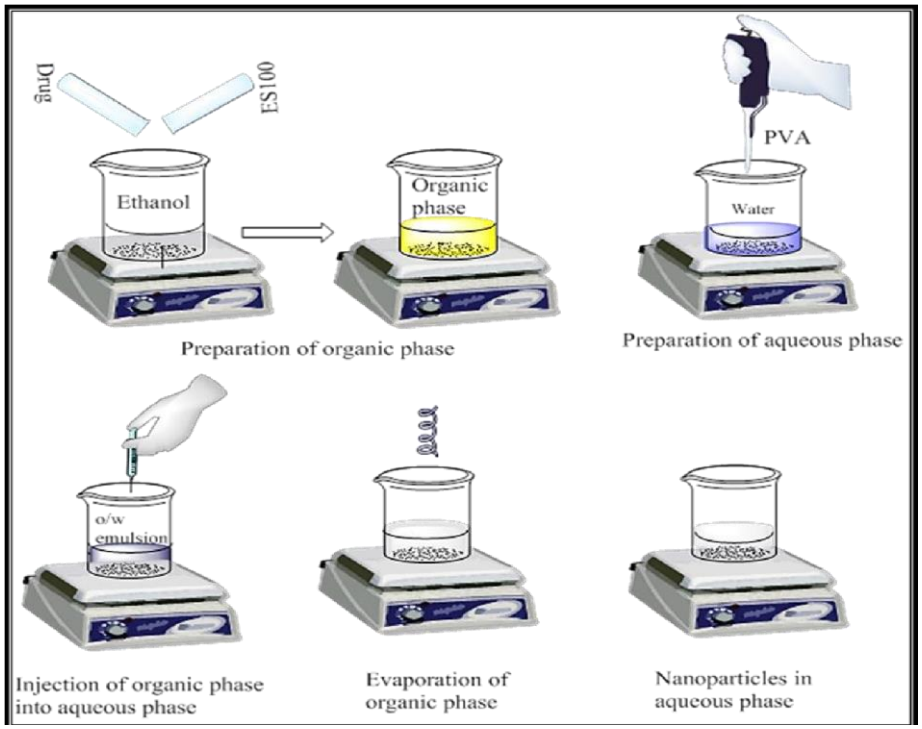


Figure 2.3 Schematic representation of nanoprecipitation technique.

2.11 Rationale of selection of resistant starch as drug carrier

Resistant starch resist digestion process in abdomen and stomach but colonic microbes can degrade it (Htoon, Uthayakumaran et al. 2010).Due to this property this starch can be used to deliver drug to specific sites in colon. There are different categories of resistant starch, retrograded starch (RS 3) is important among them because of its low solubility and thermal stability (Haralampu 2000).Preparation of this starch occur by a enzymatic processing which more organized form (crystalline) is formed from pregelatinized form (amorphous) (Thompson and Technology 2000, Chung, Shin et al. 2008).The responsible factor for this recrystallization is intra molecular forces and hydrogen bonds which is a spontaneous process (Thompson and Technology 2000, Liu, Xie et al. 2011).These changes attribute time of retrogression process, temperature, concentration of polymers and variation in botanical origin (Zhou, Wang et al. 2008)(Najafi, Baghaie et al. 2016)The chemical and physical properties of native starch make it unsuitable for controlled release of drugs. To gain more food and biomedical applications of starch its properties should be improved through different sorts of modifications like hydropyrrolation, crosslinking, chemical and blending modification and oxidation etc. have been consider by many researchers in this area of researches.(Najafi, Baghaie et al. 2016)

Starch is break down into its monomers by different types of enzymes like α-Amylase digest starch in oral cavity other enzymes like glucoamylase, sucrose isomerase, and pancreatic amylase digest starch in small intestine. Resistant starch are those which resist digestion for about 120 minutes and escape from small intestine without being digested by the enzymes are considered as resistant starch(Haralampu 2000, Dupuis, Liu et al. 2014, Lockyer and Nugent 2017)

Major health problems are caused by the rapid digestion of such starch flour i.e. elevated release of postprandial glucose and blood concentration of insulin increase(Apostolidis and Lee 2010) (Foster-Powell, Holt et al. 2002)Release of high glucose in the blood constantly force the high release of insulin which sometimes lead to chronic diseases like diabetes, metabolic disorder, obesity and in some cases it also causes cardiovascular diseases (Sekirov, Russell et al. 2010).Hence, the addition of resistant starch in our daily food will surely be a better step to control the development of harmful disease which are arising due to the rapid digestion of starch into glucose.

More insulin is released in response to glucose concentration which is harmful for health like cause obesity, cardiovascular disorder, metabolic disorders and diabetes etc (Sekirov, Russell, Antunes, & Finlay, 2010). The development of harmful disease can be controlled through the use of this starch in our daily food these disease are basically arising due to the rapid digestion of starch into glucose (Lunn and Buttriss 2007, Fuentes-Zaragoza, Sánchez-Zapata et al. 2011, Van Soest 2018).Furthermore, resistant starch upon fermentation by colonic bacteria in the large intestine produce short chain fatty acids (SCFA).Fermentation of resistant starch in the colon results in lowering of pH in large intestine (Geurts, Neyrinck et al. 2013)as well as these acts as an energy source for the dwelling bacteria of the intestine and also provides energy for colon cell. Additionally, it enhances the flow of the blood in colon which help to stop the development of colitis (Malago & Sangu, 2015).

Short chain fatty acid like butyric acid, acetic acid and propionic acid are produced in large intestine by colonic bacteria which result in lowering the pH after fermentation (Geurts, Neyrinck et al. 2013, Hoseinifar, Safari et al. 2017)

It also act as energy source for dwelling intestinal bacteria and energy is being provided for the colon cells. While colitis development are also stop as it facilitate the flow of blood in colon.(Han, Gao et al. 2013)

2.11.1 Ciprofloxacin

Bayer developed ciprofloxacin (Figure 2.11.1), in 1981.It is a floroquinolone antibiotic.drug carriers lowers the solubility of drugs.Colloidal drug carriers system like nanosuspension, microparticles, nanoparticles and liposome lower the solubility problem of drugs . Suitable delivery vehicles like chitosan nanoparticles improve ciprofloxacin’s bioavailability problem.

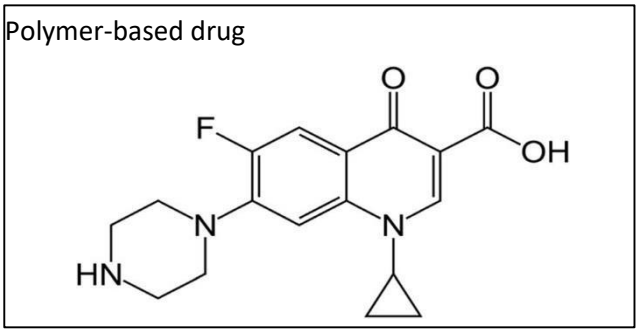


Figure 2.4 Structure of Ciprofloxacin

For easy carrier removal and appropriate release of drug biocompatible and biodegradable polymers are the best suggestion to use. In this study, ciprofloxacin was used for formulation of resistant starch nanoparticles in a method called nanoprecipitation. Further characterization were done by,SEM.FTIR,XRD.DLS, Invitro release of the drug, antibacterial activity, drug entrapment efficiency and crystallinity.(Singh, Mishra et al. 2018)**.**

Material and Methodology

Study Area:

I have performed my research at the Applied, Environmental and Geomicrobiological Laboratory (AEG), Department of microbiology, Quaid-i-Azam University Islamabad, Pakistan, during the session 2017 to 2019.

It included the synthesis and characterization of enzymatically produced resistant starch coated drug microspheres and their antimicrobial potential.

3.1 Materials:

3.1.1 Different types of starches used in experimentation

Maize flour used was purchased from the local market of Swat, KPK Pakistan, and isolated pure corn starch was purchased from Sigma chemicals limited company.Starch with high amylose content also known as Industrial Starch was purchased from Megazyme International Ireland Limited.

3.1.2 Reagents and enzymes

Pullulanase and Amylase enzymes were extracted and purified from *Bacillus licheniformis* an indigenously isolated thermophilic strain. Protease enzyme was purchased from Sigma Chemical Company (St. Louis, MO, USA).

Resistant starch content was determined by the Megazyme kit, purchased from Megazyme Ltd.Other material used in the study were 0.2M sodium phosphate buffer, dimethylsufoxide, (DMSO), tween 80, 90% DMSO,10% DMSO, Iodine crystals, deionized water, methanol, ethanol, 1N NaOH, 0.1N HCl buffer, Iodine reagent, distilled water, GOPOD reagent, Ciprofloxacin as model drug.

3.2 Methodology

3.2.1. Resistant Starch Production from Maize flour by using thermo stable enzymes:

Resistant Starch production is carried out by, the method given by Englyst *et al* with some modifications, 0.5 molar citrate buffer of pH 5 was added in 5g of sieved maize flour and gently shaken for dissolution. The starch was autoclaved at 121°C for 20mins for the process of gelatinization. Then it was cooled to 50°C after which purified protease enzyme was added for the removal of network of proteins and then incubated for about 40 minutes. After that placed in the boiling water bath for the inactivation of protease enzyme. Starch was then cooled to about 50°C by addition of a combination of both amylase and pullulanase enzyme, in RS(III) production. For the attainment of the proper degree of polymerization slurry was incubated for a time of 16hrs in shaker incubator of 50°C with the speed of 100rpm. After this the starch slurry was placed in a hot water bath for the inactivation of enzymes.1hr of autoclaving is performed again for more gelatinization and debranching of the starch. Afterwards retrogradation of 4°C for 24hrs is performed, followed by drying of the starch in an oven at 105°C for 5hrs. Finally the dried starch was milled properly to a fine powder and pass it from sieve stored in bottles in refrigerator for the determination of its RS content.

3.2.2 Determination of resistant starch content

Megazyme kit assay was used to measure the resistant starch content, measure (0.1g) of the dried powder of the enzymatically produced starch. Add 4ml of prepared solution of α-amylase and (AMG) amyloglucosidase (3U/ mL) to the falcon tube containing starch sample.Each falcon tube is capped tightly and vortexed then placed in horizontal position in a shaking water bath along the direction of motion for 16 hrs at 37 ℃**,** till the completion of incubation, then the tubes were taken out and the surface water of the tubes was cleaned with a towel or tissue paper. After that, stopping agent to stop further enzymatic reaction was added in reaction mixture such as 4.0 mL of absolute ethanol (99% v/v) and the mixture was vortexed for proper mixing, then centrifuged at about

3000 rpm for 10 minutes, soluble starch is separated in supernatant**,** and resistant starch as a pellet in the bottom, then added 2ml of 50% ethanol in pellet after the removal of supernatant,6 ml 50% ethanol was added again in same, vortexed appropriately and then centrifuging it for 10 minutes to remove the remaining soluble starch.The supernatant was wasted and the pellet was stored for further analysis.This process was repeated to carry proper washing of soluble starch.Carefully transfer the supernatants and excess water was drained by keeping the tubes in an inverted position.

3.2.3 Quantification of Resistant starch content:

The resistant portion of the starch is in the form of the pellet that is obtained at the end of the above procedure. To quantify 2 ml of 2M KOH solution added in to the pellet place it over the ice water bath and gently shaken with a magnetic stirrer for the proper dissolution of resistant starch. Addition of 8 mL of sodium acetate buffer of (pH 3.8) to each tube was completed for further dissolution and stirring on a magnetic stirrer to counterbalance the alkaline solution. Instantly added 0.1 mL of AMG solution (3300 U/mL), and vortexed for proper mixing after that the tubes were placed in a water bath at 50 ℃ for about 30 minutes with alternating mixing. Solution is then transferred to a 50 mL flask after 30 minutes adjust volume up to 50 mL by the addition of distilled water. Then centrifuged the solutions at 3000 rpm for about 10 minutes, then 3 mL of GOPOD reagent was added to 0.1mL of supernatant in glass tube, and incubating the samples for 20 minutes at 50℃. Absorbance of the samples was measured against reagent blank at 510 nm.

3.3 Solubility measurements

Solubility was determined by using simple dissolution method,RSIII and Ciprofloxacin was taken about ,5mg in 5mL of each solvent,(Dichloromethane,Acetone,Methanol,Ethanol,DMSO,Water)test tubes are then vortexed in order to enhance the solubility of RSIII and drug in solvents, placed them in shaker incubator at 50 ℃ with continuous shaking for 30 minutes, and then centrifuged the samples at 4000 rpm for 20 minutes at 4℃ then solubility is measured

by taking absorbance using uv-visible spectrophotometer at 510 nm for RRSIII and for Ciprofloxacin at 271nm.

3.4 Iodine staining index

Resistant starch (RSIII) sample and industrial starch sample of (0.125g) was taken separately, and dissolved in (1.25 mL) of 90% DMSO, cooked it for 30 minutes in a hot water bath at 90℃, (11 mL) deionized water (autoclaved distilled water) was added. Then (0.125 grams) of sample from native starch was taken and add (12.5 mL) of deionized water in it, cooked for 30 minutes in boiling water bath for 30 minutes,50 µL from above solutions was taken in different covered test tubes,5 mL of 0.6 molar solution of iodine was added in 10% DMSO.Incubated for 30 minutes. Iodine staining index of debranched starch iodine complex stated as the absorbance at 460 nm for short chains and 570 nm for long chains is observed through spectrophotometer.

3.5 Preparation of Solutions

3.5.1 Preparation of organic phase

Add 100mg of RS(III) and 10mg of Ciprofloxacin in 20 mL of DMSO with stirring and light brownish clear solution was obtained.

3.5.2 Preparation of 0.5% Tween 80 solution

For the preparation of 0.5% Tween 80 solution, 0.5 mL of tween 80 and add this into 100 mL of distilled water.

