CARBOHYDRATE – BASED MICRO AND NANO SCALE MATERIALS FROM SOYBEAN MEAL, DISTILLERS DRIED GRAINS, AND SUGAR BEET PULP FOR FOOD AND OTHER APPLICATIONS

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Title

Carbohydrate – based micro and nano scale materials from soybean meal, distillers dried grains, and sugar beet pulp for food and other applications.

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ABSTRACT

Study 1 was carried out to isolate and produce enzyme resistant micro and nano scale carbohydrates from soybean meal (SBM), distillers dried grains (DDG), and sugar beet pulp (SBP). All materials produced were in micro and nano scales, and the yields were different depending on the starting material.

Study 2 determined the physiological stability of study 1 materials for potential encapsulation applications. Both SBM and SBP were stable under simulated physiological conditions; the stability of the SBM was higher than SBP.

In study 3 the SBM nanomaterial from buffer extraction was used for in vitro fermentation by *Bifidobacterium brevis*. The *B. brevis* showed a good growth in the presence of SBM nanomaterial.

Study 4 characterized the SBM- resistant starch thin films produced with pectin and carboxymethylcelllulose. The films formed with SBM-resistant starch and pectin at 3:1 and 1:3 provided good barrier and viscoelastic properties.

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DEDICATION

To my parents, brother, and teachers

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1. INTRODUCTION

Agricultural by-products are renewable resources. Approximately, 650 million metric tons of agricultural by-products are produced annually in North America (USDA 2012). Main agricultural by-products include distillers dried grain, soybean meal, and sugar beet pulp. Annual production of distillers dried grain, soybean meal, and sugar beet pulp include 43, 38, and 29 million metric tons respectively (USDA, 2012; FAO, 2011). These abundant agricultural by-products are renewable resources, and therefore could be utilized for value added applications.

The value of agricultural by-products depends on the availability and potential uses. These uses can differ in developed and developing countries. In developed countries nearly 850 million metric tons of agricultural by-products are utilized as animal feed (IFIF 2013). In developing countries, they are used as fiber, fuel, and chemicals (Crop Diversification Division, 2003). The refinement and exploitation of the abundant agricultural by-products for bio-product development could add value to the existing uses.

The nutritional composition of bio-refined agricultural by-products can lead to value added applications in food and other sectors such as pharmaceuticals. Biodegradable agricultural by-products in nature are in the form of polysaccharides, mainly starch and dietary fiber. These polysaccharides could be processed into biodegradable products such as biodegradable packaging material for food and biomedical applications. Thus, the polysaccharide rich agricultural by-products provide substantial benefits towards value added applications.

Specifically, agricultural by-products could be used for biomedical packaging of drugs and nutrients. These bioactive packaging could increase the bioavailability of bioactive compounds under physiological conditions. The increased bioavailability could combat the most

prevalent micronutrient deficiencies such as Iron (Fe) and Zinc (Zn) deficiencies. Thus, polysaccharide rich agricultural by-products, which are comprised of starch and dietary fiber, could be used as packaging and encapsulating materials to increase the bioavailability of drugs and nutrients.

Starch and dietary fiber can be hydrolyzed during their transit through the gastro intestinal tract, thus releasing the encapsulated material. Furthermore, targeted delivery can be achieved by reducing the particle size of the encapsulating materials. In turn the reduced particle size of encapsulated material could increase the efficiency of the delivery of nutrient and drugs. Hence, polysaccharides with reduced particle size could be used as good encapsulating agents for nutrients and drugs.

Encapsulating materials reduced to nano scale materials could be used as efficient delivery systems. Nano particles as nano particulate delivery systems in food and nutraceutical application can exhibit novel and significant biomedical properties. Timely release of nutrient and drug using nano materials could increase the bioavailability at the targeted sites. Moreover, nano scale materials are potent sources to be used as delivery systems. Therefore, the current research focused on the following studies.

1.1. Feasibility of producing laboratory scale micro and nano materials from agricultural by-products; soybean meal (SBM), distillers dried grain (DDG), and sugar beet pulp (SBP)

Objective: Determine the feasibility of producing micro and nano materials from agricultural by-products.

H_0: The yield and morphological characteristics are the same for the agricultural by-products (SBM, DDG, and SBP) produced at micro and nano scale.

1.2. Examine the stability of micro and nano materials produced from enzyme resistant carbohydrates of sugar beet pulp and resistant starch of soybean meal under simulated physiological condition

Objective: Determine the stability of the micro and nano materials produced from enzyme resistant carbohydrates of sugar beet pulp and resistant starch of soybean meal under simulated physiological condition.

H_0: Both the sugar beet pulp and soybean meal have similar stabilities under simulated physiological condition.

1.3. In vitro fermentation of the *Bifidobacterium brevis* in the presence of nano scale soybean meal resistant starch under simulated physiological conditions

Objective: Determine the ability of the in vitro fermentation of *Bifidobacterium brevis* in the presence of nano scale soybean meal resistant starch under simulated physiological conditions.

H_0: The growth of *Bifidobacterium brevis* is same in the presence and absence of soybean meal resistant starch nanoparticles.

1.4. Characterize the thin films formed using soybean meal resistant starch nano particles, pectin, and carboxymethylcellulose for food and other applications

Objective: Determine the physical properties of the thin films for suitable application in food and other sectors.

H_0: The films formed with different quantities of soybean meal resistant starch nano particles, pectin, and carboxymethylcelluloses have similar barrier and viscoelastic properties.

2. LITERATURE REVIEW

2.1. Nanotechnology

Nanotechnology is a field that has had significant growth in the recent years. Nanotechnology can be defined as understanding and manipulating materials at atomic, molecular, and macro molecular scale at less than 100 nm (Cushen et al., 2012). Nano scale materials can be produced by mechanical (top-down) or chemical process (bottom-up). The mechanical process involves using shear or particle collision to break down the larger aggregates into nano scale entities. The chemical process involves producing nano materials through chemical reactions or self-assembly of materials (Acosta, 2009; Cushen et al., 2012). Production of nano materials by mechanical or chemical process leads to increased surface area per mass ratio with significant biochemical properties.

Nano materials possess novel properties due to their greater surface area to volume ratio. Nano materials are more chemically and biologically active compared to macro scale materials with the same chemical composition (Sozer & Kokini, 2009). The larger surface area of these materials could be used in increasing the bioavailability of components such as vitamins, minerals, probiotics, and prebiotics (Acosta, 2009). Enhanced bioavailability of nano scale materials can be achieved by their targeted release of active ingredients at the specified site (Acosta, 2009). Thus, increased bioavailability and targeted release of ingredients through nano scale materials facilitates many possible food and other applications such as pharmaceuticals.

2.2. Nanotechnology in food and other applications

Nanotechnology has driven the attention of food and other sectors for various applications. These applications include encapsulating and delivering bioactive compounds to the targeted sites (Yadav, 2004). Bioactive compounds are subjected to acidic conditions in stomach and alkaline conditions in intestine. Furthermore, the compounds enter the blood stream by penetrating into gastric mucosa (Russell- Jones & Luke, 2007). Encapsulation could keep the bioactive ingredients at optimum conditions until they are released at the specified site (Yadav, 2004). Thus, nano materials can be used for encapsulation to deliver the bioactive compounds to the targeted sites ensuring the active release of the compound at appropriate amounts.

Nano scale materials are potential materials to deliver bioactive ingredients in food and other sectors. Nano scale materials as carriers provide increased surface area enhancing the bioavailability of the active ingredients at the targeted sites (Rashidi et al., 2011). Increased surface area enables the nano materials to remain at the targeted site for a longer period. The retention of nano materials at the specified site increases the release of encapsulated material, which in turn enhances the bioavailability (Acosta, 2009). Thus, the nano materials can be widely used to enhance the bioavailability of the bioactive compounds.

2.2.1. Nanotechnology in food applications

Nanotechnology in food sectors provides new methods to improve nutritional value by enhancing the bioavailability of nutrients such as Iron (Fe) and Zinc (Zn). Enhancing the bioavailability at the specific site by ensuring the release of the nutrient is important in food industry (Sanguansri & Augustin, 2006). The increased surface area of the nano materials caused by the reduced particle size, releases the nutrients at the specified site thereby increasing the

bioavailability (Yadav, 2004). A key strategy involved in increasing the bioavailability of the nutrient can be achieved by encapsulation. Encapsulation involves coating of a desired nutrient within a wall material (Sanguansri & Augustin, 2006). Nano scale materials used for encapsulating increase the bioavailability of the nutrient compared to materials of larger scale (Sanguansri & Augustin, 2006).

Nano encapsulation could retain the bioactivity of nutrients until they are released at targeted site (Sozer & Kokini, 2009). In the food industry nano encapsulation is achieved by using nano scale packaging materials, which in turn can increase the bioavailability of nutrients (Sozer & Kokini, 2009). Nano scale food packaging materials keep bioactive compounds at optimum level until they are released at the targeted region (Rashidi et al, 2011). A major concern of encapsulation in the food sector is the use of food grade coatings such as carbohydrates, proteins, and lipids (Sanguansri & Augustin, 2006). These are biodegradable and in turn reduce the accumulation of waste material in the environment (Sozer & Kokini, 2009). In summary, nano encapsulation in the food sector is an important application for enhanced bioavailability of nutrients.

2.2.2. Nanotechnology in pharmaceutical applications

Nano materials are widely used as carriers for therapeutics in the pharmaceutical industry due to the specificity of nano particles. The reduced particle size of nano materials enables them to penetrate into cells through capillaries and enhance the uptake/bioavailability of therapeutic agents (Hornig et al., 2009). This property of nano material allows it to be used as an effective carrier in targeted delivery, which reduces the side effects due to therapeutics (Acosta, 2009;

Parveen et al., 2012). Nano materials in pharmaceutical applications have several advantages attributable due to their reduced particle size.

Selective and controlled release of therapeutics is an important property to be considered in pharmaceuticals and medicine. Nano particles accumulate at the targeted site and release therapeutics while minimizing the side effects (Parveen et al., 2012). The selective and controlled release depends on the type of nano materials used. Both biodegradable and non-biodegradable materials can be used in nano scale for selective and controlled release. Biodegradable nano scale material can be used for sustained drug release at a selected site for several days (Parveen et al., 2012). However, non-biodegradable materials at nano scale can be used for selective and controlled release for a shorter period compared to biodegradable material (Hornig et al., 2009). Thus, nano materials have potential functions in pharmaceutical sector for targeted, selective, and controlled release of therapeutics.

2.3. Risks associated with nano materials

Nanotechnology is a rapidly growing field in modern times. However, the reduced particle sizes of nano materials are of concern and are continually assessed for safety aspects (Bouwmeester et al., 2009). The degree of risk of nano materials in food and other application depends on the size and the chemical composition (Peralta-Videa et al., 2011). Reduced particle size has the capability to enter human body through inhalation, ingestion, and dermal penetration (Maynard, 2006). Furthermore, the potential of the nano materials to cause harm depends on the type of materials used. Synthetic materials in nano scale could be toxic to cells due to the incorporation of metal ions and radioactive isotopes (Savolainen et al., 2010; Bouwmeester et al., 2009). Bio-based materials like carbohydrates, proteins, and lipids do not harm the cells as they

are biocompatible (Peralta- Videa et al., 2011). Several risk assessment practices are implemented for both natural and man-made nano materials (Savolainen et al., 2010). In conclusion, the bio-based nano materials are at lower risk compared to man-made synthetic nano materials.

2.4. Agricultural by-products

A range of distinct agricultural by-products are produced around the world due to various farm activities (Eady et al., 2012). Agricultural by-products are bio-based in nature, which can replace a major portion of petroleum derived products (Crop Diversification Division, 2003). Increased use of bio-based products from agricultural by-products in place of petroleum- derived products could reduce significant health and environmental damage (Reddy & Yang, 2005). The abundant agricultural by-products have the potential to be used in the developments of new technologies.

The abundant agricultural by-products could be influential in food and other sectors. The main agricultural by-products are derived from corn, soybean, and sugar beet (Crop Diversification Division, 2003; Mohanty et al., 2000). The majority of these agricultural by-products are carbohydrates in nature. Carbohydrates could provide skeletal frame works for value added applications of the by-products (Crop Diversification Division, 2003). Major carbohydrates utilized in food and other sectors are starch and its derivatives (Crop Diversification Division, 2003). Therefore, agricultural by-products could be a potent source to be used in food and pharmaceutical sectors.