3.5.3 Phosphate buffer (pH 6.8 and7.8)

For this, first 0.2 M NaOH and 0.2 M KH_2PO_4 were prepared and mixed to obtain desired pH.

3.5.4 0.1 M HCL buffer (pH 1.2)

4.08 mL of HCl was added in to 500 mL of distilled water and adjust the pH 1.2

3.6 Preparation of drug loaded microspheres.

Microparticles were prepared by using single emulsion evaporation technique(nanoprecipitation).Tween 80 was taken 0.5% as stabilizer in a beaker with distilled water and stir it,100 mg of RSIII and 10 mg of Ciprofloxacin added into DMSO, and poured dropwise into the aqueous phase, with stirring.Centrifuged the above formulation at 12000 rpm for 20 minutes, microspheres were collected at the bottom then washed with distilled water and frozen before lyophilized.

3.7 Experimental Design for Optimization of Process Parameters

Various parameters were optimized to get desired size and entrapment efficiency of 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. Size of spheres and encapsulation efficacy were checked for each formulation. The formulation with lowest size and highest encapsulation efficiency was selected as the optimized one.

3.8 Characterization of Microspheres.

Characterization of microspheres was performed by using different analytical techniques.

3.8.1 Light Microscopy

Light microscopy was performed to investigate the morphology and structural features of treated starch microspheres.Samples were arranged for microscopic study by suspending the powder in a drop of 50% glycerol and spread it on a microscopic slide correctly and samples were covered by a glass coverslip. The samples were observed within 30 minutes of the slide preparation.40 X resolution was used to carry microscopic examination. Images were then captured.

3.8.2 Particle size measurements and dispersion studies.

Size of spheres and dispersity index was examined to confirm the micro range by using Dynamic light scattering (DLS).

3.8.3 Surface properties, morphology and shape of RS and drug loaded MPs.

The morphological appearance and surface features of the resistant starch coated drug loaded microspheres were observed under the SEM in dry powder form. A round aluminum stub covered by an adhesive tape from one side it was cling with the stub and from the other side stick with sample. The prepared samples were passed through electric shock and then coated with a thin layer of about 20 nm gold. Samples were ready for the examination of electron microscopy. High accelerating electron beam was then used to examine the samples,and different resolution images were taken. Where an accelerating potential of 20 kV was used for the examination.

3.8.4 Fourier transform infrared radiation (FTIR) analysis

Potential interaction and composition of the ingredients used in the formation of microspheres was studied through FTIR. For this purpose, Ciprofloxacin, RSIII and final formulation of drug loaded microspheres were fixed with KBr discs and their FTIR bands were investigated. Samples were prepared by mixing the microspheres with KBr and grind it properly for about 10 minutes in infrared lamp, and water molecules were removed from the samples, just to eliminate its interfering in the absorption peaks in generating spectra. Then peaks were generated for the samples. The 2 mg of microspheres mixed with KBr and pressed under the sheet and then scanned for the spectra. Area from 1000 to 4000 cm^{-1} was scanned at a resolution of 4cm.

3.8.5 X-ray diffraction (XRD) studies

XRD studies were performed for scanning of drug loaded microspheres. All the four samples, RSIII, Drug, their physical mixture and drug loaded microspheres were in dried powder form and were prepared for scanning before the analysis. Moistness was controlled by keeping the samples in a closed desiccator. Strong radiation of Cu generated from X-ray diffractometer with angle of 2 theta of 5°C to 550°C. XRD profiles were classified according to pattern given by zobel 1964, and relative

crystallinity was analyzed by the method used by Nara and Komiya in (1983) for measuring the crystallinity, Quantitative estimation of relative crystallinity was analyzed by software (Origin version 8.5).The graphs were plotted for RSIII, Ciprofloxacin, and their physical mixture and for the final formulation between the angles of 2ɵ of 5 and 45 degrees. Then graphs were smoothed by the adjacent tools in the origin software. The analysis was done to confirm the encapsulation of drug within resistant starch and the nature of individual ingredients used in the preparation of microspheres i.e. Ciprofloxacin, and RSIII as well as nature of Ciprofloxacin within microspheres and physical mixture of RSIII and Ciprofloxacin.

3.9 Percentage yield of Microspheres

It is very important factor it determines the capacity of polymer to entrap the drug, and calculated by measuring weight of dried microspheres divided by total amount of drug and RSIII taken.

3.10 Entrapment efficiency

3.10.1 Standard calibration curve of ciprofloxacin in distilled water.

Ciprofloxacin of about 1mg was dissolved in 1mL of distilled water for the preparation of stock solution. Dilutions of 0.1mg/mL, 0.2mg/mL, 0.3mg/mL, 0.4mg/mL, 0.5mg/mL,0.6mg/mL,0.7mg/mL,0.8mg/mL, 0.9mg/mL were made and run at 271 nm on UV-Vis spectrophotometer and distilled water was taken as blank. Calibration curve was drawn with by using Microsoft excel and then unknown concentration of drug was determined.

3.10.2 Encapsulation efficiency and drug loading capacity

Drug encapsulating and loading capacity of RSIII was determined by , centrifuging the formulation for 30 minutes at 12,000 rpm, and then 1mL of supernatant was taken, diluted with 10 mL of distilled water and analyzed in UV-Vis spectrophotometer at 271 nm. Following formula was used.

3.10.3 Encapsulation efficiency (Ali et al., 2016):

$$\%\ Encapsulation\ Efficiency(EE) = \frac{w1 - w2}{w1} \times 100$$

W1= total Ciprofloxacin added.

- W2= free Ciprofloxacin in supernatant.

$$\%\ loading\ capacity\ (LC) = \frac{w1-w2}{w3} * 100$$

W3= amount of RS added

3.11 *In Vitro* Release Studies

3.11.1. Construction of calibration curve of Ciprofloxacin in simulated gastrointestinal fluids (HCl buffer of pH 1.2 and phosphate buffer of pH 6.8 &7.8)

Drug concentration was estimated in release medium of respective pH, taken as simulated fluids, calibration curves were constructed in simulated gastric fluid (HCl buffer of pH 1.2),simulated intestinal fluid (phosphate buffer of pH 6.8) and simulated colonic fluid(phosphate buffer of pH 7.8). For this persistence, 1 mg of drug was dissolved in 10 mL of respective fluids and drug content was analyzed at 271 nm from calibration curve.

3.11.2. *In vitro* drug release from microspheres.

Amount of drug released from Simulated gastric fluid (HCL buffer of pH 1.2), intestinal fluid (phosphate buffer of pH 6.8) and colonic fluids (phosphate buffer of pH 7.8) was measured.First of all, 3 mg of microspheres were taken in each dialysis membrane add 5 mL of respective medium in it and placed this into 50 mL of release medium. Take 1 ml from each release medium after the time interval of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5,6, 12, and 24, hours.Samples were analyzed at 271 nm pH 1.2,6.8 and 7.8, mechanism of drug release from microspheres was analyzed by various kinetic models.

3.11.3. Kinetics of drug release

Overall release profile of drug from microspheres was analysed by using various models.These models were in use to predict the *in vivo* bio performance from the data provided after *in vitro* drug dissolution. Following kinetic models were applied.

A. Zero order kinetics model (Schwert and Eisenberg 1949)

Zero order kinetics defined the constant and controlled drug release.Graph between drug release and time was used to find the release mechanism.

$W1=K_1t$

W1= cumulative drug released T=
time in hrs.

K_1= zero order release constant

B. First order kinetics model (Beers and Sizer 1952)

First order kinetics describe the amount of drug present initially in the system. (Larisch, Goss et al. 2017), log of cumulative drug release and time was plotted and shows the first order kinetics.

$\ln (100-W) = \ln 100k_2t$ Where

k_2= first order release constant

C. Hixson crowell's cube root model (Hixson, Crowell et al. 1931)

Dissolution limited the drug release and it is defined by Hixon crowell's model and explained in equation bellow.

$(100)^{1/3}100^{1/3}-W=-K_3t$

Where

K_3=Hixon release constant

D <u>Korsmeyer peppas equations (Korsmeyer, Gurny et al. 1983)</u>

Drug release data is used to plot the log of time and log of cumulative drug release by using drug release data, and following equation is used to explain it.

$Mt/M^{\infty}=K5t^{n}$

Where, Mt/M^{∞}= drug fraction released at time t

K_5=rate constant, n=diffusion exponent

release mechanism of drug from polymeric system was evaluated by n value release exponent as demonstrated in table

E. Higuchi square root of time equation (Higuchi 1963)

This model describes the diffusion based release of drug from the polymeric matrix. In this model a graph is plotted between square root of time and cumulative drug release (Skardal *et al.*, 2017). Explained in equation:

$W= k_4t$

Where

K_4=Higuchi dissolution rate constant

3.12 *In Vitro* Antibacterial Assay

Antimicrobial activities of microspheres were determined by agar well diffusion method (Balouiri, Sadiki et al. 2016).

3.12.1 Agar well diffusion method

Excessively used method to estimate the antimicrobial activity,the method comprises of following steps.

3.12.2 Preparation of the sample

The assay was performed by preparing the samples for this 3mg of each fraction was dissolved in 1000 micro liters of DMSO.

3.12.3 Preparation of inoculums

Inoculums were prepared by dissolving colonies of *E.coli* and *S.aureus* in nutrient broth medium to grow bacteria.

3.12.4 Preparation of media for bacterial growth

Mueller Hinton Agar (MHA) was used to grow bacteria,it was prepared by dissolving 38 g/L and then autoclaved at 121°C for 15 minutes. The plates were incubated for 24 hours to confirm sterility.

3.12.5 McFarland turbidity standard (0.5 $BaSO_4$)

Bacterial culture turbidity was standardized by using, 0.5 McFarland standard.It was made by the addition of 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml 0f 0.36 N H_2SO_4 and then it was kept in test tubes with screw for the comparison of turbidity.

3.12.5 Bacterial strain used

Two strains of bacteria were used; which were, *Escherichia coli* (ATCC 10536), *and Staphylococcus aureus* (ATCC 6538). These strains were maintained at 4 °C for 24 hours on nutrient agar medium and then mixed with normal sterilized saline solution (0.9% w/v) and the turbidity was corrected by using McFarland turbidity standard (0.5 $BaSO_4$).For making the bacterial lawn on MHA petri plates these freshly prepared inoculums were used.

3.12.6 Sample inoculation

Bacterial lawn of different strains were prepared on MHA plates. The drug loaded microspheres samples of 100 microliters of concentrations were placed in the wells of plates with the help of sterile micropipette,Ciprofloxacin was taken as positive control, and incubated for 24 hours at 37°C.

3.12.7 Measurement of zone of inhibition

Zone of inhibition was measured with the help of scale and compared with control.

Results

Resistant starch was enzymatically prepared by the treatment of maize flour according to optimized protocols with purified thermophilic enzymes such as amylase and pullulanase.

The Invitro digestibility and different physicochemical characteristics of this enzymatically produced starch was determined and compared to the untreated maize flour. Moreover, the encapsulation of Ciprofloxacin by resistant starch(RSIII) was conducted to prove the ability of RSIII as a colon targeted drug carrier. Insights about, antimicrobial potential physical and structural characteristics of drug loaded microspheres were also given by, antimicrobial assay, light microscopy, scanning electron microscopy (SEM) X-ray diffraction studies. (XRD), Fourier transformed infrared spectroscopy (FTIR), and Dynamic light scattering (DLS) techniques.

4.1 Determination of Resistant content of untreated Maize starch and Enzymatically produced starch:

4.1(a). Resistant Starch (RSIII) Content:

The untreated maize flour as well as enzymatically treated starch samples were compared and resistant content was quantified according to the standardized protocol of Megazyme Assay kit (Ireland).The Resistant starch content of enzymatically produced starch was found to be about 19% and is shown in **Fig No. 4.1** Industrial RS obtained from Meagzyme International was used as a positive control with the highest RS content of 44%. After IR, enzymatically produced RSIII having a RS content of 19% as compared to the native maize flour. Native Maize showed the least RS content. RS showed the highest RS content because of involvement of two enzymes in its debranching and re-alignment into a more compact resistant structure.

Figure 4. 1 RS content (%) of starch samples

4.2 Solubility measurements.

4.2.1 Solubility of resistant starch in different solvents.

Solubility of RSIII was assayed in different solvents and was demonstrated by the absorbance taken at 510 nm. The solubility of RSIII ranged between absorbance values of 0.206-1.261. Highest solubility was found in DMSO and the least in distilled water and slightly soluble in acetone, methanol and water.

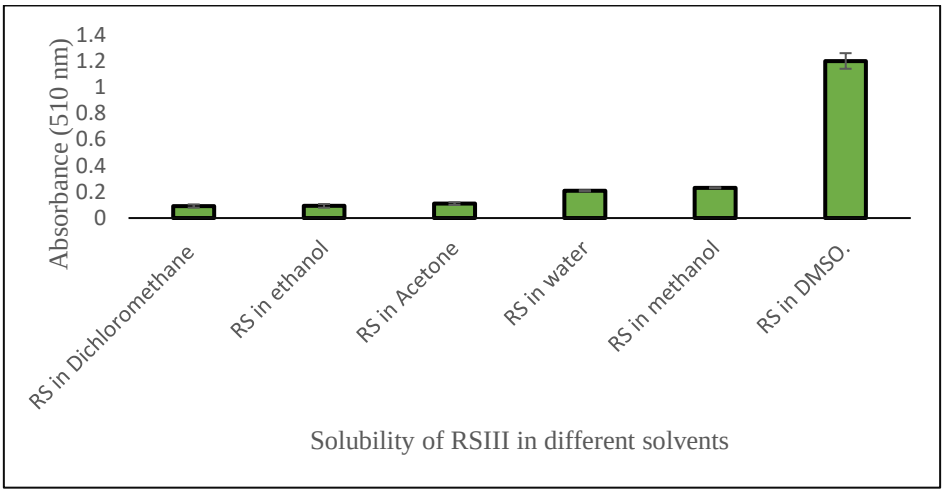


Figure 4.2 Solubility of resistant starch in different solvents.

4.2.2 Solubility of Ciprofloxacin in different solvents.

Solubility of Ciprofloxacin was assayed in different solvents and was demonstrated by the absorbance taken at 271nm.

The solubility of Ciprofloxacin ranged between 0.291-0.38. Highest solubility of the drug was found in distilled water with complete dissolution.It was only sparingly soluble in acetone with the least solubility in dichloromethane.

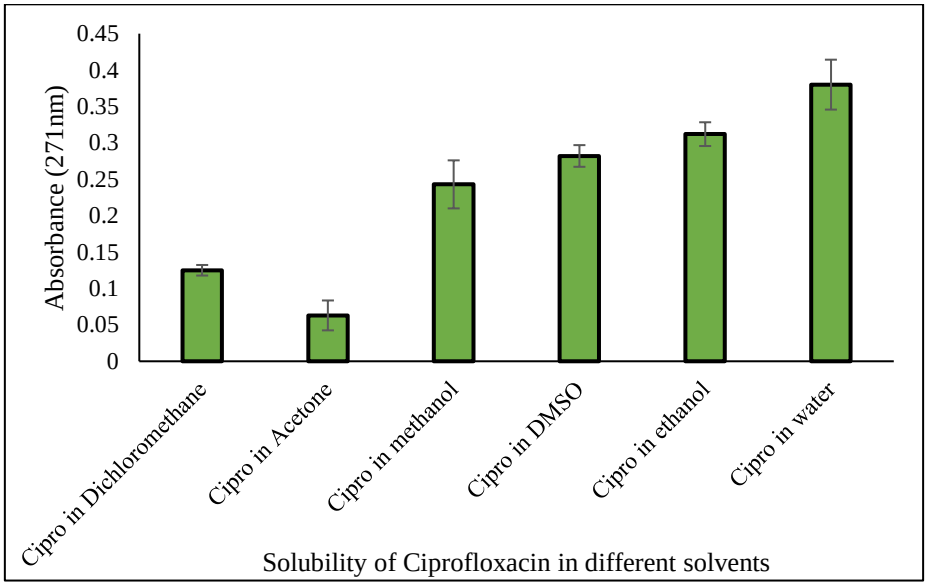


Figure 4.3 Solubility of Ciprofloxacin in different solvents.

4.3 Iodine Staining Index.

Binding ability of iodine with untreated maize flour and enzymatically produced resistant starch were performed and stated as the absorbance at 460 nm is directly related to short chains and at 570 nm with longer chains starch samples showed different absorbances at 460 nm are explained in **Fig No 4.4** and absorbance at 570 nm is presented in **Fig No 4.5** RSIII showed greater binding ability explained by absorbance at 460 nm for short chains and 570 nm for long chains, the untreated maize flour showed low values of absorbance at above mentioned wavelengths (Knutson, 1986).

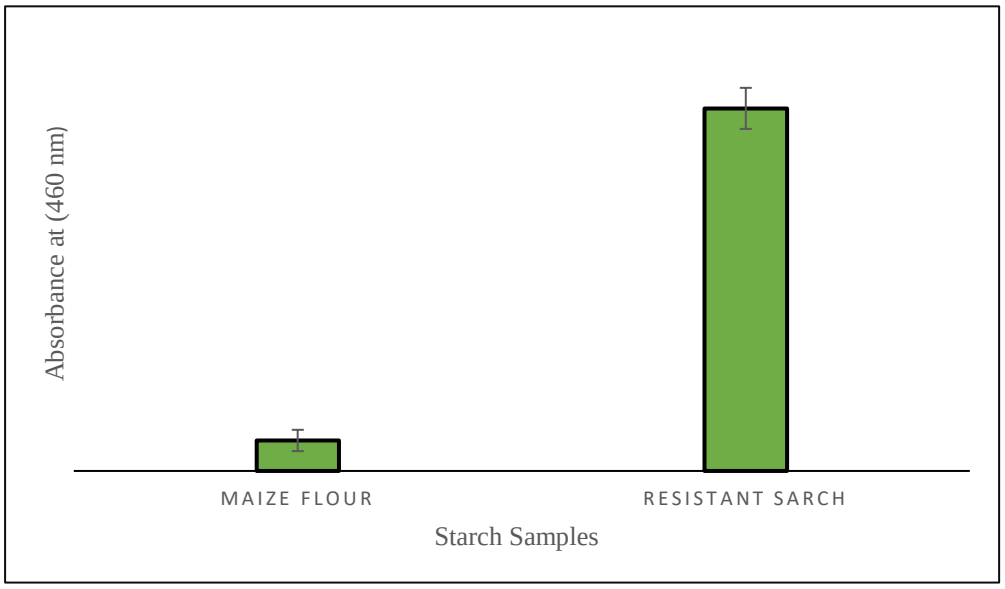


Figure 4.4 Iodine ability of different starch samples at 460 nm.

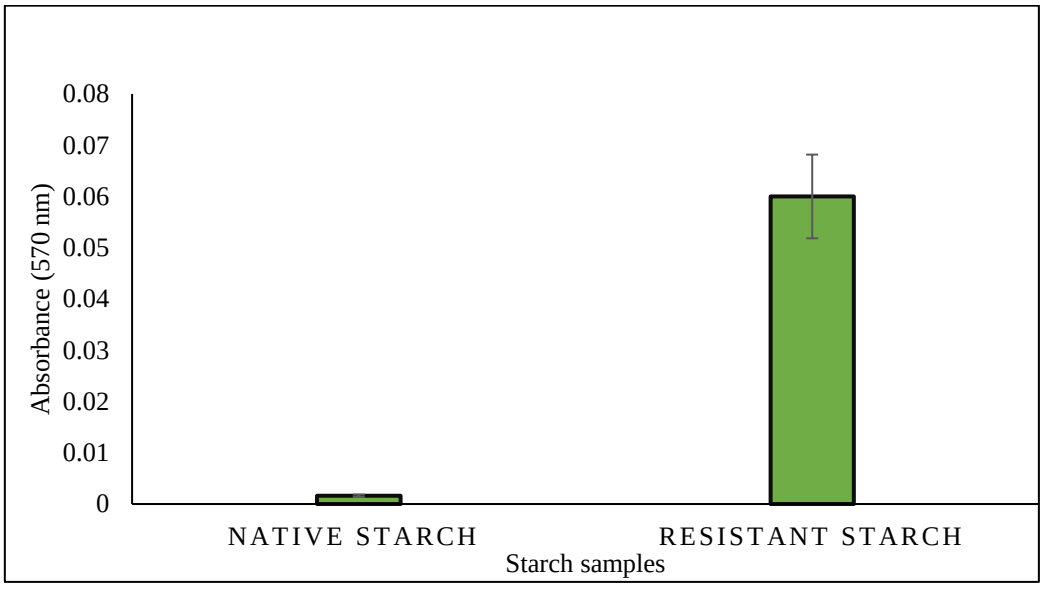


Figure 4.5 Iodine ability of different starch samples at 570 nm

4.4 Preparation and Selection of Optimized Drug Loaded Microspheres

Drug loaded microspheres were synthesized, and then an optimized formulation was selected for further characterization (Table 1). Based on the above criteria formulation (Sr.4) containing 100 mg RSIII, 10 mg Ciprofloxacin 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 %).

Table 1: Optimization of process parameter for the desired size and encapsulation efficiency of drug loaded microspheres.

Sr no	Stirring speed in rpm	Stirring time Hrs	Injection rate mL/min	Organic to aqueous phase ratio	Resistant starch in mg	Drug in mg	Size in nm	EE (%)
1	200	1	2.5	1:2	100	10	4156	53.3
2	400	2	2	1:2	100	10	1548	58.6
3	600	3	1.5	1:4	100	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

4.5 Characterization of drug loaded microspheres

4.5.1 Light Microscopy

Figure No 4.6 (A) and **Figure No 4.6 (B):** are the particular light micrographs of drug loaded microspheres and enzyme treated starch of maize flour. Samples were observed under 40 X magnification.Both of the micrograph have clear differences in RSIII shows compact granular,flat block like structures while microspheres are round in shape and smooth in textue.

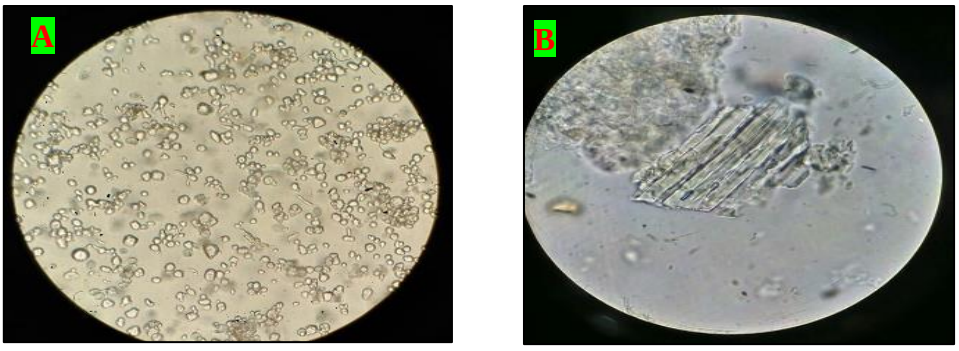


Figure 4.6 Light Micrographs of (A) Drug loaded microspheres ,(B) Enzymatically produced RSIII.

4.5.2. Particle size analysis of drug loaded microspheres.

Dynamic light scattering (DLS) technique was used to evaluate the particle size and poly dispersity indexn(PDI) of optimized drug loaded microspheres.Size was found 964 nm about 1µm and PDI of 0.452 showing the ideal monodispersity of the system **Figure No 4.7**

Results				
Size (d.nm):	**% Intensity:**			**St Dev (d.n...**
Z-Average (d.nm): 964	**Peak 1:**	**964.6**	**100.0**	**161.2**
PdI: 0.452	**Peak 2:**	**0.000**	**0.0**	**0.000**
Intercept: 0.844	**Peak 3:**	**0.000**	**0.0**	**0.000**

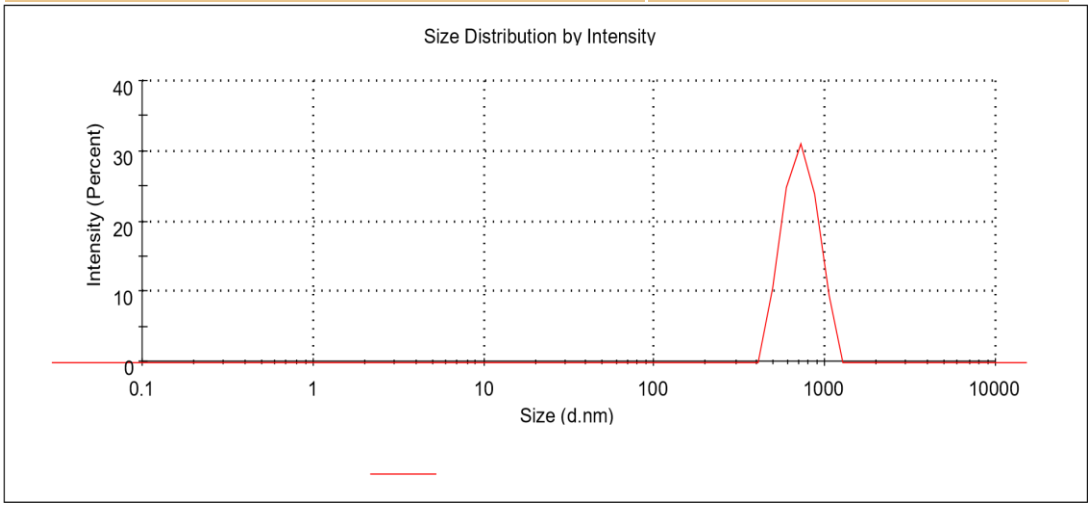


Figure 4.7 shows the size of spheres 964 nm about 1µ and PDI of 0.452.

4.5.3 Morphology of resistant starch and drug loaded Microspheres

SEM analysis in **Figure No 4.8 (A)** showed compact block like clusterious aggregates of resistant starch and **Figure No 4.8(B)** showed spherical, dispersed drug loaded microspheres.

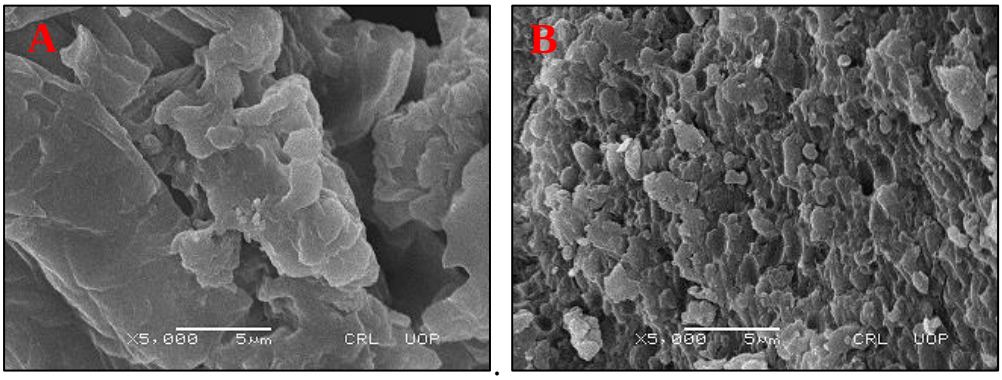


Figure 4.8 SEM Analysis of (A) Resistant starch, (B) Drug loaded microspheres

4.5.4 Fourier transform infrared (FTIR) analysis

FTIR spectra of Ciprofloxacin was showing a prominent characteristic peak between 3500 and 3450 cm^{-1} and it was due to OH stretching vibration (intermolecular hydrogen bonding).