2.4.1. Soybean meal (SBM)

Soybean (*Glycine max*), a dicotyledonous plant belonging to the family Leguminosae, is an important crop in the US (Hoover & Zhou, 2003; Stevenson et al., 2007). Production of soybean in the US has increased from 8.5 million metric tons in 2004 to 43.3 million metric tons in 2010 (Steveson et al., 2007; FAO, 2012). The US soybean accounts for nearly 44% of the world's soybean trade (Soy Stats, 2011). The increased production of soybean both in the US and globe may be due to the high nutritive value of soybeans.

The uniqueness of soybeans is due to their rich nutrient content. Soybean is composed of 40% fat, 35% carbohydrates, 20% protein, and 1-5% of minerals (Chen et al., 2010). The high content of oil in soybean is used for oil extraction in soybean industry (Huisman et al., 1998). The by-product of oil extraction of soybean is soybean meal (Huisman et al., 1998). Soybean meal contains a higher amount of carbohydrates (40%) than soybean, which has only 35% carbohydrate (Lilienthal et al., 2005). Soybean meal with higher carbohydrate content is mainly used as feed for livestock.

Carbohydrate contents of soybean meal are predominant components in animal feed. The carbohydrates of soybean meal could be categorized as structural or non-structural carbohydrates (Johnson et al., 2008). The non-structural carbohydrates include low-molecular weight sugars, oligosaccharides, and storage polysaccharides (Lilienthal et al., 2005). Starch is the storage polysaccharides of soybean meal, which comprises 1-5% of total non-structural carbohydrates (Johnson et al., 2008). A fraction of starch in SBM is resistant towards hydrolytic enzymes in the digestive system of mono gastric animals (Hoover & Sosulski, 1990). Therefore, this fraction of

starch can be used as an encapsulating material to deliver the bioactive compounds in the GI tract.

2.4.2. Distillers dried grain (DDG)

Corn (*Zea mays*) is a cereal grown widely in North America (Hoseney, 1986; Cheesbrough et al., 2008). Approximately 35-70 million metric tons of corn is produced in the US mainly for ethanol production (USDA, 2012; Cheesbrough et al., 2008). Ethanol from corn can be produced in two ways: dry-grind and wet milling (Liu, 2011a). The dry-grind process of ethanol production (**Figure 1**) gives rise to a cereal by-product known as distillers dried grains (DDG) (Liu, 2011a). The DDG is a nutrient rich component, which is currently used as an animal feed (Liu et al., 2011b).

The major use of DDG as an animal feed is due to the high nutritive value, which replaces some corn components in the diet. High protein content of the DDG is the main component that makes it a high value use in livestock feed (Cheesbrough et al., 2008). The nutritional components of DDG include 31% protein, 12% fat, 4% ash, and 5% starch (Saunders & Rosentrater, 2009). Starch in DDG is in the form of resistant starch after subjecting it to dry-grind with enzymes such as α- amylase and glucoamylases (Liu et al., 2011b; Bothast & Schlicher, 2005). The presence of other components in DDG could be utilized for alternate paths for value-added uses.

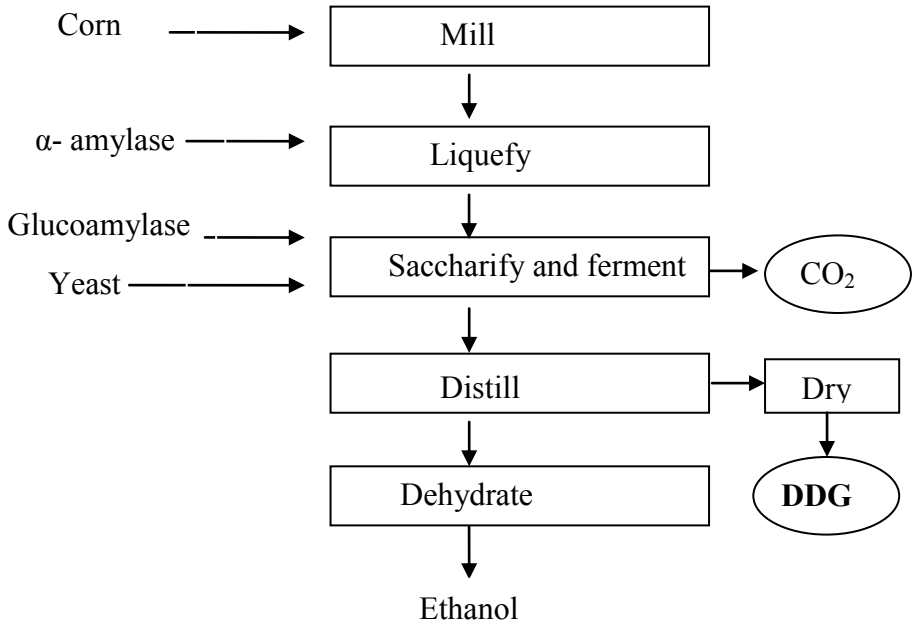


Figure 2.1. Dry grind ethanol production (Adapted from Bothast & Schlicher, 2005)

Increased demands for ethanol have exacerbated the needs for uses of DDG in different fields, by isolating the components of DDG. Isolation of different components of DDG and implementing these components in food and other sectors can increase the utility of DDG beyond animal feed (Bothast & Schlicher, 2005; Zarrinbakhsh & Mohanty, 2013). A major concern in incorporating components of DDG into food and other applications is the safety issue due to mycotoxins (Liu et al., 2011b). However, bio-refined DDG could be integrated into food and other sectors as a potential resource in the near future.

2.4.3. Sugar beet pulp (SBP)

The sugar beet (*Beta vulgaris*) constitutes 55% of total sucrose production of the US sugar beet industry (FAO, 2012). The sucrose production results in the co-production of approximately 29 million metric tons of sugar beet pulp (FAO, 2012). Sugar beets are major

agricultural product in the Great Lakes region (Michigan and Ohio), the Upper Midwest (Minnesota and North Dakota), the Great Plains (Colorado, Montana, Nebraska, and Wyoming), and the West (California, Idaho, Oregon, and Washington). Among different regions, the Red River valley of western Minnesota and eastern North Dakota leads the nation in sugar beet production (Sugar beet research, 2011).

The SBP is a by-product of sucrose extraction (**Figure 2.2**). This by-product is widely used as animal feed; however, novel value added opportunities may exist due to SBP's rich nutrient content, which makes it an ideal material for food and feed applications towards improved nutrition. Nutritionally, the SBP contains non-sucrose carbohydrates (75-80%), fat (1.4%), protein (10.3%), ash (3.7%), and lignin (5.9%) (Dinand et al., 1999). Cellulose (22-24%), hemicelluloses (30%), and pectin (25%) are the main non-sucrose carbohydrates of SBP (Dinand et al., 1999). These carbohydrate polymers could be further developed for unique food and non-food applications.

Non-sucrose carbohydrate polymers of SBP have unique chemical and physical properties (Elleuch et al., 2011). The carbohydrate fraction of the SBP is not susceptible to hydrolytic enzymes in the upper gastro intestinal tract (Zhou et al., 2013). The stability of the carbohydrates in the upper gastro intestinal tract can enhance the health of gut micro-flora in the lower gastro intestinal tract (Liu et al., 2011). Furthermore, these carbohydrates are physically stable due to their rigidity and thermal stability (Peng et al., 2012). The combination of specific chemical and physical stability of these carbohydrates could be incorporated for value added uses in nutraceutical and food applications.

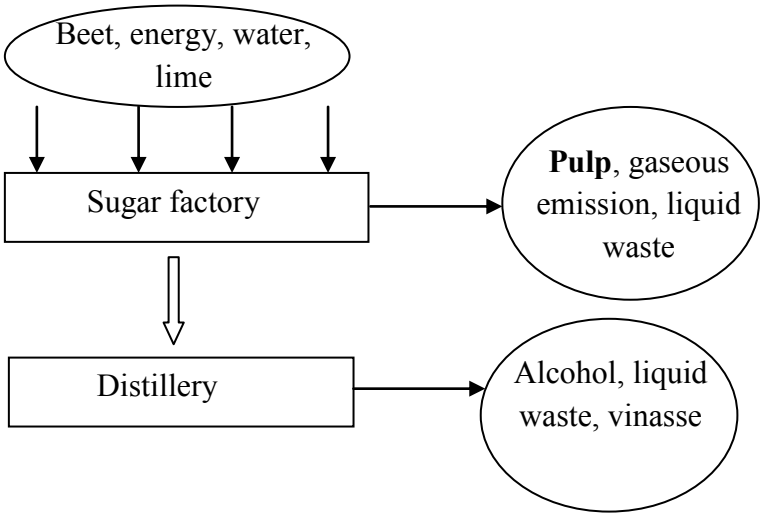


Figure 2.2. Sugar beet pulp as a by-product in the sugar distillery (Adapted from Elleuch et al., 2011)

2.5. Carbohydrate as a major nutrient

Carbohydrates are naturally occurring organic compounds. They play a central role in metabolism of organisms. Carbohydrates are one of the major nutrient components, which are essential as energy sources (Belitz et al., 2009). Approximately 40-45% of calories for Western population are supplied from carbohydrate diets (Blaak & Saris, 1995). Carbohydrates are composed of carbon, hydrogen and oxygen with 2:1 ratio of hydrogen and oxygen (Blaak & Saris, 1995). However, different combinations of molecular species give rise to different types of carbohydrates, which in turn possesses various health benefits.

The classification of carbohydrates is an important step to understand the chemical and nutritional properties. Carbohydrates can be classified based on chemistry and physiology, which is shown in **Table 2.1** and **Table 2.2** respectively (Asp, 1996). However, classifications of some carbohydrates such as resistant starch have contradictions depending on whether they are viewed chemically or physiologically. A more generalized classification of carbohydrates in terms of

physiochemical properties and nutritional values is shown in **Table 2.3** (Englyst et al., 1996). Classification is important in understanding the health benefits of different types of carbohydrates.

Table 2.1. Classification of carbohydrate by chemistry (Adapted from Asp, 1996)

Types of carbohydrates	Examples
Monosaccharides	Glucose, fructose, galactose
Disaccharides	Sucrose, lactose
Oligosaccharides	
Galactooligosaccharides	Raffinose, stachyose, verbascose
Fructooligosaccharides	Fructan, inulin
Polysaccharides	Starch, modified starch
Non-starch polysaccharides	Cellulose, hemicelluloses, pectin

Western countries, which are at potential risk of developing diseases due to higher consumption of fat are recommended to replace fat with complex carbohydrates (Blaak & Saris, 1995). Complex carbohydrates, also known as dietary carbohydrates and low digestible carbohydrates comprise non-starch polysaccharide, resistant starch, and oligosaccahrides (Scheppach et al., 2001; Cummings et al., 2004). These are a complex group of components, which are resistant to digestion in the small intestine but could be metabolized by gut micro-flora (Scheppach et al., 2001). Furthermore, the complex carbohydrates provide diverse physiological health benefits in the organisms.