In the FTIR spectra of the final formulation drug loaded microspheres, the prominent band in the region of 3550 and 3500 cm^{-1} were due to single bridge hydrogen bonding. While the bands in the range of 3450 to 3400 cm^{-1} were assigned to polymeric hydrogen bonds, the strong hydrogen bonding was represented by bands between 2650 and 2600 cm^{-1}.The bands from 1650 to 1600 cm^{-1} was shown by carbonyl stretching vibration. The peak between 1100 and 1000 cm^{-1} represented C-F groups, while the band at 800 cm^{-1} was indication of the meta distribution of aromatic group FTIR spectrum confirm the presence of chemical and electrostatic interactions of the components.

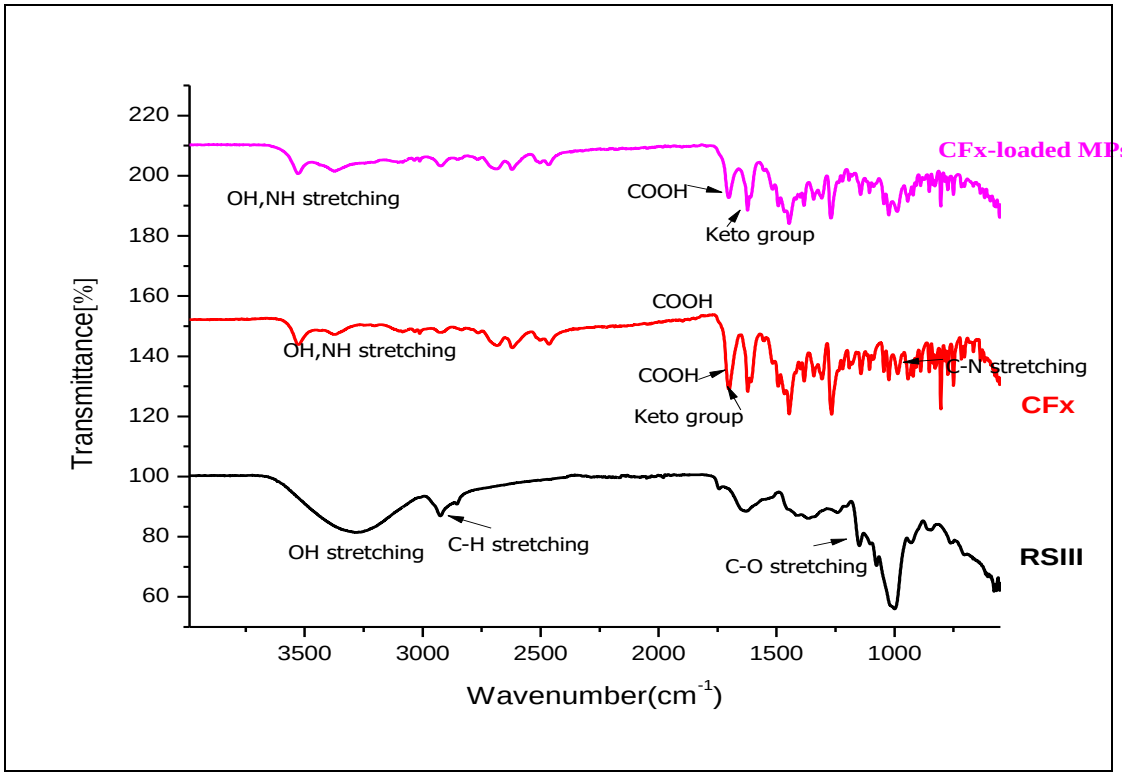


Figure 4.9 FTIR Spectrum of drug loaded microspheres, drug. RS(III).

4.5.5. X-Ray diffraction (XRD) analysis of drug loaded microspheres

Ciprofloxacin spectrum was showing sharp peaks in the range of 20-40° confirming the crystalline nature of drug can be seen in the **Figure No 4.10**. However, when it was loaded into enzymatically produced resistant starch microspheres, there were no peaks in the range of 20-40°, thus confirming the loading of drug into RSIII and crystalline nature of pure drug was completely masked within the polymeric microspheres.

.

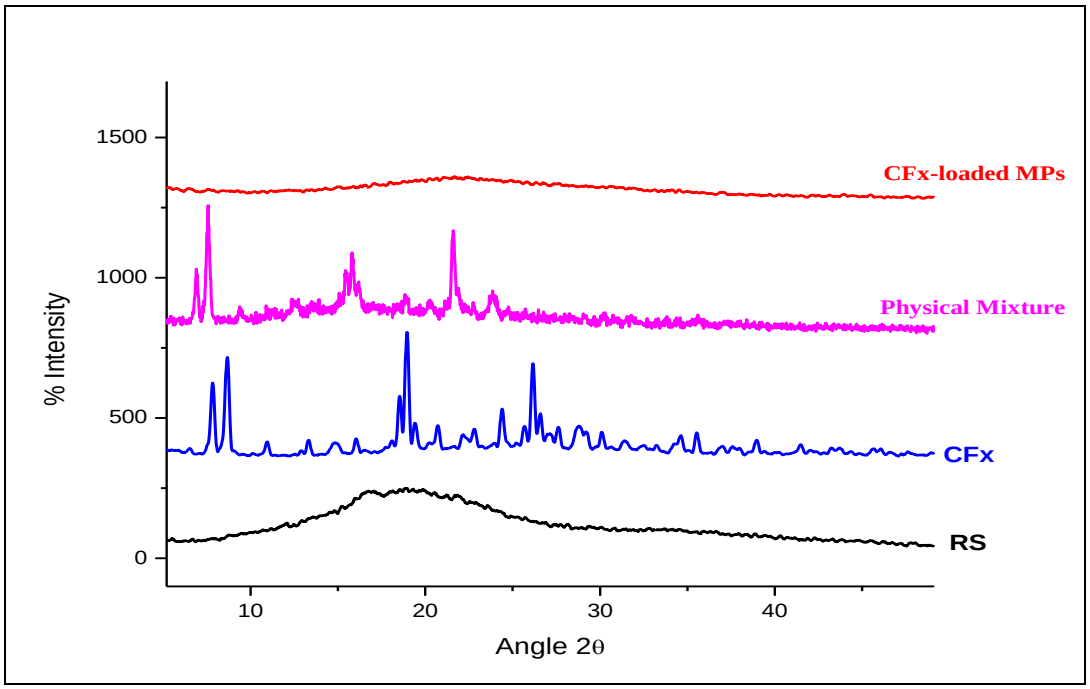


Figure no 4.10 XRD spectrum of RS, Drug, Physical mixture and drug loaded microspheres.

4.6 Encapsulation efficiency

Encapsulation efficiency was found to be 86.73%.

Table:2

Absorbance (271nm)	% EE	Average EE
0.136	88.6%	86.73± 0.013597
0.165	86.2%	
0.174	85.4%	

4.7 Percentage yield of microspheres.

Percentage yield was found to be 79.3% for drug loaded microspheres as shown below

Table 3 Percentage yield of dried microspheres

Weight of dried Microspheres	**Weight of polymer used + Drug in mg**	**Percentage yield**	**Average± STD**
86	110	78.1 %	79.3± 0.0143
89	110	80.9 %	
87	110	79 %	

4.8 *In Vitro* Drug Release Studies

4.8.1 Calibration curve of ciprofloxacin in simulated gastric fluid (HCL buffer of pH 1.2)

Calibration curve of Ciprofloxacin in HCL buffer of pH 1.2 has been shown in **Figure No 4.11**

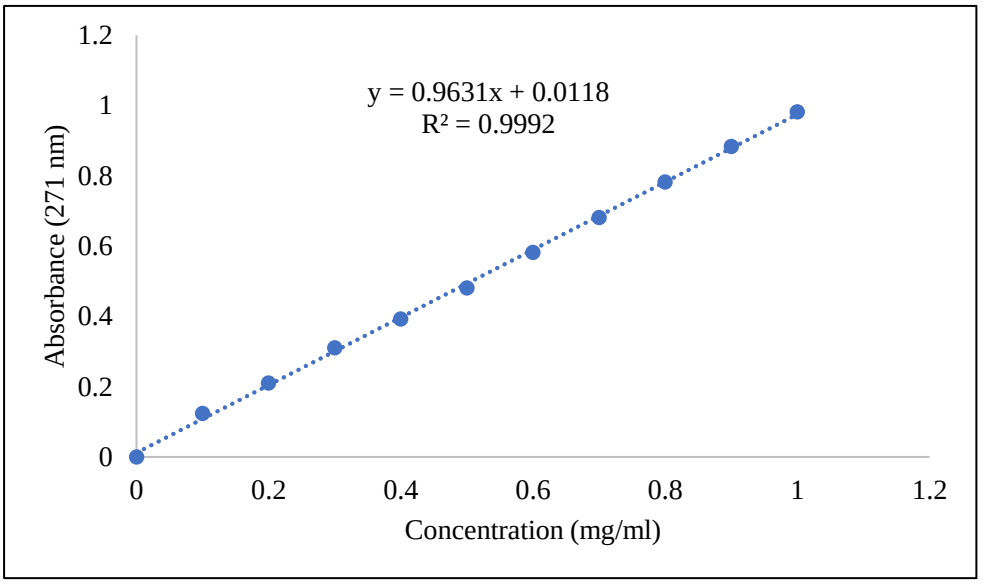


Figure 4.11 Standard curve for ciprofloxacin in HCL buffer of pH 1.2

4.8.2. Calibration curve of Ciprofloxacin in simulated intestinal fluid (phosphate buffer of pH 6.8)

Ciprofloxacin in phosphate buffer of pH 6.8. Calibration curve has been shown bellow

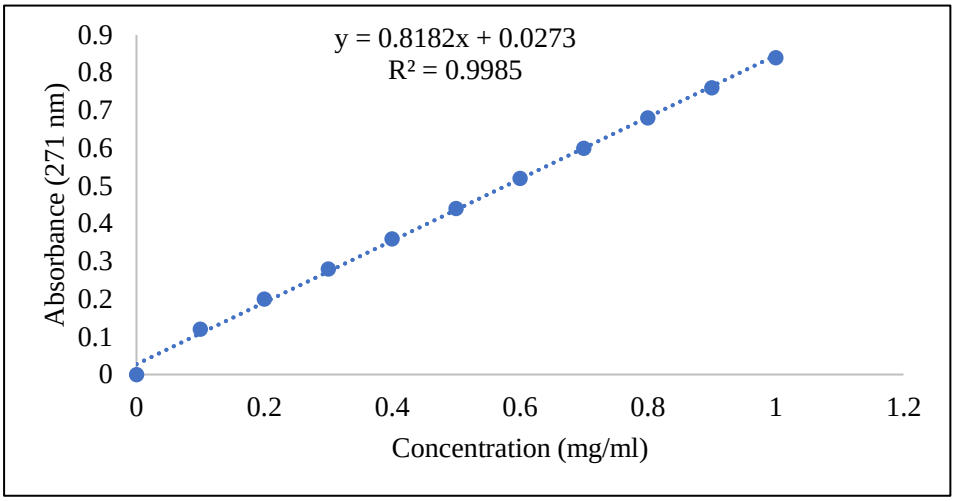
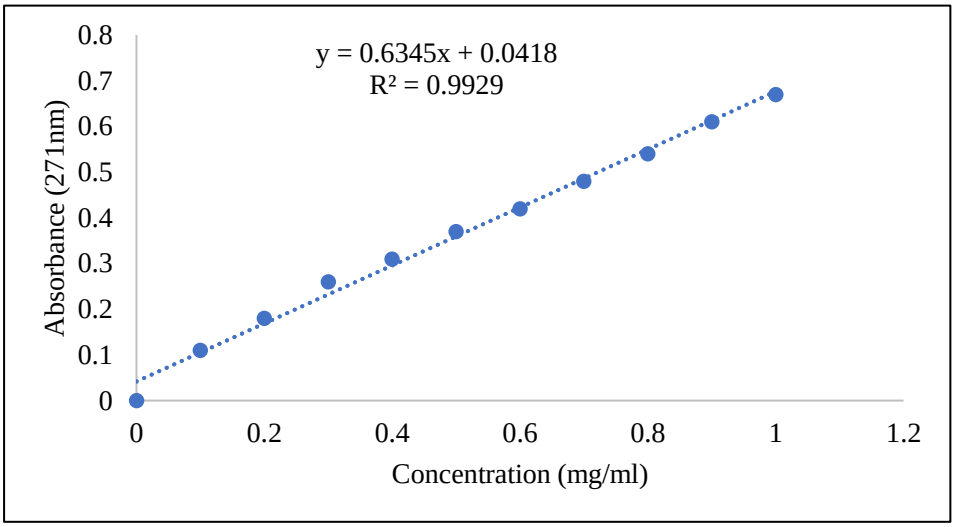


Figure 4.12 Standard curve for cipro in phosphate buffer of pH 6.8

4.8.3 Calibration curve of ciprofloxacin in simulated colonel fluid (phosphate buffer of pH 7.8)

Table is showing the values of concentration and absorbance of Ciprofloxacin in phosphate buffer of pH 7.8.



Figur 4.13 Standard curve for cipro in phosphate buffer of pH 7.8

4.8.4. *In vitro* release of drug from microspheres.

Release profile of Ciprofloxacin from enzymatically produced resistant starch based microspheres was determined by using shaking water bath method in simulated gastro

intestinal fluids of respective pH upheld at 37°C. From the **Figure 4.14,** microspheres showed increase release at pH 7.8 and almost 52% of drug was released within 24 hours, while 48% of drug was released within 6 hours, showed sustained drug release.However, in case of simulated gastric and intestinal fluids, only 5 to 8% of the drug was released from microspheres, confirming that drug has been successfully encapsulated in RSIII.

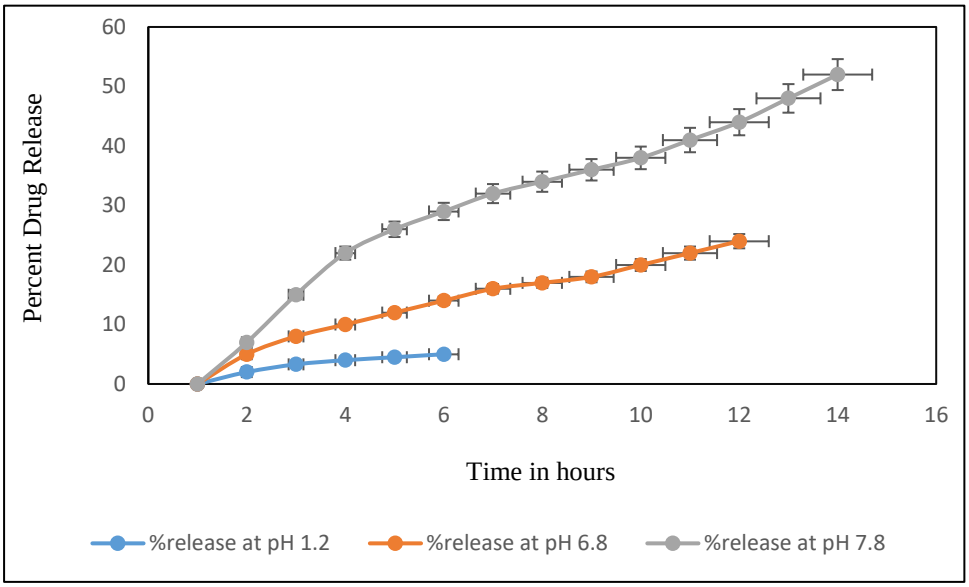


Figure 4.14 Commulative release profile of Ciprofloxacin

4.9. Kinetics of drug release from Microspheres.

To evaluate release profile of drug from microspheres,various kinetic models like zero order, first order, Higuchi, Korsmeyer peppa's and Hixon-Crowel were applied on release data.

4.9.1. Kinetics of drug release from microspheres in simulated gastric fluid

R^2 value (as shown in table) obtained in the case of simulated gastric fluid of pH 1.2 and suggests first order kinetics model to be followed in stomach.

Table 4 R^2 value of drug release from microspheres

Zero Order	1st order	Korsmeyerpeppas	Higuchi	Hixon-Crowel
0.900	0.9109	0.567	0.833	0.87

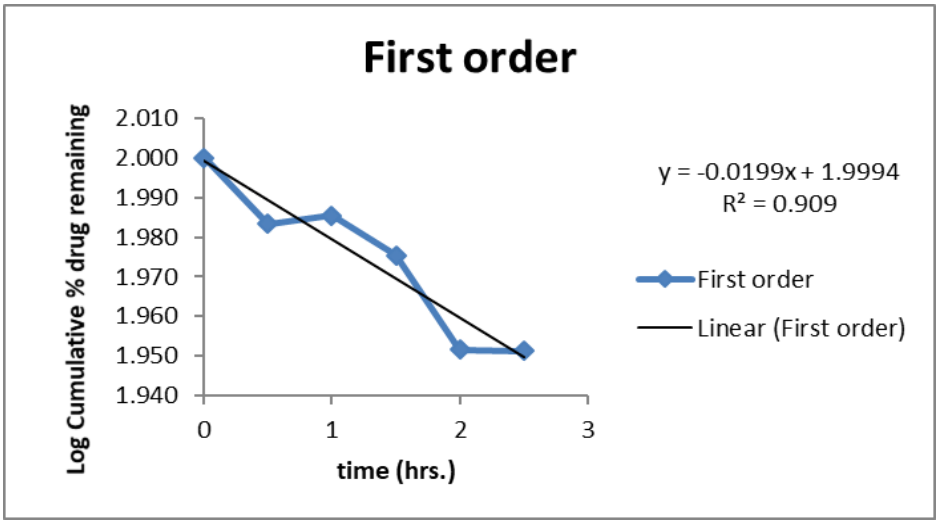


Figure 4.15 First order kinetics model to be followed in stomach

4.9.2. Kinetics of drug release from microspheres in simulated intestinal fluid

Similarly, kinetic models were applied on release profile from simulated intestinal and colonic fluids. R^2 value obtained in that case of drug release denotes that Higuchi release model has been followed.

Table 5. R^2 value of drug release from microspheres.

Zero Order	1st order	Korsmeyerpeppas	Higuchi	Hixon-Crowel
0.909	0.91 3	0.616	0.953	0.911

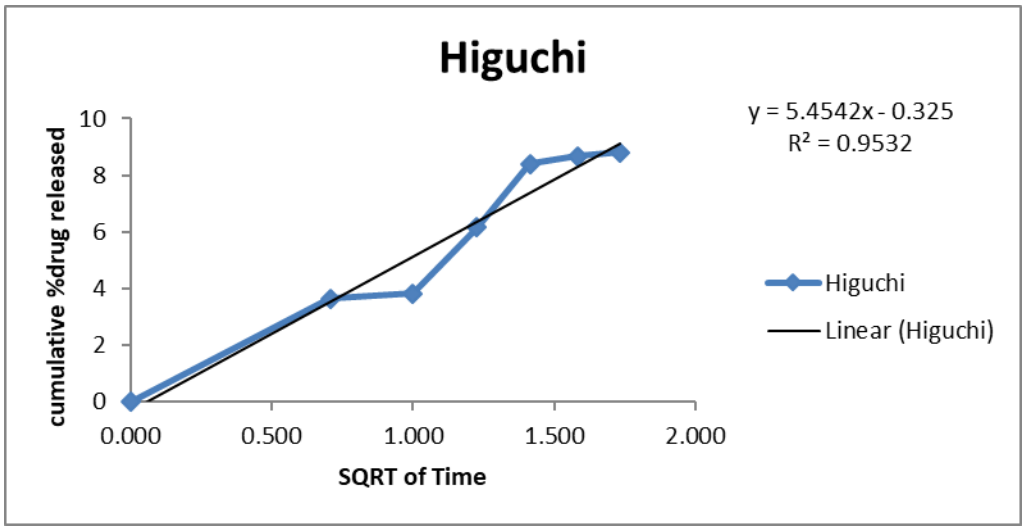


Figure 4.16 Higuchi kinetics model of drug release was followed in simulated intestinal fluid.

4.9.3 Kinetics of drug release from microspheres in simulated colonic fluid

Table 6. R^2 value of drug release from microspheres.

Zero Order	1st order	Korsmeyer peppas	Higuchi	Hixon-Crowel
0.630	0.658	0.214	0.859	0.648

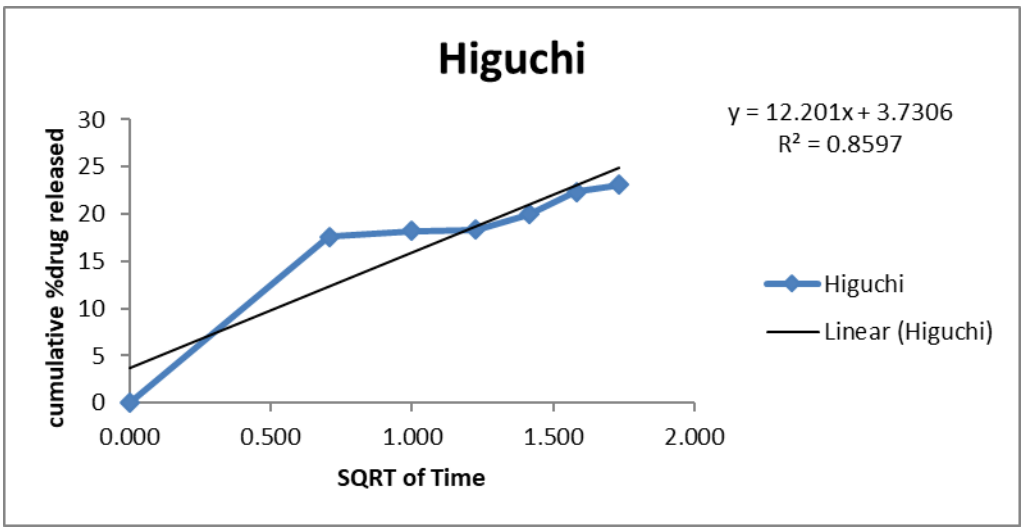


Figure 4.17 Higuchi kinetics model of drug release to be followed simulated colonic fluid.

4.10 *In Vitro* Antibacterial Assay

Antimicrobial activities of microparticles were determined by agar well diffusion method(Balouiri, Sadiki et al. 2016).Microspheres has been shown to possess antibacterial activity against *E.Coli* and *S.aureus.* The activity has been attributed to the encapsulation of drug by resistant starch.Ciprofloxacin was the positive control with drug loaded microspheres. **Figure 4.18** antibacterial effects from the polymer and drug can be seen from drug loaded microspheres in the present study.A ZOI of 22 mm for pure drug and 20 mm for drug loaded microspheres has been seen.

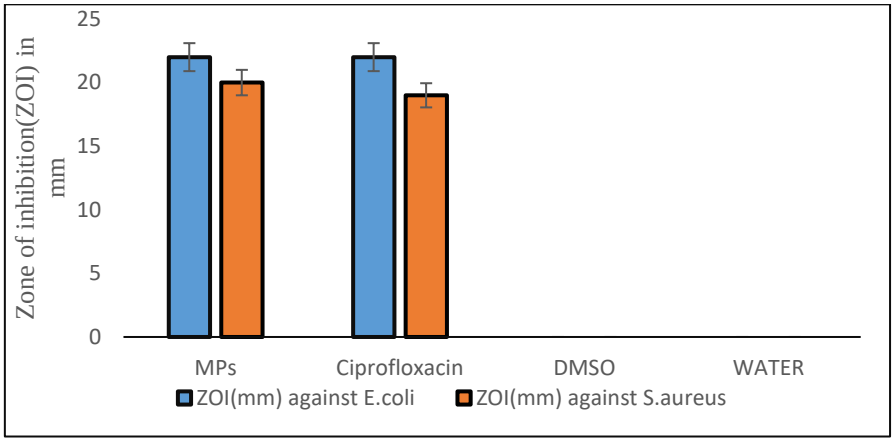


Figure 4.18 Antibacterial potential of Drug loaded Microspheres against *E. coli* and *S.aureus.*

Figure 4.19 Antibacterial activities against *E. coli* and *S.aureus*

Discussion

The current research study concentrate to explore the ability of enzymatically modified resistant starch (RSIII) from maize flour as a drug carrier for colon targeted drug delivery by exploiting the GIT track conditions.Natural biodegradable micro carriers were designed to deliver model drug i.e. Ciprofloxacin to the colon via oral route for achieving the advantage of stimuli-responsive ability of RSIII coated microspheres on one side and to minimize toxicity on the other side. Resistant starch is one of the biopolymers, is a biodegradable and is familiar as important prebiotic for stimulation growth of probiotic bacteria in colon.(Roth and Lowe 2017).Safety of nanocarriers is the important concerned.(Dunphy Guzman, Taylor et al. 2006) Metallic nanoparticles are toxic and causes inflammatory disorders(Dunphy Guzman, Taylor et al. 2006).So natural biodegradable carriers were prepared to deliver drugs through oral route to minimize danger of toxicity.Natural and biodegradable drug carriers are attracting the interest in the field of drug delivery. Starch has attained considerable importance in this field.Native starch is rapidly degraded by the intestinal enzymes after ingestion.And content of starch that is resistant to degradation by intestinal enzymes and it is only degraded by colonic bacteria can be used as potential drug carrier.(Rodrigues and Emeje 2012).RSIII coated drug microspheres can control the pH based drug release (Sahle, Giulbudagian et al. 2017).The aim was fulfilled by using single emulsion evaporation technique and drug loaded microspheres were prepared. This method was selected because it is simple,cost effective and easy method. (Chorny, Fishbein et al. 2002)(Bilati, Allémann et al. 2005).On the other side, encapsulation efficiency of this was 86.73±0.013 estimated. (Feczkó *et al*., 2011; Wang *et al*., 2009).

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. Concentration of RSIII and tween 80 has a great impact on encapsulation efficiency. It was observed that upon increasing the concentration of RSIII, encapsulation efficiency was increased and vice versa. The possible reason for improving encapsulation efficiency is that the hydrophilic nature of both RSIII and Ciprofloxacin and higher viscosity of oil phase, which promotes the escape of drug from oil phase to aqueous phase. Along with the optimization of

polymer and emulsifier concentrations, other additional 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 scrutinized. 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 formation of aggregates hence producing larger size microspheres. Rate at which organic phase is injected into the aqueous phase is very crucial factor that play major role in 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 improper mixing of two phases, while slow injection promotes the prolonged contact time of organic phase with aqueous phase that can result in smaller particle size.Process parameters were optimized to get the highest encapsulation efficiency and minimum particle size (Dong *et al*., 2015). (Budhian *et al*., 2007). High stirring speed is also a helpful parameter for proper mixing and getting smaller particle size,because it allow rapid diffusion of oil phase into aqueous phase resulting in diffusion of more drug into aqueous phase and ultimately effect the encapsulation efficiency.(Budhian *et al*., 2007: Mehrotra and Pandit 2012). (Asadi *et al*., 2011). SEM images showed that RSIII has compact, block like structure, while the drug loaded RSIII microspheres have spherical and uniform distribution and also has 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 drug (Ciprofloxacin). 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 in the range of 1750 to 1700 cm^{-1} were not detected in the final formulation most probably due to interaction

with polymer 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.(Ramesh, Ranganayakulu et al. 2010,Sahoo, Chakraborti et al. 2011Tom, Suryanarayanan et al.2004) In Dynamic light scattering (DLS) analysis size of about 964 nm almost 1µm and poly dispersity index (PDI) of 0.452 was measured,results are showing ideal monodispersity system.