Table 2.2. Classification of carbohydrate by physiology (Adapted from Asp, 1996)

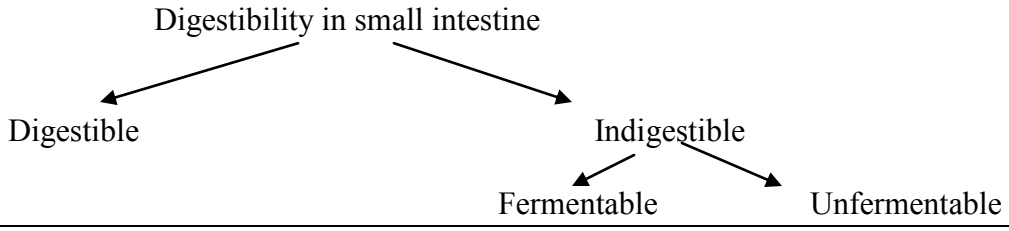


Table 2.3. Classification of food carbohydrates (Adapted from Englyst & Hudson, 1996)

Types of carbohydrates	Components
Sugars	Glucose, fructose, sucrose, lactose
Sugar alcohols	Sorbitol, xylitol, maltitol
Short chain carbohydrates	fructo and galacto oligosaccharides
Starch	Rapidly digestible starch (RDS)
	Slowly digestible starch (SDS)
	Resistant starch (RS)
Non-starch polysaccharides	Dietary fiber (present in plant cell wall)
	Other NSP (gums, mucilages)

2.5.1. Resistant starch (RS)

Resistant starch is a fraction of starch that escapes digestion in the small intestine and enters the large intestine for fermentation (Sharma & Yadav, 2008). Resistant starch can be regarded as a component of dietary fiber due to the similar physiological abilities of both resistant starch and dietary fiber (Sharma & Yadav, 2008; Murphy et al., 2008). However, RS does not possess all the properties of dietary fiber, thus could be considered as a "functional fiber" (Sharma & Yadav, 2008). Resistant starch can be classified as RS 1 (physically inaccessible starch), RS 2 (ungelatinized starch), RS 3 (retrograded starch), and RS 4 (chemically modified starch) depending on different features for enzyme resistance (Haralampu, 2000). Enzyme resistance is an important property of RS to influence beneficial health effects in organisms.

Resistant starch can act as a prebiotic by selectively fermenting the gut micro-flora and conferring benefits to the host health. Prebiotic effects of RS promote the production of short chain fatty acids (SCFA), which in turn enhances colonic health (Fuentes- Zaragoza et al., 2011). Recommended daily intake of RS to maintain a healthy human colonic environment is set as 8-40 g/day (Sharma & Yadav, 2008). Furthermore, the production of SCFA could decrease the pH

of the colon and facilitate the absorption of minerals such as Calcium (Ca), Magnesium (Mg), Iron (Fe), and Zinc (Zn) (Yeung et al., 2005). Overall, RS is a functional fiber, which produces diverse health benefits when fermented by gut micro-flora.

2.5.2. Non-starch polysaccharide (NSP)

Non-starch polysaccharides are complex polysaccharides, which are either homopolymeric or heteropolymeric in nature with predominant β-glycosidic linkages (Kumar et al., 2012). Non-starch polysaccharides can be classified as soluble or insoluble NSP based on the solubility in water. However, a more generalized classification of NSP includes three main classes of NSP namely, cellulose, non-cellulose, and pectin (Englyst et al., 1987; Kumar et al., 2012). Presence of diverse compounds of NSP possesses different physio-chemical properties, which in turn plays an important role in nutritional benefits for organisms.

Health benefits of NSP are attributable to their non-digestibility in the small intestine. The NSP that escapes the digestion in the small intestine can be broken down by gut micro-flora to produce short chain fatty acids such as acetate, propionate, and butyrate (Kendall et al., 2010). Furthermore, NSP could also selectively stimulate the beneficial micro-flora in the intestine by exerting prebiotic effect through the production of SCFAs (Kumar et al., 2012). However, intake of NSP in diets could also modify the peristaltic movement of the gastrointestinal tract and lower the risk of cholesterol formation (Voet & Voet, 1996). Therefore, NSP could diminish the health risks prevalent in the Western world.

2.5.3. *Bifidobacteria* for enhanced health benefits

Probiotics are organisms that benefit the hosts by influencing the intestinal micro-flora (WGO, 2011). Health benefits of probiotics include both nutritional benefits and therapeutic

benefits. Synthesis of vitamins and enhancement of the bioavailability of minerals, Calcium (Ca), Magnesium (Mg), Iron (Fe), and Zinc (Zn), are attributed to the nutritional benefits (Puneeth et al., 2012). The therapeutic benefits include reducing serum cholesterol levels and promoting anti-cancer activities in colonocytes (Puneeth et al., 2012). Most abundant probiotics in human gut include *Lactobacillus* and *Bifidobacteria*. The *Bifidobacteria* is the second most abundant probiotics found in human GI tract (Kantha & Arunachalam, 1999).

Approximately 33 different species of *Bifidobacteria* are found in human origin (WGO, 2011). One of the abundant *Bifidobacteria* species found in human GI tract is *Bifidobacterium brevis*. The *B. brevis* is commonly found in infants but retains in the gut and vagina throughout the life. However, the richness of the population declines after the age of 50 (Wong et al., 2006). Thus, the potential existence of the species is recommended by the consumption of such probiotics as supplements or as a part of food component.

The *B. brevis* can ferment 20 different types of carbohydrates including dietary fiber and resistant starch (WGO, 2011). The fermentation possesses multiple levels of health benefits. End products of fermentation include SCFAs; acetate, propionate, and butyrate (Wong et al., 2006). In addition *B. brevis* can also produce lactic acid (Kantha & Arunachalam, 1999). The production of SCFAs can lower the pH in the gut and enhance the absorption and bioavailability of Ca, Mg, Fe, and Zn. Furthermore, the fermentation can reverse the atrophy by promoting the cell proliferations of colonocytes (Wong et al., 2006). Therefore, predominant substrates for the fermentation by *Bifidobacteria* can influence the host health at multiple levels.

3. MATERIALS AND METHODS

3.1. Study 1

Feasibility of producing laboratory scale micro and nano materials from agricultural by-products; soybean meal (SBM), distillers dried grain (DDG), and sugar beet pulp (SBP)

3.1.1. Materials

All the chemicals and solvents for analytical analyses were purchased from Sigma Aldrich Co. (St. Louis, MO), VWR International (Radnor, PA), EMD Serono Inc. (Rockland, MA), and J.T. Baker Chemicals Co. (Phillipsburg, NJ) and used without further purification. Millipore water with 18 MΩ or greater (Millipore Corporation, Bedford, MA) was used for all the experiments.

3.1.2. Samples

Agricultural by-products SBM, DDG, and SBP were obtained from Northern Crop Institute (NCI), Fargo, North Dakota. All the samples were collected during August 2012-January 2013. Collected samples were finely ground using a cyclone sample mill (UDY Corporation, Fort Collins, CO). Ground samples were defatted with Soxhlet apparatus using hexane at 1: 20 (w/v).

3.1.3. Nano material production by sulfuric acid hydrolysis

The procedure for nano material production by sulfuric acid hydrolysis was adapted from Neethirajan et al., 2012. The samples were deproteinated by steeping in 0.2 % sodium hydroxide at 45°C for 90 minutes. The samples were then centrifuged at 3000 rpm (Beckman Coulter Inc, Brea, CA) for 20 minutes at room temperature (25°C). The supernatant was discarded and the precipitate was subjected to continuous washing with 18 MΩ Millipore water (Millipore

Corporation, Bedford, MA) until no traces of yellow color were visible to the naked eye. The remaining samples were re-suspended in millipore water and pH was adjusted to 7.0 using 50 mM hydrochloric acid. The residues were air dried.

Air dried samples were used to isolate human digestive enzyme carbohydrates by a method reported by AOAC, 2002. Approximately 1.0 g of the pre-treated material was wetted using 2.0 mL of 80% ethanol. Volume of 30.0 mL thermostable α-amylase (3000 U/mL) and 90.0 mL sodium acetate buffer (100 mM, pH 4.5) were added and the mixture was incubated in a water bath at 95°C for 6 minutes with continuous stirring on a magnetic stirrer (Henry Troemnor LLC, Thorofare, NJ). The samples were then placed in a 50°C water bath after which 1.0 mL of amyloglucosidase (AMG) (350 U/mL) was added and the mixture incubated at 50°C for 30 minutes at 300 rpm on a mini vortex incubator (VWR international LLC, Radnor, PA). The solutions were then centrifuged at 3000 rpm for 15 minutes at room temperature (25°C). The supernatants were removed and the residues were air dried for 24 hours.

One gram of air dried residues was hydrolyzed with 10.0 mL of 3.16 M sulfuric acid for 5 days at 100 rpm and 40°C in a mini vortex incubator. At the end of fifth day, the solutions were cooled to room temperature (25°C). Solutions were neutralized with successive washings with millipore water and were freeze dried using liquid nitrogen. The yield (%) was calculated by,

$$\text{Yield (\%)} = (\text{Initial dry weight} / \text{Final dry weight}) \times 100$$

3.1.4. Nano material production by complex formation with ethanol

Samples were deproteinated and were subjected to enzyme assay by the previously mentioned procedure (3.1.1). The samples subjected to enzyme assay were used for the complex formation to ethanol in the following manner by a procedure reported by Neethirajan et al., 2012.

Approximately 1.0 g of isolated enzyme resistant carbohydrates of SBM, DDG, and SBP were added in to 18.0 mL of Millipore water. The solutions were heated to 90°C for 90 minutes in a water bath (VWR international LLC, Radnor, PA) with constant stirring over magnetic stirrer. Then 18.0 mL of ethanol was added drop wise over a period of an hour and cooled to room temperature (25°C). On cooling to room temperature an additional portion of 18.0 mL was added drop wise in a similar manner as before. The suspension was centrifuged at 8000 rpm for 25 minutes. The residues were oven (Thermo electron Corporation, Marietta, OH) dried over night at 50°C on Petri-dishes. The yield was calculated as mentioned in 3.1.4.

3.1.5. Nano material production using an extraction buffer

The nano material production using extraction buffer was adapted from a previously reported procedure (Hilz et al., 2006). Defatted sample were mixed with 150.0 mL extraction buffer (50 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium acetate, and 50 mM sodium oxalate at pH 5.2). The mixture was stirred for 60 minutes at 70°C using a magnetic stirrer plate (VWR International LLC, West Chester, PA). After cooling to room temperature (25°C), the samples were centrifuged for 15 minutes at 5000 rpm in a Beckman J2-HS (Beckman Coulter Inc., Brea, CA) centrifuge, with the precipitate discarded. The supernatants were mixed with ethanol to a final alcohol concentration of 70%. The samples were centrifuged again under the same conditions and the resulting precipitates were collected and dissolved in 50 mM sodium

hydroxide with heating to 70°C. Non-soluble particles were removed by filtration through Whatman filter paper number 4 (Whatman International Ltd, Maidstone, UK) and pectin was precipitated from clear solution by addition of solid barium chloride. The samples were then centrifuged for 10 minutes at 6000 rpm and the supernatants were mixed with ethanol to a final alcohol concentration of 70%. The samples were again centrifuged, with the precipitate air dried.

The resulted samples were subjected to enzymatic assay to isolate the enzyme resistant carbohydrates as mentioned in 3.1.1. The isolated enzyme resistant carbohydrates were used to prepare nano scale materials by mechanical agitations. Mechanical agitations were carried out by subjecting the materials to sonication using an ultra sonicator (Branson Inc, Chicago, IL) at 40°C for 5 hours. The yield was calculated as mentioned in 3.1.4.

3.1.6. Characterization of the particle nature by Scanning electron microscopy (SEM), Field emission scanning electron microscopy (FESEM), and Dynamic light scattering (DLS)

Imaging of nano materials with SEM was carried out by crushing the samples gently between the ground-glass ends of clean microscope slides. The samples were applied to adhesive carbon tabs on cylindrical aluminum mounts; the excess was blown off gently with a stream of nitrogen gas. Samples then were sputter coated with gold-palladium (Model SCD 030, Balzers, Liechtenstein) to make them electrically conductive. The samples were viewed and images obtained with a JEOL JSM-6490LV scanning electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 15 kV.

Imaging of nano materials with FESEM was carried out by crushing the samples gently between the ground-glass ends of clean microscope slides. The samples were applied to adhesive carbon tabs on cylindrical aluminum mounts; the excess was blown off gently with a

stream of nitrogen gas. Samples then were coated with carbon (Cressington Carbon Coater 208, Ted Pella Inc., Redding, CA) to make them electrically conductive. The samples were viewed and images obtained with a JEOL JSM-7600F scanning electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 2.0 kV.

The Dynamic light scattering (DLS) was done by dissolving the nano materials in 50 mM sodium hydroxide solutions and subjecting them for the particle size analysis using a DLS Nicomp 380 particle sizing system (PSS, Port Richey, FL).