XRD results illustrated that Ciprofloxacin have sharp peaks in the range of 20-40° confirming the crystalline nature of drug. But, when it was loaded into RSIII, there were no characteristics peaks in the range of 20-40°; however, the spectrum of EM-RSIII remains same before and after loading of drug into polymer. Physical mixture of drug and RSIII also confirmed that complete encapsulation of drug only occur by single emulsion evaporation method. Hence confirming the loading of drug into microspheres and crystalline nature of pure drug was completely masked within the microspheres. Key advantage of this is an increase in solubility and thus enhanced bioavailability of drug. These results are in accordance with the results reported by Eesfandiarpour Boroujeni, as the drug changed its nature from crystalline to amorphous form.(Esfandiarpour-Boroujeni *et al*., 2017: Qindeel, Ahmed et al. 2019)

Percent yield of microspheres is critically important from industrial and economic point of view. Higher the percent yield of micropaticles 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 amount of reactants lost during the preparation method was much less than product produced. Hence, the preparation method is highly beneficial. Higher encapsulation efficiency is the most important features 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 previous studies where encapsulation efficiency was found to be 67 % ± 8% in which they prepared pH sensitive curcumin loaded NPs(Beloqui *et al*., 2014).

In vitro release studies of the drug encapsulated microspheres revealed a faster and higher 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 RSIII is resistant to gastric conditions and sensitive to higher pH and is also fermented by colonic bacteria. In the absences of colonic bacteria the outer coating of RSIII impart additional stability to 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 as colonic bacteria will also digest the polymer so drug released will be more enhanced at colon (Chen *et al*., 2017). While in case of simulated gastric and intestinal fluids only 5 to 8% of the drug was released from microspheres, confirming that drug has been successfully encapsulated in RSIII, similar results were also concluded.The *in vitro* release of Ciprofloxacin from microspheres 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 polymer 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

where, pH sensitive nanoparticles exhibited significant difference in release profile at acidic and neutral pH (Beloqui *et al*., 2014; Sahle *et al*., 2017) (Thakral *et al*., 2010). To investigate release mechanism of drug from microspheres various kinetic models were applied on the release data,like zero order, first order, Higuchi, Korsmeyer peppa's and Hixon-Crowel were applied in release data. In simulated gastric buffer R^2 value closer to 1 explains that drug release form microspheres follows first order kinetics which explains concentration dependent release of drug form polymer matrix. On the other hand, in simulated intestinal fluid and colonic fluid it follows Higuchi release kinetics with $R^2 = 0.951$. Higuchi model explains diffusion based drug release from matrix system (Fernández-Colino *et al*., 2016).Antibacterial assay was carried out to have strong evidence that drug was encapsulated into RSIII. The antibacterial effect seen in case of drug loaded microspheres was the confirmation of encapsulation ability of RSIII..

Conclusion

- It was concluded from the current study that enzymatically produced resistant starch can be used as a potential drug carrier in oral delivery of drugs.
- Microspheres were successfully prepared by single emulsion evaporation technique.
- Characterization studies including ingredients compatibility, particle size, polydispersity index, morphology, encapsulation efficiency and antibacterial assay of drug loaded microspheres explains their compatibility for targeted delivery of drug.
- *In vitro* release study showed 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.

Future Prospects

- *In vivo* study should be conducted for the further permeation of drug loaded microspheres.
- Polymer utilized in the preparation of formulation is a prebiotic, natural, biodegradable resistant to enzymatic degradation, and highly stable, so formulations of this drug carrier system can be scaled up to commercial level..
- Method utilized for the preparation of microspheres is suitable for hydrophobic and hydrophilic drugs as well.
- Variety of drugs, probiotics, proteins and enzymes can be encapsulated in resistant starch.

References

Carbinatto, F.M., de Castro, A.D., Cury, B.S., Magalhães, A. and Evangelista, R.C., 2012. Physical properties of pectin–high amylose starch mixtures cross-linked with sodium trimetaphosphate. *International journal of pharmaceutics*, *423*(2), pp.281288.

Prezotti, F.G., Meneguin, A.B., Evangelista, R.C. and Ferreira Cury, B.S., 2012. Preparation and characterization of free films of high amylose/pectin mixtures crosslinked with sodium trimetaphosphate. *Drug development and industrial pharmacy*, *38*(11), pp.1354-1359.

Wang, Q., Hu, X., Du, Y. and Kennedy, J.F., 2010. Alginate/starch blend fibers and their properties for drug controlled release. *Carbohydrate Polymers*, *82*(3), pp.842847.

Abbas, O. and Baeten, V., 2016. Advances in the Identification of Adulterated Vegetable Oils. In *Advances in Food Authenticity Testing* (pp. 519-542).

Soares, G.A., de Castro, A.D., Cury, B.S. and Evangelista, R.C., 2013. Blends of crosslinked high amylose starch/pectin loaded with diclofenac. *Carbohydrate polymers*, *91*(1), pp.135-142.

Ashwar, B.A., Gani, A., Shah, A., Wani, I.A. and Masoodi, F.A., 2016.

Preparation, health benefits and applications of resistant starch—A review. *StarchStärke*, *68*(3-4), pp.287-301.

Singh, B., Dhiman, A. and Kumar, A., 2016. Slow release of ciprofloxacin from βcyclodextrin containing drug delivery system through network formation and supramolecular interactions. *International journal of biological macromolecules*, *92*, pp.390-400.

Mura, P., Cirri, M., Mennini, N., Casella, G. and Maestrelli, F., 2016. Polymeric mucoadhesive tablets for topical or systemic buccal delivery of clonazepam: Effect of cyclodextrin complexation. *Carbohydrate polymers*, *152*,.755. ppda Rosa Zavareze, E. and Dias, A.R.G., 2011. Impact of heat-moisture treatment and annealing in starches: A review. *Carbohydrate Polymers*, *83*(2), pp.317-328.

Marinich, J.A., Ferrero, C. and Jiménez-Castellanos, M.R., 2012. Graft copolymers of ethyl methacrylate on waxy maize starch derivatives as novel excipients for matrix tablets: Drug release and fronts movement kinetics. *European Journal of Pharmaceutics and Biopharmaceutics*, *80*(3), pp.674-681.

Teacă, C.A., Bodîrlău, R. and Spiridon, I., 2013. Effect of cellulose reinforcement on the properties of organic acid modified starch microparticles/plasticized starch biocomposite films. *Carbohydrate polymers*, *93*(1), pp.307-315.

Htoon, A.K., Uthayakumaran, S., Piyasiri, U., Appelqvist, I.A., López-Rubio, A., Gilbert, E.P. and Mulder, R.J., 2010. The effect of acid dextrinisation on enzymeresistant starch content in extruded maize starch. *Food chemistry*, *120*(1), pp.140-149.

Afifi, S.A., Mandour, W.M. and Elkhodairy, K.A., 2015. Optimization of a novel oral colon delivery system of indomethacin using full factorial design. *Tropical Journal of Pharmaceutical Research*, *14*(5), pp.761-768.

Chen, H.Y., Yu, S.L., Chen, C.H., Chang, G.C., Chen, C.Y., Yuan, A., Cheng, C.L., Wang, C.H., Terng, H.J., Kao, S.F. and Chan, W.K., 2007. A five-gene signature and clinical outcome in non–small-cell lung cancer. *New England Journal of Medicine*, *356*(1), pp.11-20.

Yang, S.H., Salas, R., Krivoy, E.M., Nair, H.P., Bank, S.R. and Jarrahi, M., 2016. Characterization of ErAs: GaAs and LuAs: GaAs superlattice structures for continuouswave terahertz wave generation through plasmonic photomixing. *Journal of Infrared, Millimeter, and Terahertz Waves*, *37*(7), pp.640-648.

Dunphy Guzman, K.A., Taylor, M.R. and Banfield, J.F., 2006. Environmental risks of nanotechnology: National nanotechnology initiative funding, 2000− 2004.

Dunphy Guzman, K.A., Finnegan, M.P. and Banfield, J.F., 2006. Influence of surface potential on aggregation and transport of titania nanoparticles. *Environmental science & technology*, *40*(24), pp.7688-7693.

Chen, L., Pu, H., Li, X. and Yu, L., 2011. A novel oral colon-targeting drug delivery system based on resistant starch acetate. *Journal of Controlled Release*, *152*, pp.e51-e52.

Dunphy Guzman, K.A., Taylor, M.R. and Banfield, J.F., 2006. Environmental risks of nanotechnology: National nanotechnology initiative funding, 2000− 2004.

Chen, J., Li, X., Chen, L. and Xie, F., 2018. Starch film-coated microparticles for oral colon-specific drug delivery. *Carbohydrate polymers*, *191*, pp.242-254.

Chen, J., Li, X., Chen, L. and Xie, F., 2018. Starch film-coated microparticles for oral colon-specific drug delivery. *Carbohydrate polymers*, *191*, pp.242-254.

Chouhan, R. and Bajpai, A.K., 2010. Release dynamics of ciprofloxacin from swellable nanocarriers of poly (2-hydroxyethyl methacrylate): an in vitro study. *Nanomedicine: Nanotechnology, Biology and Medicine*, *6*(3), pp.453-462.

Chen, J., Liang, Y., Li, X., Chen, L. and Xie, F., 2016. Supramolecular structure of jackfruit seed starch and its relationship with digestibility and physicochemical properties.
Carbohydrate polymers, *150*, pp.269-277.

(LR) Mollania, N., Khajeh, K., Hosseinkhani, S. and Dabirmanesh, B., 2010.

Purification and characterization of a thermostable phytate resistant α-amylase from Geobacillus sp. LH8. *International journal of biological macromolecules*, *46*(1), pp.27-
36.

Li, S., Yang, X., Yang, S., Zhu, M. and Wang, X., 2012. Technology prospecting on enzymes: application, marketing and engineering. *Computational and Structural Biotechnology Journal*, *2*(3), p.e201209017.

Gurung, N., Ray, S., Bose, S. and Rai, V., 2013. A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. *BioMed research international*, *2013*.

Dhar, S., Banerjee, R. and Malakar, R., 2012. Neonatal erythroderma: Diagnostic and therapeutic challenges. *Indian journal of dermatology*, *57*(6), p.475.

Rigoldi, F., Donini, S., Redaelli, A., Parisini, E. and Gautieri, A., 2018. Engineering of thermostable enzymes for industrial applications. *APL Bioengineering*, *2*(1), p.011501.

Van Der Maarel, M.J., Van der Veen, B., Uitdehaag, J.C., Leemhuis, H. and

Dijkhuizen, L., 2002. Properties and applications of starch-converting enzymes of the αamylase family. *Journal of biotechnology*, *94*(2), pp.137-155.

Van Der Maarel, M.J., Van der Veen, B., Uitdehaag, J.C., Leemhuis, H. and

Dijkhuizen, L., 2002. Properties and applications of starch-converting enzymes of the αamylase family. *Journal of biotechnology*, *94*(2), pp.137-155.

Burhan, A., Nisa, U., Gökhan, C., Ömer, C., Ashabil, A. and Osman, G., 2003. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator

resistant amylase from an alkaliphilic Bacillus sp. isolate ANT-6. *Process Biochemistry*, *38*(10), pp.1397-1403.

Yang, S.T., El-Ensashy, H. and Thongchul, N. eds., 2013. *Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers*. John Wiley & Sons.

Cherubini, F., 2010. The biorefinery concept: using biomass instead of oil for producing energy and chemicals. *Energy conversion and management*, *51*(7), pp.14121421.

Streb, S. and Zeeman, S.C., 2012. Starch metabolism in Arabidopsis. *The Arabidopsis book/American Society of Plant Biologists*, *10*.

Wandee, Y., Uttapap, D., Puncha-arnon, S., Puttanlek, C., Rungsardthong, V. and Wetprasit, N., 2017. In vitro fermentabilities of raw and cooked canna starches and their derivatives. *Journal of Functional Foods*, *34*, pp.461-469.

Parada, J. and Aguilera, J.M., 2009. In vitro digestibility and glycemic response of potato starch is related to granule size and degree of gelatinization. *Journal of Food Science*, *74*(1), pp.E34-E38.

Kong, X., Zhu, P., Sui, Z. and Bao, J., 2015. Physicochemical properties of starches from diverse rice cultivars varying in apparent amylose content and gelatinisation temperature combinations. *Food chemistry*, *172*, pp.433-440.

Karmakar, R., Ban, D.K. and Ghosh, U., 2014. Comparative study of native and modified starches isolated from conventional and nonconventional sources. *International Food Research Journal*, *21*(2), p.597.

Karmakar, R., Pahari, P. and Mal, D., 2014. Phthalides and phthalans: Synthetic methodologies and their applications in the total synthesis. *Chemical reviews*, *114*(12), pp.6213-6284.

Kalinga, D.N., Bertoft, E., Tetlow, I. and Seetharaman, K., 2014. Structure of clusters and building blocks in amylopectin from developing wheat endosperm. *Carbohydrate polymers*, *112*, pp.325-333.

Whistler, R.L. and BeMiller, J.N., 1997. *Carbohydrate chemistry for food scientists*. Eagan press.

Syahariza, Z.A., Sar, S., Hasjim, J., Tizzotti, M.J. and Gilbert, R.G., 2013. The importance of amylose and amylopectin fine structures for starch digestibility in cooked rice grains. *Food chemistry*, *136*(2), pp.742-749.

Lehmann, U. and Robin, F., 2007. Slowly digestible starch–its structure and health implications: a review. *Trends in Food Science & Technology*, *18*(7), pp.346-355.

Lockyer, S. and Nugent, A.P., 2017. Health effects of resistant starch. *Nutrition bulletin*, *42*(1), pp.10-41.

Zhang, J., Tang, M. and Viikari, L., 2012. Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases. *Bioresource technology*, *121*, pp.8-12.

Juntunen, K.S., Niskanen, L.K., Liukkonen, K.H., Poutanen, K.S., Holst, J.J. and Mykkänen, H.M., 2002. Postprandial glucose, insulin, and incretin responses to grain products in healthy subjects. *The American journal of clinical nutrition*, *75*(2), pp.254-262.

Sekirov, I., Russell, S.L., Antunes, L.C.M. and Finlay, B.B., 2010. Gut microbiota in health and disease. *Physiological reviews*, *90*(3), pp.859-904.

Fuentes-Zaragoza, E., Riquelme-Navarrete, M.J., Sánchez-Zapata, E. and PérezÁlvarez, J.A., 2010. Resistant starch as functional ingredient: A review. *Food Research International*, *43*(4), pp.931-942.

Paterson, A.H., Wendel, J.F., Gundlach, H., Guo, H., Jenkins, J., Jin, D., Llewellyn, D., Showmaker, K.C., Shu, S., Udall, J. and Yoo, M.J., 2012. Repeated polyploidization of Gossypium genomes and the evolution of spinnable cotton fibres. *Nature*, *492*(7429), p.423

Simsek, S. and El, S.N., 2012. Production of resistant starch from taro (Colocasia esculenta L. Schott) corm and determination of its effects on health by in vitro methods. *Carbohydrate polymers*, *90*(3), pp.1204-1209.

Reddy, C.K., Suriya, M. and Haripriya, S., 2013. Physico-chemical and functional properties of Resistant starch prepared from red kidney beans (Phaseolus vulgaris. L) starch by enzymatic method. *Carbohydrate polymers*, *95*(1), pp.220-226.

Singh, J., Dartois, A. and Kaur, L., 2010. Starch digestibility in food matrix: a review. *Trends in Food Science & Technology*, *21*(4), pp.168-180.

Alsaffar, A.A., 2011. Effect of food processing on the resistant starch content of cereals and cereal products–a review. *International journal of food science & technology*, *46*(3), pp.455-462.

Lehmann, U. and Robin, F., 2007. Slowly digestible starch–its structure and health implications: a review. *Trends in Food Science & Technology*, *18*(7), pp.346-355.

Jyothsna, E. and Hymavathi, T.V., 2017. Resistant starch: Importance, categories, food sources and physiological effects. *Journal of Pharmacognosy and Phytochemistry*, *6*(2), pp.67-69.

Yuan, J.H., Xu, R.K. and Zhang, H., 2011. The forms of alkalis in the biochar produced from crop residues at different temperatures. *Bioresource technology*, *102*(3), pp.34883497.

Zięba, T., Szumny, A. and Kapelko, M., 2011. Properties of retrograded and acetylated starch preparations: Part 1. Structure, susceptibility to amylase, and pasting characteristics. *LWT-Food Science and Technology*, *44*(5), pp.1314-1320.

Ng, M., Fleming, T., Robinson, M., Thomson, B., Graetz, N., Margono, C., Mullany, E.C., Biryukov, S., Abbafati, C., Abera, S.F. and Abraham, J.P., 2014. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980– 2013: a systematic analysis for the Global Burden of Disease Study 2013. *The lancet*, *384*(9945), pp.766-781.

Raturi, V., 2016. *Studies on the effect of Integrated Nutrient Management on growth, yield and quality of apple (Malus x domestica Borkh.) cv. Red Delicious* (Doctoral dissertation, College of Horticulture, Bharsar Campus, VCSG Uttarakhand University of Horticulture and Forestry).

Zhou, X., Baik, B.K., Wang, R. and Lim, S.T., 2010. Retrogradation of waxy and normal corn starch gels by temperature cycling. *Journal of cereal science*, *51*(1), pp.57-65.

Ashwar, B.A., Gani, A., Wani, I.A., Shah, A., Masoodi, F.A. and Saxena, D.C.,

2016. Production of resistant starch from rice by dual autoclaving-retrogradation

Hidayat, B., Muslihudin, M. and Akmal, S., 2018, January. Application of autoclavingcooling cycling treatment to improve resistant starch content of corn-based rice analogues. In *Journal of Physics: Conference Series* (Vol. 953, No. 1, p. 012010). IOP Publishing.

Rafiq, S.I., Singh, S. and Saxena, D.C., 2016. Effect of heat-moisture and acid treatment on physicochemical, pasting, thermal and morphological properties of Horse Chestnut (Aesculus indica) starch. *Food Hydrocolloids*, *57*, pp.103-113.

Ma, Q., Shen, Q., Xu, Q., Li, D., Shu, L. and Weber, B., 2011. Empathic responses to others' gains and losses: an electrophysiological investigation. *Neuroimage*, *54*(3), pp.2472-2480.

Guo, L., 2018. In vitro amylase hydrolysis of amylopectins from cereal starches based on molecular structure of amylopectins. *Food Hydrocolloids*, *77*, pp.238-247.

Ooms, N., Vandromme, E., Brijs, K. and Delcour, J.A., 2018. Intact and Damaged Wheat Starch and Amylase Functionality During Multilayered Fermented Pastry Making. *Journal of food science*, *83*(10), pp.2489-2499.

Jeong, D., Han, J.A., Liu, Q. and Chung, H.J., 2018. Effect of processing, storage, and modification on in vitro starch digestion characteristics of food legumes: A review. *Food Hydrocolloids*.

Shi, J., Sun, Z. and Shi, Y.C., 2019. Improved in vitro assay of resistant starch in crosslinked phosphorylated starch. *Carbohydrate Polymers*.

Jeong, D., Han, J.A., Liu, Q. and Chung, H.J., 2018. Effect of processing, storage, and modification on in vitro starch digestion characteristics of food legumes: A review. *Food Hydrocolloids*.

Shah, A., Masoodi, F.A., Gani, A. and Ashwar, B., 2018. Dual enzyme modified oat starch: Structural characterisation, rheological properties, and digestibility in simulated GI tract. *International journal of biological macromolecules*, *106*, pp.140147.

Ai, Y. and Jane, J.L., 2018. Understanding starch structure and functionality. In *Starch in Food* (pp. 151-178). Woodhead Publishing.

Saito, H., Tamura, M. and Ogawa, Y., 2019. Starch digestibility of various Japanese commercial noodles made from different starch sources. *Food Chemistry*.

Knutson, C.A., 2000. Evaluation of variations in amylose–iodine absorbance spectra. *Carbohydrate polymers*, *42*(1), pp.65-72.

Lei, Z., Shao, Y., Yin, X., Yin, D., Guo, Y. and Yuan, J., 2016. Combination of xylanase and debranching enzymes specific to wheat arabinoxylan improve the growth performance and gut health of broilers. *Journal of agricultural and food chemistry*, *64*(24), pp.4932-4942.

Yotsawimonwat, S., Sriroth, K., Kaewvichit, S., Piyachomkwan, K., Jane, J.L. and Sirithunyalug, J., 2008. Effect of pH on complex formation between debranched waxy rice starch and fatty acids. *International journal of biological macromolecules*, *43*(2), pp.94-
99.

Klaochanpong, N., Puttanlek, C., Rungsardthong, V., Puncha-arnon, S. and Uttapap, D., 2015. Physicochemical and structural properties of debranched waxy rice, waxy corn and waxy potato starches. *Food Hydrocolloids*, *45*, pp.218-226.

Oliveira, I., Sousa, A., Ferreira, I.C., Bento, A., Estevinho, L. and Pereira, J.A., 2008. Total phenols, antioxidant potential and antimicrobial activity of walnut (Juglans regia L.) green husks. *Food and chemical toxicology*, *46*(7), pp.2326-2331.

Ashwar, B.A., Gani, A., Shah, A., Wani, I.A. and Masoodi, F.A., 2016. Preparation, health benefits and applications of resistant starch—A review. *Starch-Stärke*, *68*(3-4), pp.287-301.

Dhital, S., Warren, F.J., Butterworth, P.J., Ellis, P.R. and Gidley, M.J., 2017.

Mechanisms of starch digestion by α-amylase—Structural basis for kinetic properties.

Critical reviews in food science and nutrition, *57*(5), pp.875-892. Maughan, R., 2009. Carbohydrate metabolism. *Surgery (Oxford)*, *27*(1), pp.6-10.

Alsaffar, A.A., 2011. Effect of food processing on the resistant starch content of cereals and cereal products–a review. *International journal of food science & technology*, *46*(3), pp.455-462.

Singh, J., Dartois, A. and Kaur, L., 2010. Starch digestibility in food matrix: a review. *Trends in Food Science & Technology*, *21*(4), pp.168-180.

Fässler, C., Arrigoni, E., Venema, K., Hafner, V., Brouns, F. and Amadò, R., 2006.

Digestibility of resistant starch containing preparations using two in vitro models.

European journal of nutrition, *45*(8), pp.445-453.

Pongjanta, J., Utaipattanaceep, A., Naivikul, O. and Piyachomkwan, K., 2009.

Debranching enzyme concentration effected on physicochemical properties and αamylase hydrolysis rate of resistant starch type III from amylose rice starch. *Carbohydrate polymers*, *78*(1), pp.5-9.

Giuberti, G., Gallo, A., Cerioli, C., Fortunati, P. and Masoero, F., 2015. Cooking quality and starch digestibility of gluten free pasta using new bean flour. *Food Chemistry*, *175*, pp.43-49.

Cevc, G. and Vierl, U., 2010. Nanotechnology and the transdermal route: A state of the art review and critical appraisal. *Journal of controlled release*, *141*(3), pp.277-299.

Han, J., Wang, M., Hu, Y., Zhou, C. and Guo, R., 2017. Conducting polymer-noble metal nanoparticle hybrids: Synthesis mechanism application. *Progress in Polymer Science*, *70*, pp.52-91.

González-Rubio, G. and Liz-Marzán, L.M., 2018. Peptides used to make lighttwisting nanoparticles.

Joos, M., Ding, C., Loo, V., Giacobino, E., Bramati, A. and Glorieux, Q., 2017,

April. Single nanocrystal quantum dot coupled to a nanofiber as a tool for chiral Quantum Optics. In *Quantum Information and Measurement* (pp. QT2B-3). Optical Society of America.

Kumari, M., Pandey, S., Giri, V.P., Bhattacharya, A., Shukla, R., Mishra, A. and Nautiyal, C.S., 2017. Tailoring shape and size of biogenic silver nanoparticles to enhance antimicrobial efficacy against MDR bacteria. *Microbial pathogenesis*, *105*, pp.346-355.

Stebbins, G.L., 2014. *The basis of progressive evolution*. UNC Press Books. Uchegbu, I.F. and Schatzlein, A.G., 2006. *Polymers in drug delivery*. CRC Press.

Baghel, S., Cathcart, H. and O'Reilly, N.J., 2016. Polymeric amorphous solid dispersions: a review of amorphization, crystallization, stabilization, solid-state characterization, and aqueous solubilization of biopharmaceutical classification system class II drugs. *Journal of pharmaceutical sciences*, *105*(9), pp.2527-2544.

Bou-Chacra, N., Melo, K.J.C., Morales, I.A.C., Stippler, E.S., Kesisoglou, F., Yazdanian, M. and Löbenberg, R., 2017. Evolution of choice of solubility and dissolution media after two decades of biopharmaceutical classification system. *The AAPS journal*, *19*(4), pp.989-1001.

de Sousa, I.P. and Bernkop-Schnürch, A., 2014. Pre-systemic metabolism of orally administered drugs and strategies to overcome it. *Journal of Controlled Release*, *192*, pp.301-309.

Wilkinson, G.R., 2005. Drug metabolism and variability among patients in drug response. *New England Journal of Medicine*, *352*(21), pp.2211-2221.

Farokhzad, O.C. and Langer, R., 2009. Impact of nanotechnology on drug delivery.

ACS nano, *3*(1), pp.16-20.

Akerib, D.S., Araújo, H.M., Bai, X., Bailey, A.J., Balajthy, J., Beltrame, P., Bernard, E.P., Bernstein, A., Biesiadzinski, T.P., Boulton, E.M. and Bradley, A., 2016. Improved limits on scattering of weakly interacting massive particles from reanalysis of 2013 LUX data. *Physical review letters*, *116*(16), p.161301.

Surendiran, A., Sandhiya, S., Pradhan, S.C. and Adithan, C., 2009. Novel applications of nanotechnology in medicine. *Indian Journal of Medical Research*, *130*(6).

Nahar, M., Dutta, T., Murugesan, S., Asthana, A., Mishra, D., Rajkumar, V., Tare, M., Saraf, S. and Jain, N.K., 2006. Functional polymeric nanoparticles: an efficient and promising tool for active delivery of bioactives. *Critical Reviews™ in Therapeutic Drug Carrier Systems*, *23*(4).

Jawahar, N. and Meyyanathan, S.N., 2012. Polymeric nanoparticles for drug delivery and targeting: A comprehensive review. *International Journal of Health & Allied Sciences*, *1*(4), p.217.

Sumit, G., 2012. Nanotechnology in food packaging a critical review. *Russian Journal of Agricultural and Socio-Economic Sciences*, *10*(10).

Jawahar, N. and Meyyanathan, S.N., 2012. Polymeric nanoparticles for drug delivery and targeting: A comprehensive review. *International Journal of Health & Allied Sciences*, *1*(4), p.217.

Dadwal, M., Solan, D. and Pradesh, H., 2014. Polymeric nanoparticles as promising novel carriers for drug delivery: an overview. *Journal of Advanced Pharmacy Education & Research Jan-Mar*, *4*(1).

Rao, J.P. and Geckeler, K.E., 2011. Polymer nanoparticles: preparation techniques and size-control parameters. *Progress in polymer science*, *36*(7), pp.887-913.