3.1.7. Characterization of crystalline patterns of nano materials by X-ray diffractometry (XRD)

Nano materials were subjected to XRD. X-ray powder diffraction analysis was performed using X'pert MPD (Phillip, Brussels. Belgium), operating at 40 mA and 45 kV with Cu. Freeze-dried micro scale materials were acquired at an angular range of 2θ from 4° to 99° with step-size of 0.05°. Counting time was 2s per step.

3.1.8. Statistical analysis

Sample layout was a single-factor experiment with completely randomized design in which combinations of samples and methods were considered as individual treatments. Extraction buffer method was duplicated for DDG and soybean meal and other methods were repeated three times for SBP, SBM and DDG. Analysis of variance was performed using the 'GLM' procedure in SAS for Window (Version 9.2, SAS Institute Inc., Cary, NC). Analysis of variance was done for the initial yield data after transformation of data to the natural logarithm ($\log_e$) since error variance was not homogeneous among treatments.

3.2. Study 2

Determination of the stability of nano materials produced from enzyme resistant carbohydrates of sugar beet pulp and resistant starch of soybean meal under simulated physiological condition

3.2.1. Samples

Samples used for this study were obtained from study 1. The SBP produced by sulfuric acid hydrolysis and SBM produced by buffer extraction was used for this study.

3.2.2. Characterization of nano materials under simulated physiological conditions by dissolution and digestion tests

Soybean meal nano scale materials produced by extraction buffer and micro scale materials of sugar beet pulp produced by sulfuric acid hydrolysis were subjected to dissolution and digestion tests under simulated stomach and intestine conditions. Dissolution and digestion studies were adapted from Dimantov et al., 2004a; Dimantov et al., 2004b.

Dissolution studies under simulated stomach environment were conducted by using 0.1g of the nano materials. Nano materials were subjected to buffer containing 0.1M hydrochloric acid at pH 1.5 and 6.5 individually for 3 hours at 37°C and 100 rpm. The samples were withdrawn at every 30 minutes time intervals centrifuged at 1500 rpm and dried at 40°C over night and weighed. The dissolution was calculated as percent weight loss of the material. The analysis was carried out in duplicates.

Dissolution studies under simulated intestine environment were conducted by using 0.1g of the nano materials. Nano materials were subjected to buffer containing 0.1M phosphate at pH 4.0 and 8.5 individually for 5 hours at 37°C and 100 rpm. The samples were withdrawn at every

one hour time intervals, centrifuged at 1500 rpm and dried at 40°C over night and weighed. The dissolution was calculated as percent weight loss of the material. The analysis was carried out in duplicates.

Continuous dissolution tests were performed for the simulation of the changes in the gastrointestinal tract pH conditions. The samples (0.1 g) were incubated with 0.1 M (pH 1.5) hydrochloric acid buffer for two hours at 37°C simulating stomach conditions. Then, the buffer solutions were drawn by vacuum oven. Further, the samples were incubated with 0.1 M phosphate buffer (pH 7) for 3 hours at 37°C simulating intestine conditions. The buffer solution was withdrawn and dried in an oven at 40°C overnight.

Digestion studies under simulated physiological conditions were conducted by using 0.1g of the nano materials. Nano materials were subjected to enzymatic solution containing pancreatic α-amylase (30 U/mL) and amyloglucosidase (300 U/mL) for 16 hours at 37°C at 100 rpm. The samples were withdrawn at the end of 16 hours, centrifuged at 1500 rpm and dried at 40°C over night and weighed. The digestion was calculated as percent weight loss of the material. The analysis was carried out in duplicates.

3.2.3. Thermal stability of the nano materials by Differential scanning calorimetry (DSC)

Differential scanning calorimetry analysis was performed on a Pyris 6 DSC system (Perkin Elmer, Waltham, MA). Approximately 5-10 mg of samples was filled into pre-weighed aluminum sample pans. The DSC curve was obtained at a temperature range of 0- 180°C at a heating rate of 10°C/ minute under dry nitrogen flow.

3.2.4. Statistical analysis

Experimental design for both simulated stomach and intestine was completely randomized design with factorial sample layout, involving three factors; sample, pH, and treatment time with two replications. Analysis of variance was performed using 'GLM' procedure in SAS for Window (Version 9.2, SAS Institute Inc., Cary, NC).

3.3. Study 3

In-vitro fermentation of soybean meal resistant starch nano particle by *Bifidobacterium brevis*

3.3.1. Materials

All the chemicals and solvents for analytical analyses were purchased from VWR International (Radnor, PA). *Bifidobacterium* broth and agar were purchased from Hi-Media laboratory Pvt, Ltd (Mumbai, India). Gas generating anaerobic packs was purchased from VWR International (Batavia, IL). *Bifidobacterium* strains were obtained from Veterinary Diagnostic lab at the North Dakota State University (Fargo, ND). Pack rectangular jars of 2.5 L and 7.5 L were purchased from Mitsubishi gas chemical Inc (Tokyo, Japan).

3.3.2. Culture conditions of *Bifiobacterium brevis*

Bifidobacterium brevis (ATCC No 15700) were maintained at -80 °C as glycerol stock solution. The bacteria were thawed and revived by culturing in *Bifidobacterium* broth. The broth contained following ingredients in grams per liter (g/l); peptic digest of animal tissue (10), casein enzymic hydrosylate (20), yeast extract (10), glucose (20), tomato juice solids (16.65), and polysorbate 80 (2). The final pH of the medium was 6.8. The strains were incubated in an anaerobic incubator (VWR, Bridgeport, NJ) at 37°C for 72 hours.

3.3.3. Growth experiments and analysis of growth characteristics

Growth experiments and analysis of growth characteristics were adapted from Zampa et al., 2004; Wronkowska et al., 2006. Growth experiments were carried out in sterilize tubes. Each tube contained 3.0 mL of broth with (1% v/v) cultured cells. One milliliter of filter sterilized soybean meal resistant starch nano material (5 mg/mL) was added into each tube as a carbon source. Medium with no resistant starch was used as a control. Growth was monitored by measuring the optical density using a spectrophotometer (Bio Rad smart spec 3000, Philadelphia, PA) at 600 nm (OD_{600}) at every one hour interval for 16 hours (hour 0- hour 16). Growth was monitored in duplicates.

The growth was further characterized by measuring the total viable counts of the *Bifidobacteruin brevis* by spreading 10 fold dilutions of the original samples (10^{-1}- 10^{-8}) on *Bifidobacterium* agar. The analysis was performed at every four hours including at the 0^{th} hour (immediately after inoculation). The agar plates were spread in duplicates and were incubated in anaerobic conditions at 37°C for 72 hours (3 days).

3.3.4. Short chain fatty acid (SCFA) analysis

Short chain fatty acid produced by the anaerobic fermentation of *Bifidobacterium brevis* were quantified by Gas Chromatography (Agilent Technology 6890N, Santa Clara, CA). Standards used for the quantification in mM were; acetic acid (73.5), propionic acid (18.9), isobutyric acid (7.98), butyric acid (15.89), isovaleric acid (6.86), and valeric acid (6.86). The SCFA analysis was performed at every 4 hour interval. Samples were analyzed in duplicates.

3.3.5. Statistical analysis

Sample layout was a single-factor experiment with completely randomized design in which individual incubation time was considered as a treatment. Experiment was duplicated for individual treatments. Analysis of variance was performed using the 'GLM' procedure in SAS for Window (Version 9.2, SAS Institute Inc., Cary, NC) after transformation of data to the natural logarithm ($\log_e$) scale since error variance was not homogeneous among treatments.

3.4. Study 4

Semi-synthetic thin film formation using soybean meal resistant starch (RS) nano particles, pectin, and carboxymethylcellulose

3.4.1. Materials

All the chemicals and solvents for analytical analyses were purchased from Sigma Aldrich Co. (St. Louis, MO), VWR International (Radnor, PA), EMD Serono Inc. (Rockland, MA), and J.T. Baker Chemicals Co. (Phillipsburg, NJ) and used without further purification. Glass slides (3”x 6”) for coating were obtained from Center for Polymeric and coating Materials at North Dakota State University (Fargo, ND). Millipore water with 18 MΩ or greater (Millipore Corporation, Bedford, MA) was used for all the experiments.

3.4.2. Formation of semi-synthetic thin films with different composition of soybean meal resistant starch nano particles, pectin, and carboxymethylcellulose

Film forming solutions were prepared as shown in **Table 3.1**. Solutions were casted on glass slides using 75 microns drag down metal bar. The casted films were oven dried at 40°C for 5 hours. Glass slide with soybean meal resistant starch nano particle alone was used as a control.

Table 3.1. Single factorial design of film forming solutions

Film type	Film forming ratios
F_a- [+]Control	
F_b- [*]R: P: C	1: 1: 1
F_c- R: P: C	1: 1: 3
F_d- R: P: C	1: 3: 1
F_e- R: P: C	3: 1: 1
F_f- R: P: C	3: 3: 1
F_g- R: P: C	3: 1: 3
F_h- R: P: C	1: 3: 3
F_i- [‡]R: P	1: 3
F_j- R: P	3: 1
F_k- R: P	1: 1
F_l- [ŧ]R: C	1: 3
F_m- R: C	3: 1
F_n- R: C	1: 1

[+] Control= soybean meal resistant starch (RS)
[*]R: P: C= soybean meal RS: pectin: carboxymethylcellulose
[‡] R: P= soybean meal RS: pectin; [ŧ]R: C= soybean meal RS: carboxymethylcellulose

3.4.3. Surface characterization of the films by atomic force microscopy (AFM)

Coated glass slides were used to study the surface properties of the films. Atomic force microscopy (Veeco technologies 3100, Santa Clara, CA) measurements were obtained in the tapping mode. Scans were performed in air medium. The cantilever had a resonance frequency of 47-76 kHz and a force constant of 12.64 N/m. Sample images were obtained at 20 x 20 μm^2 on representative spots of the film surface. The images are on a 3-dimensional representation with brighter color representing the higher spot.

3.4.3.1. Surface roughness analysis

Surface roughness is an important surface property of the thin films. The surface roughness is presented by (a) root-mean square roughness (R_q); (b) peak- to- valley height (R_z). The surface skewness (R_{sk}) and kurtosis (R_{ku}) were also calculated (Stawikowska & Livingston, 2013).

$$R_q = \sqrt{\frac{1}{n}\sum_{i=1}^{n} Zi^2}$$

$$R_z = Zmax - Zmin$$

$$R_{sk} = \frac{1}{nRq^3}\sum_{i=1}^{n} Zi^3$$

$$R_{ku} = \frac{1}{nRq^4}\sum_{i=1}^{n} Zi^4$$

3.4.3.2. Phase lag image analysis

The phase lag images were quantified by using Eq. 1 and 2 for root-mean square roughness phase lag (P_q) and (b) peak- to- valley phase lag (P_z) respectively.

3.4.4. Thermal stability of the films by Differential scanning calorimetry

Thermal analysis of the films was performed on a Pyris 6 DSC system (Perkin Elmer, Waltham, MA). Approximately 5-10 mg of samples was filled into pre-weighed aluminum sample pans. The DSC curve was obtained at a temperature ramp of 0- 180°C at a heating rate of 10°C/ minute under dry nitrogen flow.

3.4.5. Statistical analysis

Sample layout was a single-factor experiment with completely randomized design in which individual incubation time was considered as a treatment. Experiment was duplicated for individual treatments. Analysis of variance was performed using the 'GLM' procedure in SAS

for Window (Version 9.2, SAS Institute Inc., Cary, NC) after transformation of data to the natural logarithm ($\log_e$) scale since error variance was not homogeneous among treatments.

4. RESULTS AND DISCUSSION

4.1. Study 1

Feasibility of producing laboratory scale micro and nano materials from agricultural by-products; soybean meal (SBM), distillers dried grain (DDG), and sugar beet pulp (SBP)

Micro and nano scale materials are widely used in food and pharmaceutical applications for encapsulating vitamins, minerals, probiotics, prebiotics, and drugs. The reduced particle size of micro and nano scale materials possess enhanced biochemical activities compared to macro scale materials. The enhanced biochemical activity of micro and nano scale materials can be used in encapsulating bioactive compounds such as vitamins, minerals, probiotics, prebiotics, and drug for targeted delivery in the gastro intestinal (GI) tract. Furthermore, the micro and nano materials designed for targeted delivery in the GI tract should be non-toxic. The non-toxic encapsulating materials can be derived from bio-based sources such as agricultural by-products, which are widely used as animal feed. Therefore, in study 1 the micro and nano scale materials were produced by isolating the enzyme resistant carbohydrates from agricultural by-products such as soybean meal, distillers dried grain, and sugar beet pulp. Furthermore, the materials were characterized to evaluate their suitability in designing encapsulating materials for food and pharmaceutical applications.