Mahdavian, A.R., Ashjari, M. and Makoo, A.B., 2007. Preparation of poly (styrene– methyl methacrylate)/SiO2 composite nanoparticles via emulsion polymerization. An investigation into the compatiblization. *European Polymer Journal*, *43*(2), pp.336-344.

Landfester, K., 2009. Miniemulsion polymerization and the structure of polymer and hybrid nanoparticles. *Angewandte Chemie International Edition*, *48*(25), pp.4488-4507.

Hu, F.Q., Hong, Y. and Yuan, H., 2004. Preparation and characterization of solid lipid nanoparticles containing peptide. *International Journal of Pharmaceutics*, *273*(1-2), pp.29-
35.

Galindo-Rodriguez, S., Allemann, E., Fessi, H. and Doelker, E., 2004. Physicochemical parameters associated with nanoparticle formation in the salting-out, emulsification-diffusion, and nanoprecipitation methods. *Pharmaceutical research*, *21*(8), pp.1428-1439.

Desgouilles, S., Vauthier, C., Bazile, D., Vacus, J., Grossiord, J.L., Veillard, M. and Couvreur, P., 2003. The design of nanoparticles obtained by solvent evaporation: a comprehensive study. *Langmuir*, *19*(22), pp.9504-9510.

Banik, B.L., Fattahi, P. and Brown, J.L., 2016. Polymeric nanoparticles: the future of nanomedicine. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, *8*(2), pp.271-299.

Sane, A. and Limtrakul, J., 2009. Formation of retinyl palmitate-loaded poly (llactide) nanoparticles using rapid expansion of supercritical solutions into liquid solvents (RESOLV). *The Journal of Supercritical Fluids*, *51*(2), pp.230-237.

Guo, H., Liu, Y., Wang, Y., Wu, J., Yang, X., Li, R., Wang, Y. and Zhang, N., 2014. pH-sensitive pullulan-based nanoparticle carrier for adriamycin to overcome drugresistance of cancer cells. *Carbohydrate polymers*, *111*, pp.908-917.

Miladi, K., Sfar, S., Fessi, H. and Elaissari, A., 2016. Nanoprecipitation process: from particle preparation to in vivo applications. In *Polymer Nanoparticles for Nanomedicines* (pp. 17-53). Springer, Cham.

Maaz, A., Abdelwahed, W., Tekko, I.A. and Trefi, S., 2014. Influence of nanoprecipitation method parameters on nanoparticles loaded with gatifloxacin for ocular drug delivery. *Int. J. Acad. Sci. Res*, *3*(1), pp.1-12.

Budhian, A., Siegel, S.J. and Winey, K.I., 2007. Haloperidol-loaded PLGA nanoparticles: systematic study of particle size and drug content. *International journal of pharmaceutics*, *336*(2), pp.367-375.

McManamey, W.J. and Woollen, J.M., 1973. The diffusivity of carbon dioxide in some organic liquids at 25 and 50 C. *AIChE Journal*, *19*(3), pp.667-669.

Mora-Huertas, C.E., Fessi, H. and Elaissari, A., 2010. Polymer-based nanocapsules for drug delivery. *International journal of pharmaceutics*, *385*(1-2), pp.113-142.

Bilensoy, E., Sarisozen, C., Esendağlı, G., Doğan, A.L., Aktaş, Y., Şen, M. and Mungan, N.A., 2009. Intravesical cationic nanoparticles of chitosan and polycaprolactone for the delivery of Mitomycin C to bladder tumors. *International journal of pharmaceutics*, *371*(1-2), pp.170-176.

Govender, T., Stolnik, S., Garnett, M.C., Illum, L. and Davis, S.S., 1999. PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. *Journal of Controlled Release*, *57*(2), pp.171-185.

Almoustafa, H.A., Alshawsh, M.A. and Chik, Z., 2017. Technical aspects of preparing PEG-PLGA nanoparticles as carrier for chemotherapeutic agents by nanoprecipitation method. *International journal of pharmaceutics*, *533*(1), pp.275-284.

Bukhari, A., Idris, A. and Atta, M., 2014. Effect of organic and aqueous dispersion medium on the development of polystyrene nanoparticles in nanoprecipitation method.
Malaysian Journal of Fundamental and Applied Sciences, *10*(1).
Noor, N., Shah, A., Gani, A., Gani, A. and Masoodi, F.A., 2018.

Microencapsulation of caffeine loaded in polysaccharide based delivery systems. *Food Hydrocolloids*, *82*, pp.312-321.

Arica, B. and Lamprecht, A., 2005. In vitro evaluation of betamethasone-loaded nanoparticles. *Drug development and industrial pharmacy*, *31*(1), pp.19-24

Allémann, E., Gurny, R. and Doelker, E., 1992. Preparation of aqueous polymeric nanodispersions by a reversible salting-out process: influence of process parameters on particle size. *International Journal of Pharmaceutics*, *87*(1-3), pp.247253.

Fonseca, F.C., Goya, G.F., Jardim, R.F., Muccillo, R., Carreno, N.L.V., Longo, E. and Leite, E.R., 2002. Superparamagnetism and magnetic properties of Ni nanoparticles embedded in SiO 2. *Physical Review B*, *66*(10), p.104406.

Htoon, A.K., Uthayakumaran, S., Piyasiri, U., Appelqvist, I.A., López-Rubio, A., Gilbert, E.P. and Mulder, R.J., 2010. The effect of acid dextrinisation on enzymeresistant starch content in extruded maize starch. *Food chemistry*, *120*(1), pp.140-149.

Haralampu, S.G., 2000. Resistant starch—a review of the physical properties and biological impact of RS3. *Carbohydrate polymers*, *41*(3), pp.285-292.

Chung, H.J., Shin, D.H. and Lim, S.T., 2008. In vitro starch digestibility and estimated glycemic index of chemically modified corn starches. *Food research international*, *41*(6), pp.579-585.

Thompson, D.B., 2000. Strategies for the manufacture of resistant starch.

Trends in Food Science & Technology, *11*(7), pp.245-253.

Chung, H.J., Liu, Q. and Hoover, R., 2009. Impact of annealing and heatmoisture treatment on rapidly digestible, slowly digestible and resistant starch levels in native and gelatinized corn, pea and lentil starches. *Carbohydrate Polymers*, *75*(3), pp.436-447.

Zhou, Y., Wang, D., Zhang, L., Du, X. and Zhou, X., 2008. Effect of polysaccharides on gelatinization and retrogradation of wheat starch. *Food Hydrocolloids*, *22*(4), pp.505512.

Najafi, S.H.M., Baghaie, M. and Ashori, A., 2016. Preparation and characterization of acetylated starch nanoparticles as drug carrier: Ciprofloxacin as a model. *International journal of biological macromolecules*, *87*, pp.48-54.

Dupuis, J.H., Liu, Q. and Yada, R.Y., 2014. Methodologies for increasing the resistant starch content of food starches: A review. *Comprehensive reviews in food science and food safety*, *13*(6), pp.1219-1234.

Haralampu, S.G., 2000. Resistant starch—a review of the physical properties and biological impact of RS3. *Carbohydrate polymers*, *41*(3), pp.285-292.

Lockyer, S. and Nugent, A.P., 2017. Health effects of resistant starch. *Nutrition bulletin*, *42*(1), pp.10-41.

Apostolidis, E. and Lee, C.M., 2010. In vitro potential of Ascophyllum nodosum phenolic antioxidant-mediated α-glucosidase and α-amylase inhibition. *Journal of food science*, *75*(3), pp.H97-H102.

Foster-Powell, K., Holt, S.H. and Brand-Miller, J.C., 2002. International table of glycemic index and glycemic load values: 2002. *The American journal of clinical nutrition*, *76*(1), pp.5-56.

Sekirov, I., Russell, S.L., Antunes, L.C.M. and Finlay, B.B., 2010. Gut microbiota in health and disease. *Physiological reviews*, *90*(3), pp.859-904.

Reutrakul, S. and Van Cauter, E., 2018. Sleep influences on obesity, insulin resistance, and risk of type 2 diabetes. *Metabolism*.

Fuentes-Zaragoza, E., Sánchez-Zapata, E., Sendra, E., Sayas, E., Navarro, C., Fernández-López, J. and Pérez-Alvarez, J.A., 2011. Resistant starch as prebiotic: a review. *Starch-Stärke*, *63*(7), pp.406-415.

Geurts, L., Neyrinck, A.M., Delzenne, N.M., Knauf, C. and Cani, P.D., 2013. Gut microbiota controls adipose tissue expansion, gut barrier and glucose metabolism: novel insights into molecular targets and interventions using prebiotics. *Beneficial microbes*, *5*(1), pp.3-17.

Hoseinifar, S.H., Safari, R. and Dadar, M., 2017. Dietary sodium propionate affects mucosal immune parameters, growth and appetite related genes expression: Insights from zebrafish model. *General and comparative endocrinology*, *243*, pp.78-83.

Geurts, L., Neyrinck, A.M., Delzenne, N.M., Knauf, C. and Cani, P.D., 2013. Gut microbiota controls adipose tissue expansion, gut barrier and glucose metabolism:

novel insights into molecular targets and interventions using prebiotics. *Beneficial microbes*, *5*(1), pp.3-17.

Han, F., Gao, C. and Liu, M., 2013. Fabrication and characterization of sizecontrolled starch-based nanoparticles as hydrophobic drug carriers. *Journal of nanoscience and nanotechnology*, *13*(10), pp.6996-7007.

Singh, K., Mishra, A. and Singh, A., 2018. Synthesis Characterization and In Vitro Release Study of Ciprofloxacin-Loaded Chitosan Nanoparticle. *BioNanoScience*, *8*(1), pp.229-236.

Schwert, G.W. and Eisenberg, M.A., 1949. The kinetics of the amidase and esterase activities of trypsin. *J. biol. Chem*, *179*, pp.665-672.

Beers, R.F. and Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol chem*, *195*(1), pp.133-140.

Larisch, W. and Goss, K.U., 2017. Calculating the first-order kinetics of three coupled, reversible processes. *SAR and QSAR in Environmental Research*, *28*(8), pp.651659.

Hixson, A.W. and Crowell, J.H., 1931. Dependence of reaction velocity upon surface and agitation. *Industrial & Engineering Chemistry*, *23*(8), pp.923-931.

Korsmeyer, R.W., Gurny, R., Doelker, E., Buri, P. and Peppas, N.A., 1983. Mechanisms of solute release from porous hydrophilic polymers. *International journal of pharmaceutics*, *15*(1), pp.25-35.

Higuchi, T., 1963. Mechanism of sustained-action medication. Theoretical analysis of rate of release of solid drugs dispersed in solid matrices. *Journal of pharmaceutical sciences*, *52*(12), pp.1145-1149.

Roth, P.J. and Lowe, A.B., 2017. Stimulus-responsive polymers. *Polymer Chemistry*, *8*(1), pp.10-11.

Dunphy Guzman, K.A., Taylor, M.R. and Banfield, J.F., 2006. Environmental risks of nanotechnology: National nanotechnology initiative funding, 2000− 2004.

Rodrigues, A. and Emeje, M., 2012. Recent applications of starch derivatives in nanodrug delivery. *Carbohydrate Polymers*, *87*(2), pp.987-994.

Sahle, F.F., Giulbudagian, M., Bergueiro, J., Lademann, J. and Calderón, M., 2017. Dendritic polyglycerol and N-isopropylacrylamide based thermoresponsive nanogels as smart carriers for controlled delivery of drugs through the hair follicle. *Nanoscale*, *9*(1), pp.172-182.

.

Bilati, U., Allémann, E. and Doelker, E., 2005. Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles. *European Journal of Pharmaceutical Sciences*, *24*(1), pp.67-75.

Budhian, A., Siegel, S.J. and Winey, K.I., 2007. Haloperidol-loaded PLGA nanoparticles: systematic study of particle size and drug content. *International journal of pharmaceutics*, *336*(2), pp.367-375.

Chorny, M., Fishbein, I., Danenberg, H.D. and Golomb, G., 2002. Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. *Journal of controlled release*, *83*(3), pp.389-400.

Mehrotra, A. and Pandit, J.K., 2012. Critical process parameters evaluation of modified nanoprecipitation method on lomustine nanoparticles and cytostatic activity study on L132 human cancer cell line. *J Nanomed Nanotechnol*, *3*, p.8.

Mehrotra, A. and Pandit, J.K., 2012. Critical process parameters evaluation of modified nanoprecipitation method on lomustine nanoparticles and cytostatic activity study on L132 human cancer cell line. *J Nanomed Nanotechnol*, *3*, p.8.

Qindeel, M., Ahmed, N., Sabir, F., Khan, S. and ur. Rehman, A.2019. Development Novel pH-sensitive Nanoparticles Loaded Hydrogel for Transdermal Drug Delivery.

Drug development and industrial pharmacy, (just-accepted), pp.1-29.

Appendices

Table 1 Resistant Starch (RS) Content of Starch samples:

Starch Sample	RS1	RS2	RS3	Avg RS	STD
Native	1.88	1.54	1.99	1.80	0.23
RS(P+A)	18.95	19.01	19.05	19.00	0.05
IR	42.99	45.05	43.97	44.00	1.03

Table 2 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 3 Solubility of drug Ciprofloxacin 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 4 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 5 Calibration curve of ciprofloxacin 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 6 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 7 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 8 Calibration curve of ciprofloxacin in phosphate buffer of (pH 7.8)

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

Table:9 Antibacterial activity of drug loaded microspheres against *E.coli*

Samples	Zone of inhibition
DMSO	0mm
Ciprofloxacin positive control	22mm
Drug loaded microspheres	20mm

Table:10 Antibacterial activity of drug loaded microspheres against *S.aureus*

Samples	Zone of inhibition
DMSO negative control.	0
Ciprofloxacin positive control	20mmm
Drug loaded microspheres	19 mm