4.1.1. Yield component of the materials SBM, DDG, and SBP by three different procedures

Study 1 was to determine the yield of the nano materials produced from soybean meal (SBM), distillers dried grain (DDG), and sugar beet pulp (SBP) with different nano material preparation methods. The analysis of variance in **Table 4.1** shows the interaction between sample and method. The yield obtained for SBM, DDG, and SBP with sulfuric acid hydrolysis

showed a significant difference with higher yields obtained for SBM and SBP. The complex formation to ethanol yielded higher values for SBP compared to SBM and DDG. However, the yield for buffer extraction was obtained only for SBM and DDG as shown in **Table 4.1**.

Table 4.1. Yield of micro and nano particles of SBM, DDG, and SBP from starting material

Sample	Method	Yield (%)
SBM	Sulfuric acid hydrolysis	15.7 ab
DDG	Sulfuric acid hydrolysis	10.9 b
SBP	Sulfuric acid hydrolysis	16.8 ab
SBM	Complex formation to ethanol	20.8 ab
DDG	Complex formation to ethanol	12.4 b
SBP	Complex formation to ethanol	37.1 a
DDG	Extraction buffer	7.5 b
SBM	Extraction buffer	2.8 c

* Different letters on the column imply statistically significant differences at $p < 0.05$; LSD (2.67)

Yield is an important parameter that should be taken into consideration when utilizing materials for various applications. Previous studies (Angellier et al., 2004; Neethirajan et al., 2012; Wang et al., 2008) performed with sulfuric acid hydrolysis in nano materials preparations have produced a yield component of 15%, which is in good agreement with the present study. However, for SBP and SBM the yield with sulfuric acid hydrolysis was higher. The higher value obtained in the present study could be attributed to the use of different plant origins for nano scale material preparation by sulfuric acid hydrolysis. The higher yields could be used as potential starting materials for food, nutraceutical, and other applications.

Literature to date has not reported the yields that could be obtained from complex formation to ethanol and buffer extraction. However, the yield obtained with complex formation to ethanol was comparable to the yield obtained with sulfuric acid hydrolysis. Furthermore, the yields with buffer extraction were lower for both DDG and SBM with no yields obtained for

SBP. Arguably, the inability of the production of nano materials of SBP could be attributed to the chemical composition of the material. The SBP is mainly composed of NSPs such as cellulose, pectin, and hemicellulose. Since the extraction buffer used in this method removes all the alcohol insoluble solids (AIS) such as NSP and proteins (Hilz et al., 2006) the result was a turbid solution for SBP, instead of a solid precipitate. Overall, the yield obtained suggests that there could be possibilities for the utilization of SBM and DDG produced by buffer extraction for various applications.

4.1.2. Scanning electron microscopy (SEM) and field emission scanning electron microscopy (FESEM) of SBM, DDG, and SBP nano materials produced by three different procedures

Nano materials produced by sulfuric acid hydrolysis, complex formation to ethanol, and buffer extraction are shown in **Figures 4.1, 4.2,** and **4.3** respectively. The images clearly show that the morphology of the materials SBM, DDG, and SBP are different both within the procedures and among the procedures. However, the acid hydrolysis and complex formation to ethanol for SBM and DDG (**Figure 4.1 and 4.2**) showed similar aggregates. The acid hydrolysis of SBP clearly showed a distinct morphology (**Figure 4.1**). Complex formation to ethanol in SBP showed aggregates similar to SBM and DDG in the same procedure. The buffer extraction produced well resolved spherical structures for SBM while giving a poorly resolved structure for DDG.

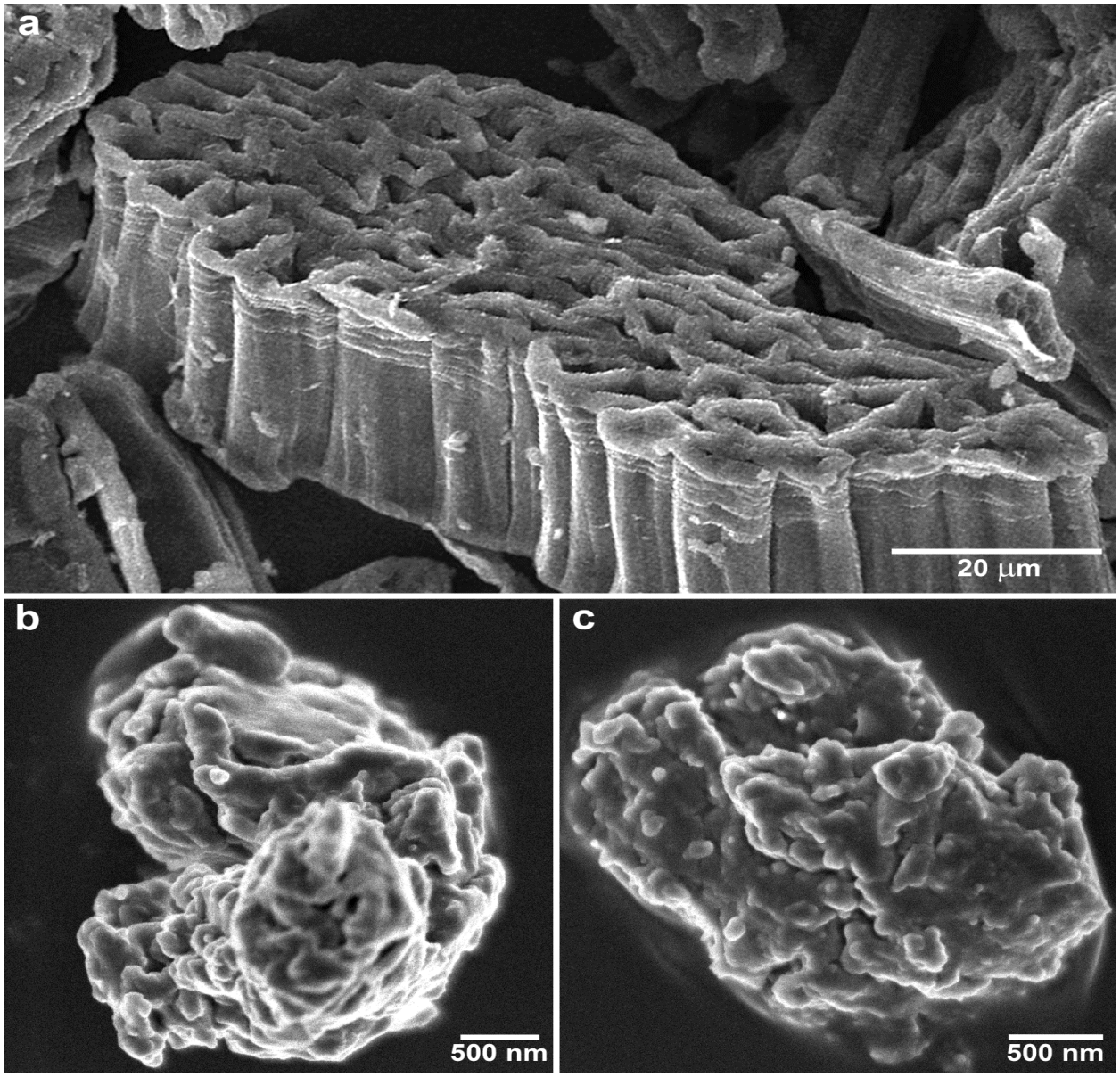


Figure 4.1. Field emission scanning electron microscopy images of sulfuric acid hydrolyzed (a) sugar beet pulp; (b) distillers dried grain; and (c) soybean meal

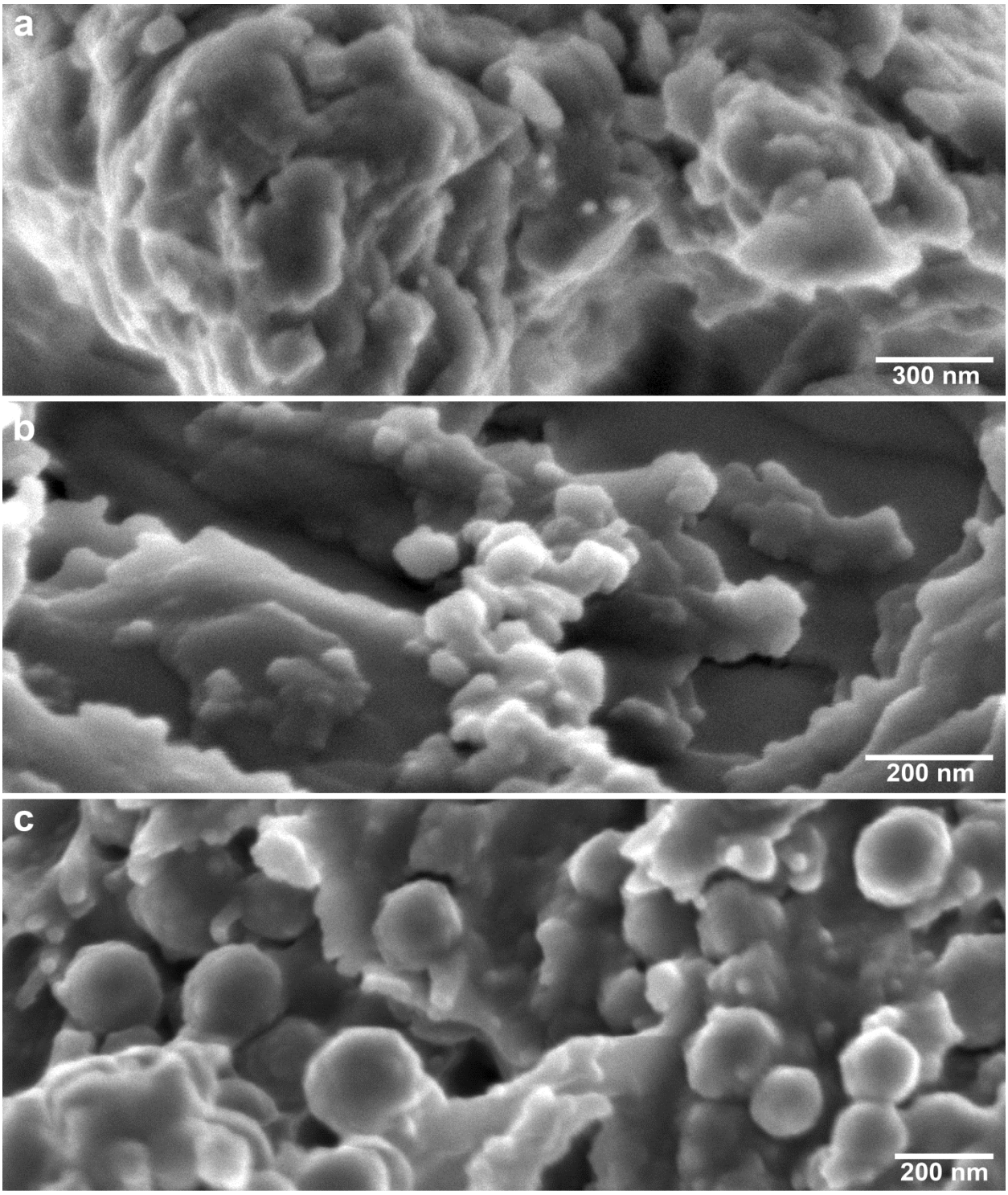


Figure 4.2. Field emission scanning electron microscopy images of complex formation to ethanol (a) sugar beet pulp; (b) distillers dried grain; and (c) soybean meal

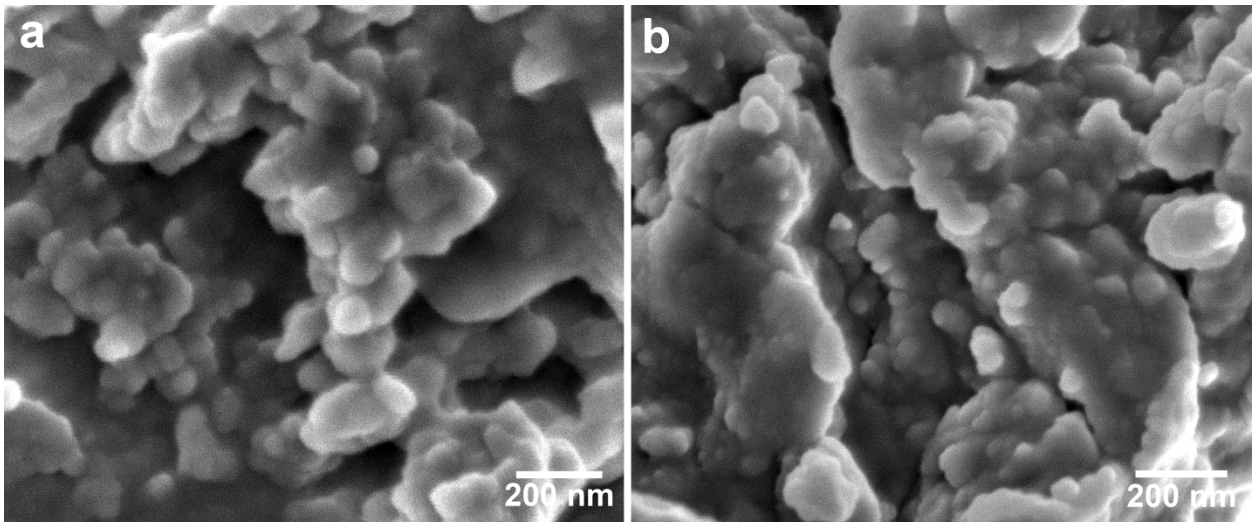


Figure 4.3. Field emission scanning electron microscopy images of buffer extraction (a) soybean meal; (b) distillers dried grain

The morphology is important to design nano materials for food and pharmaceutical applications. The SBP produced by sulfuric acid hydrolysis showed a unique tubular structure, which has not been reported in literature to date. The distinct feature of the SBP suggests that the SBP could be used as an encapsulating material in both food and nutraceutical sectors. However, the aggregates formed by SBM and DDG by sulfuric acid hydrolysis and complex formation to ethanol are not promising in either food or nutraceutical applications. Furthermore, the well-resolved spherical structure of SBM, compared to poorly resolved DDG, could be a potential material to be used as an encapsulating agent. The morphology of the materials produced by these three different procedures suggests that the SBP and SBM, produced by sulfuric acid hydrolysis and buffer extraction respectively, could be used for encapsulating minerals, vitamins, probiotics, enzymes, and drugs.

4.1.2. Particle size of SBM, DDG, and SBP nano materials produced by three different procedures

The particle size distribution of the materials was characterized by dynamic light scattering (DLS). The particle sizing was done for SBM and DDG, which was produced by sulfuric acid hydrolysis, complex formation with ethanol, and buffer extraction. The particle sizing was not performed for SBP by DLS due to the insolubility of SBP in solvents commonly used for DLS. However, the dimensions of the SBP materials were measured using SEM and FESEM. The particle size distribution of SBM and DDG are shown in **Table 4.2**. As shown in **Table 4.2** the particles are all at nano scale, while smaller particles are produced in SBM by buffer extraction.

Table 4.2. Particle size distribution of the nano materials produced by different procedures

Sample	Method	Particle size distribution (nm)
SBM	Sulfuric acid hydrolysis	55.8 ± 29.1
DDG	Sulfuric acid hydrolysis	49.0 ± 26.5
SBM	Complex formation to ethanol	104.4 ± 72.9
DDG	Complex formation to ethanol	57.5 ± 35.0
SBM	Buffer extraction	40.0 ± 33.2
DDG	Buffer extraction	75.3 ± 38.7

*Each value is the mean of three replicates experiments ± standard deviation

Reduced particle size at micro and nano scale is an important parameter because of the higher surface area to volume ratio of the reduced particle size. This increased surface area of the particles at micro and nano scale levels possess enhanced physical, chemical, and biological reactivity. In this study, the sulfuric acid hydrolysis gave rise to particles of micro scale in SBP, which was characterized by SEM and FESEM. The unique tubular structure of SBP by sulfuric acid hydrolysis showed tubules with lengths of 22.5 ±0.9 μm and cavities with diameter of 3.9 ±1.2 μm (**Figure 4.1**). In contrast, for SBM and DDG the particle sizes were in nano scale

(**Table 4.2**). The micro and nano scale material production by sulfuric acid is due to the penetration of the H^+ ions into the surface and inner amorphous regions simultaneously (Wang et al., 2008). Micro and nano scale materials possess substantial benefits, such as enhanced physical, chemical, and biological reactivity, due to the increased surface area compared to macro scale materials with the same chemical composition.

The particles in nano scale are widely used for food, nutraceutical, and pharmaceutical applications. The nano scale materials produced from SBM and DDG (**Table 4.2**) indicates that these materials could be used for food and other applications. However, considering the morphology of the materials (**Figures 4.1, 4.2,** and **4.3**) the well resolved spherical structure of SBM produced by buffer extraction could be a more potent source for encapsulation in food and other applications. The particle size of SBM produced by buffer extraction was the smallest in diameter as seen in **Table 4.2**. Furthermore, the smaller size of the SBM produced by buffer extraction could be attributed to the high energy vibration of ultrasonication generated from piezoelectric effect in a mechanical agitation (Bel Haaj et al., 2013). Previous study (Bel Haaj et al., 2013) performed with sonication for nano material preparation has resulted in particles of 100-200 nm. The reduced particle size obtained in SBM (**Table 4.2**) suggests that the SBM produced by buffer extraction could be used as encapsulating agents for various applications in nutraceuticals and food sectors.

4.1.3. Crystalline nature of SBM, DDG, and SBP nano materials produced by three different procedures

Crystalline patterns of the nano materials produced from the above mentioned three different procedures were characterized by X-ray diffractometry (XRD). **Table 4.3** shows the degree of crystallinity and the crystalline patterns of SBM, DDG, and SBP produced by the three

different procedures discussed above. The degree of crytsallinity for all the materials with all the three methods was in the range of 9-17% as seen in **Table 4.3**. The crystalline patterns of the materials were mostly A (2θ= 15, 25, and 27) and B (2θ= 5.5, 17, and 23) types (Shamai et al., 2003). The crystalline pattern of sulfuric acid hydrolysis was predominantly A- type. The SBM showed both A- and B- type crystalline patterns for both complex formation to ethanol and buffer extraction. The DDG showed A- and B- type patterns for buffer extraction; however, the corresponding peaks were weaker for DDG compared to SBM.

Crystalline patterns are indicators of degree of crystallinity and different crystalline patterns, which in turn could predict the thermal stability and the susceptibility to pancreatic enzymes. Previous study (Shujun et al., 2007) has shown that sulfuric acid hydrolysis gives A-type crystalline pattern, which is in accordance with our study. In addition, A type patterns are more susceptible to hydrolysis compared to B- type by α-amylase (Shujun et al., 2007). Thus, nano materials of SBM, DDG, and SBP produced by sulfuric acid hydrolysis could be susceptible to enzymatic hydrolysis. However, co-existence of the B- type pattern in SBM indicates the nano materials of SBM may be less susceptible to α-amylase hydrolysis compared to DDG and SBP. The susceptibility of the materials to pancreatic α-amylase is an important feature to be considered in designing materials for delivery in the gastro intestinal (GI) tract. In conclusion, our study emphasizes that SBM from buffer extraction could be a potential source for encapsulating material to deliver nutraceutical and food component to the GI tract.

Table 4.3. Degree of crystallinity and crystalline pattern of SBM, DDG, and SBP produced by different procedures

Sample	Method	Crystallinity (%)	Crystalline pattern
SBM	Sulfuric acid hydrolysis	11.07	A
DDG	Sulfuric acid hydrolysis	11.49	A
SBP	Sulfuric acid hydrolysis	16.51	A
SBM	Complex formation to ethanol	9.44	A and B
DDG	Complex formation to ethanol	10.45	A
SBP	Complex formation to ethanol	10.43	A
SBM	Buffer extraction	10.95	A and B
DDG	Buffer extraction	12.58	A and B

*Each value is the mean of duplicates

4.2. Study 2

Determination of the stability of nano materials produced from enzyme resistant carbohydrates of sugar beet pulp and resistant starch of soybean meal under simulated physiological condition

Micro and nano scale materials can be used as encapsulating materials for vitamins, minerals, probiotics, and prebiotics in the GI tract. Biodegradable materials are potential sources for designing encapsulating materials for targeted delivery in the GI tract. The encapsulating materials should be stable under physiological conditions, especially for acidic and alkaline conditions in the stomach and intestine respectively (Dimantov et al., 2004a). Furthermore, the encapsulating materials should be stable under physiological temperature (40^{o}C). The stability of the encapsulating materials under physiological condition could retain the bioactivity of vitamins, minerals, probiotics, and prebiotics until they are delivered to the targeted site. Therefore, the present study was to examine the stability of micro scale SBP and nano scale SBM produced by sulfuric acid hydrolysis and buffer extraction respectively, for targeted delivery. Both materials, SBP and SBM, were subjected to simulated in vitro stomach and

intestine conditions and the degrees of dissolution were characterized qualitatively and quantitatively. Furthermore, the thermal stability was characterized by DSC thermograms.

4.2.1. Dissolution tests of nano materials produced from SBP (by sulfuric acid hydrolysis) and SBM (by buffer extraction) under simulated physiological condition

Dissolution tests are widely used in studying the stability of the materials under simulated physiological conditions. Dissolution tests were performed with the materials used in study 1; SBP and SBM nano materials produced by sulfuric acid hydrolysis and buffer extraction respectively. **Table 4.4** shows the dissolution of SBP and SBM under simulated in vitro stomach conditions. Analysis of variance (**Table 4.4**) showed that the sample, pH, time, and the interaction between sample, pH and time are not significantly different ($p<0.05$). Furthermore, **Figure 4.2** shows that the degree of dissolution in SBM is higher at pH 6.5 compared to pH 1.5, although there is no significant difference ($p<0.05$). The similar behavior is seen in the degree of dissolution of SBP (**Figure 4.2.1**). However, the dissolution is high in SBP compared to SBM.

The degree of dissolution is further evidenced by SEM images. **Figure 4.4** clearly shows that the dissolution of SBM is high at pH 1.5 in 3 hours compared to pH 1.5 in 1 hour. The formation of irregularities or cracks on the surface (**Figure 4.4**) represents the difference in the degrees of dissolution. The dissolution at pH 6.5 also shows a similar behavior. Furthermore, the degree of dissolution in SBP is seen by the dissolution of tubular structures (**Figure 4.4**). Higher dissolutions are seen at pH 1.5 and 6.5 in 3 hours. The SEM images of dissolution studies are in good agreement with the quantitative analysis done for dissolution tests as seen in **Table 4.4**.

Table 4.4. Dissolution under simulated stomach conditions

Sample	pH	Time/minutes	Dissolution (%)
SBM	1.5	30	20.9 ij
		60	22.8 g-j
		90	22.4 g-j
		120	22.2 g-j
		150	25.2 f-i
		180	28.9 f
SBP	1.5	30	45.4 de
		60	48.2 c-e
		90	49.9 b-d
		120	49.9 b-d
		150	50.4 b-d
		180	52.9 a-c
SBM	6.5	30	18.3 j
		60	21.6 h-j
		90	23.6 g-i
		120	24.1 f-i
		150	26.6 f-h
		180	27.0 fg
SBP	6.5	30	44.6 e
		60	49.8 b-d
		90	53.3 a-c
		120	53.8 ab
		150	54.4 ab
		180	55.6 a

*****Each value is the mean of duplicate. Different letters on the column imply statistically significant differences at $p < 0.05$

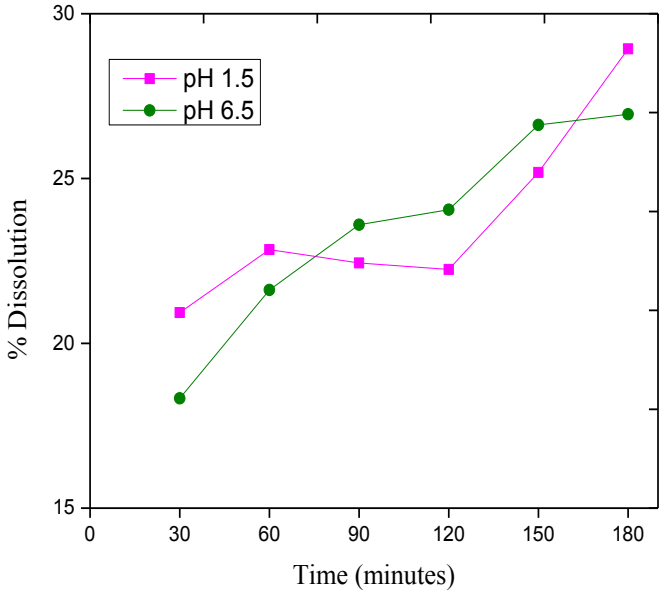


Figure 4.4. Chemical digestion of SBM under simulated stomach condition (each value is the mean of duplicates)

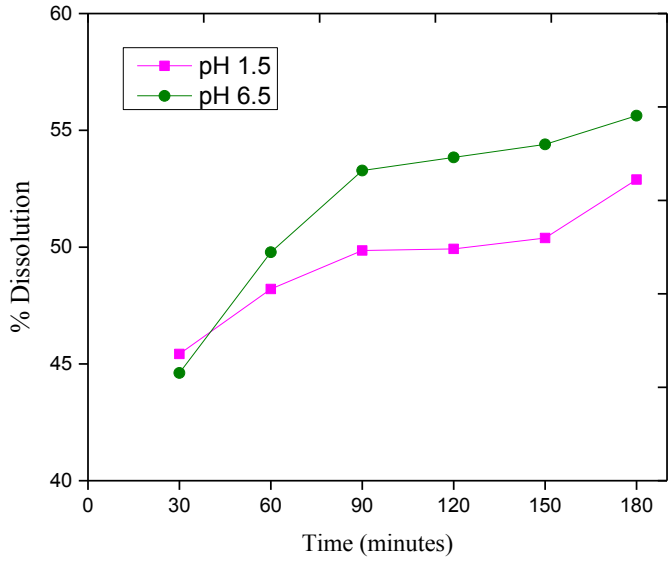


Figure 4.5. Chemical digestion of SBP under simulated stomach condition (each value is the mean of duplicates)

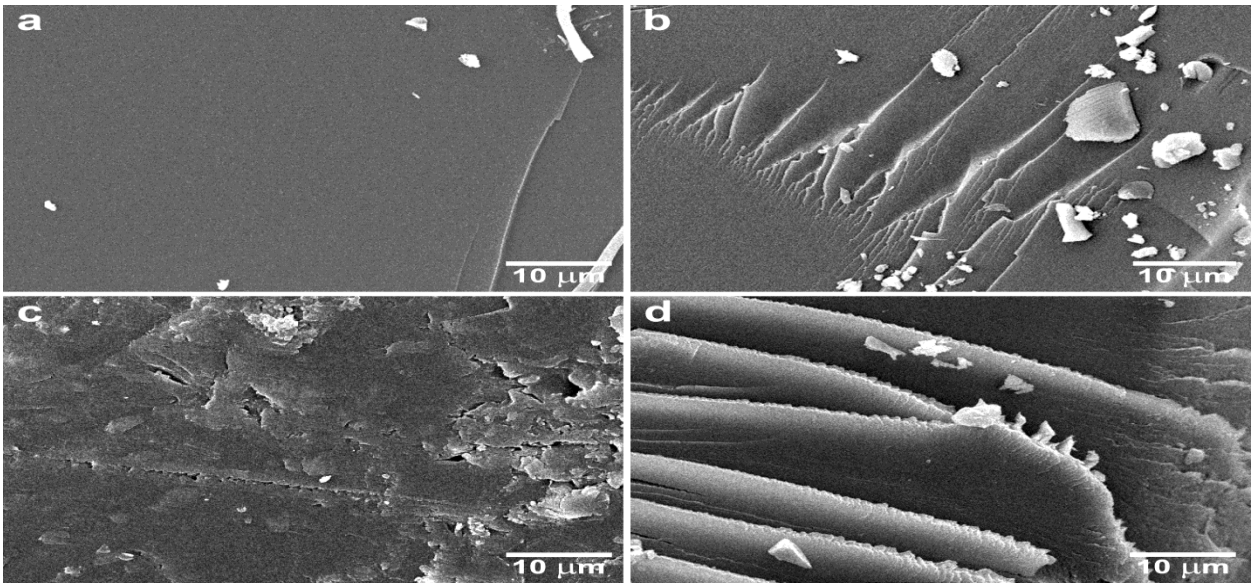


Figure 4.6. Scanning electron microscopy images for chemical digestion of SBM under simulated stomach conditions (A) pH 1.5 for 1 hour; (B) pH 1.5 for 3 hours; (C) pH 6.5 for 1 hour; (D) pH 6.5 for 3 hours

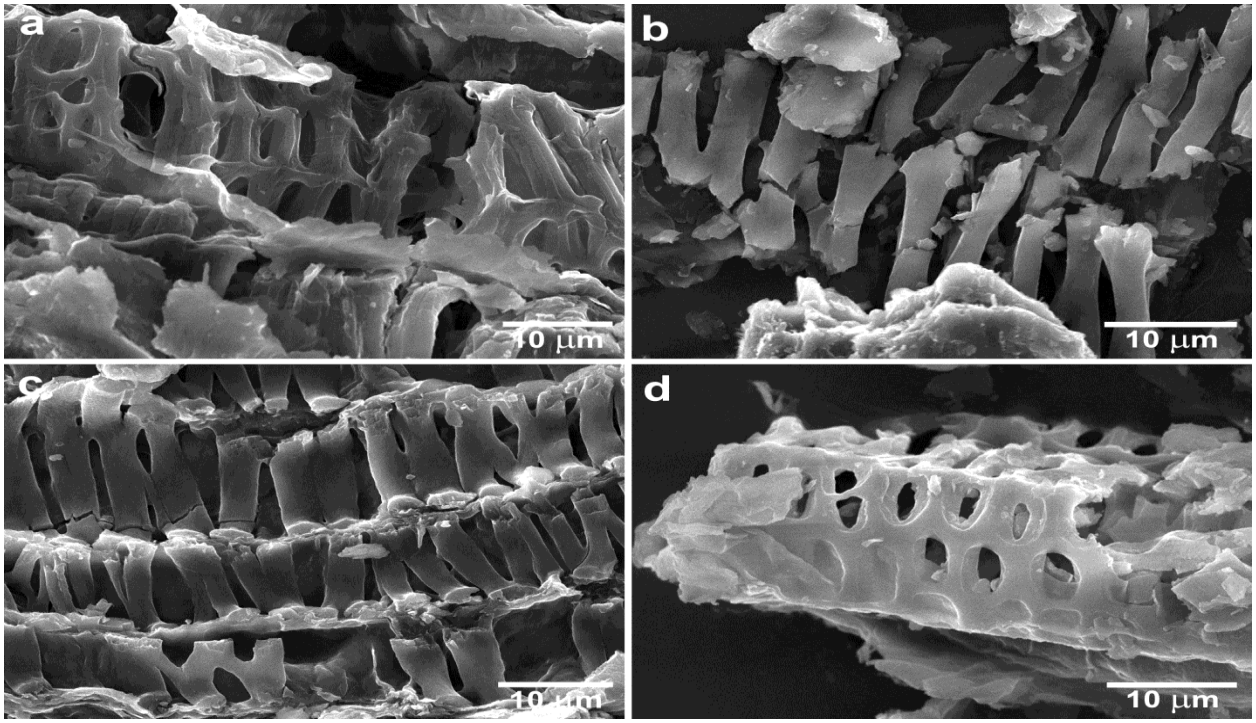


Figure 4.7. Scanning electron microscopy images for chemical digestion of SBP under simulated stomach conditions (A) pH 1.5 for 1 hour; (B) pH 1.5 for 3 hours; (C) pH 6.5 for 1 hour; (D) pH 6.5 for 3 hours

Table 4.5. Dissolution under simulated intestine conditions

Sample	pH	Time/hours	Dissolution (%)
SBM	4.0	1	19.6 de
		2	19.0 de
		3	17.4 de
		4	18.3 de
		5	16.6 de
SBP	4.0	1	44.9 c
		2	46.2 bc
		3	48.0 bc
		4	53.2 a-c
		5	55.6 ab
SBM	8.5	1	11.9 e
		2	13.6 e
		3	15.0 e
		4	17.8 de
		5	25.7 d
SBP	8.5	1	44.3 c
		2	46.0 bc
		3	49.9 a-c
		4	49.5 a-c
		5	58.5 a

*Each value is the mean of duplicate. Different letters on the column imply statistically significant differences at $p < 0.05$

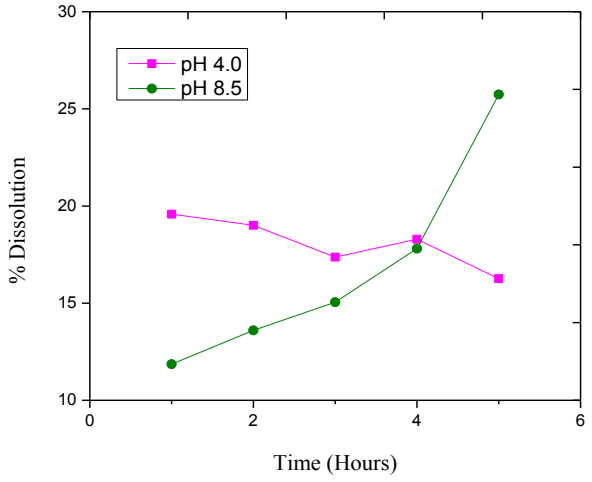


Figure 4.8. Chemical digestion of SBM under simulated intestine condition (each value is the mean of duplicates)

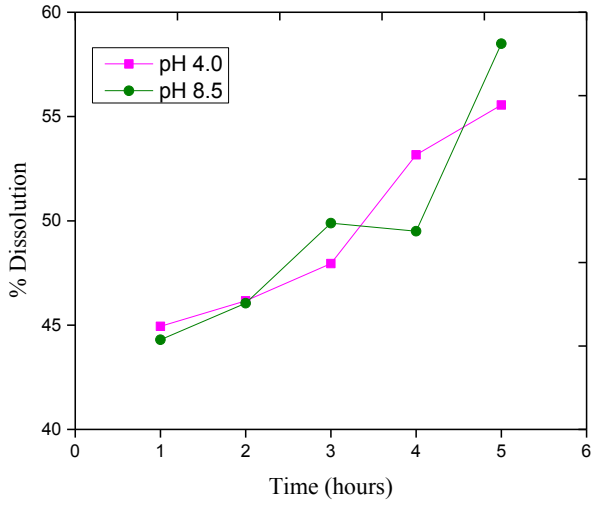


Figure 4.9. Chemical digestion of SBP under simulated intestine environment (each value is the mean of duplicates)

The dissolutions of SBP and SBM are further evidenced by SEM images shown in **Figure 4.10 and 4.11** respectively. The higher dissolution at pH 8.5 at the fifth hour in SBM is clearly seen by formation of perforations on the surface. The perforations were not seen in any other pH at different time intervals one and three hours. The perforations are a clear evident of higher dissolution of SBM at pH 8.5. Furthermore, the dissolution of SBP is seen by the dissolution of the tubular structures. At pH 8.5 the tubules are dissolved more compared to pH 4.0. The SEM images are in good agreement with the quantitative analysis of degree of dissolution.

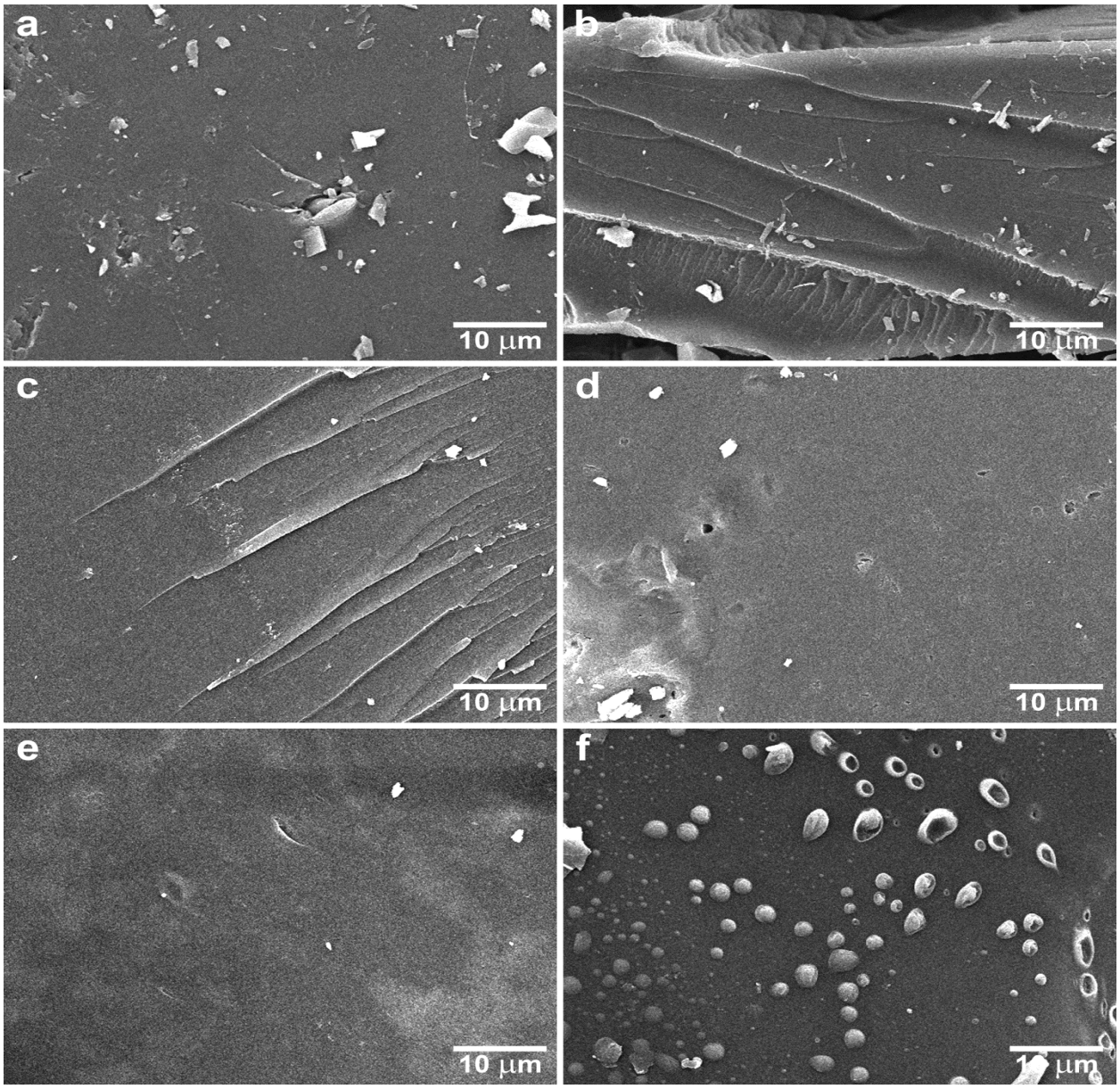


Figure 4.10. Scanning electron microscopy images for chemical digestion of SBM under simulated intestine conditions (A) pH 4.0 for 1 hour; (B) pH 4.0 for 3 hours; (C) pH 4.0 for 5 hours; (D) pH 8.5 for 1 hour; (E) pH 8.5 for 3 hours; (F) pH 8.5 for 5 hours

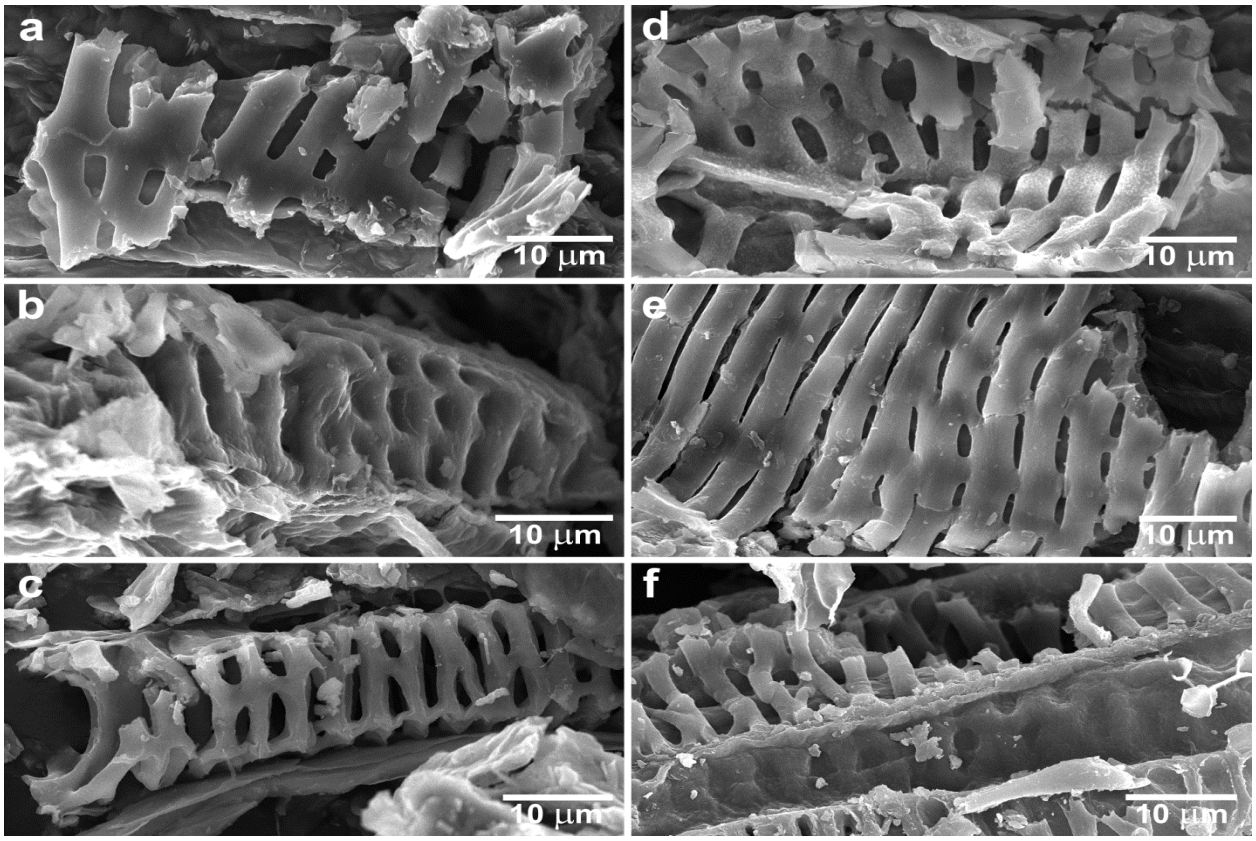


Figure 4.11. Scanning electron microscopy images for chemical digestion of SBP under simulated intestine conditions (A) pH 4.0 for 1 hour; (B) pH 4.0 for 3 hours; (C) pH 4.0 for 5 hours; (D) pH 8.5 for 1 hour; (E) pH 8.5 for 3 hours; (F) pH 8.5 for 5 hours

Degree of dissolutions of nano materials under simulated stomach and intestine conditions are important in using these materials for different applications in food, nutraceuticals, and pharmaceuticals. Previous studies have shown (Wakerly et al., 1997; Macleod et al., 1997; Dimantov et al., 2004a; Dimantov et al., 2004b) that the materials with 20-30% dissolution in the simulated stomach and intestine conditions could be used in targeted delivery for colon. In our study, the dissolution of SBM is 20-30% in both stomach and intestine suggesting the possibility of SBM nano material use in colon specific delivery. In contrast, the dissolution of SBP is higher (>30%) in both stomach and intestine. The increased dissolution of

SBP could be potential in targeted delivery of nutraceutical and pharmaceutical components to stomach and intestine. Overall, both, SBP and SBM could be used as coating substances for specified delivery in the gastrointestinal (GI) tract.

4.2.2. Continuous dissolution tests for SBP and SBM

In addition to individual dissolution tests under simulated stomach and intestine conditions, continuous dissolution was carried out for SBP and SBM. The continuous dissolution for SBP and SBM were 70.2% and 20.3%. The analysis of variance showed that the degree of dissolutions were significantly different at $p<0.05$. The continuous dissolution was further evidenced by SEM studies. According to **Figure 4.12**, the degree of dissolution for SBP is seen by distorted tubular structure. Distorted tubular structure was seen when SBP was subjected to individual dissolutions under simulated stomach and intestine conditions (**Figure 4.12 and 4.13**. However, the degree of distortion is higher for continuous dissolution. In SBM the continuous dissolution was evidenced by the formation of cracks on the surface with few perforations (**Figure 4.13**). The cracks and perforations were observed in individual dissolutions as well (**Figure 4.10 and 4.11**). However, the appearance of cracks and perforations together are seen in continuous dissolution studies.

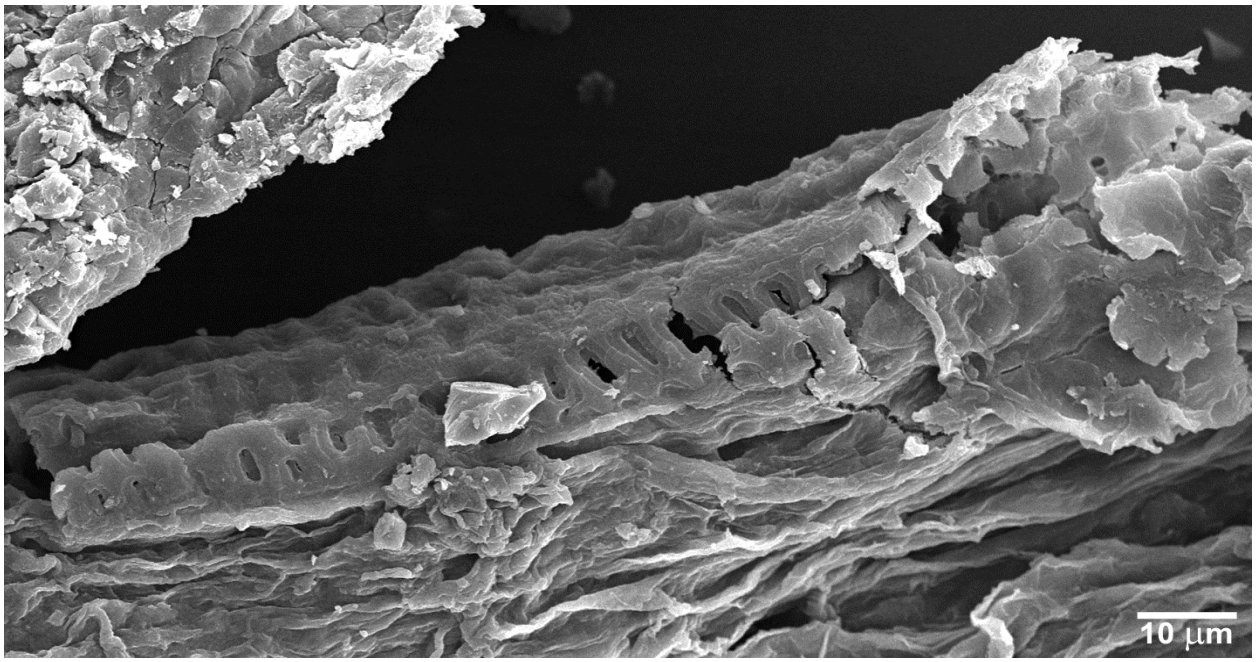


Figure 4.12. Scanning electron microscopy images for continuous dissolution of SBP

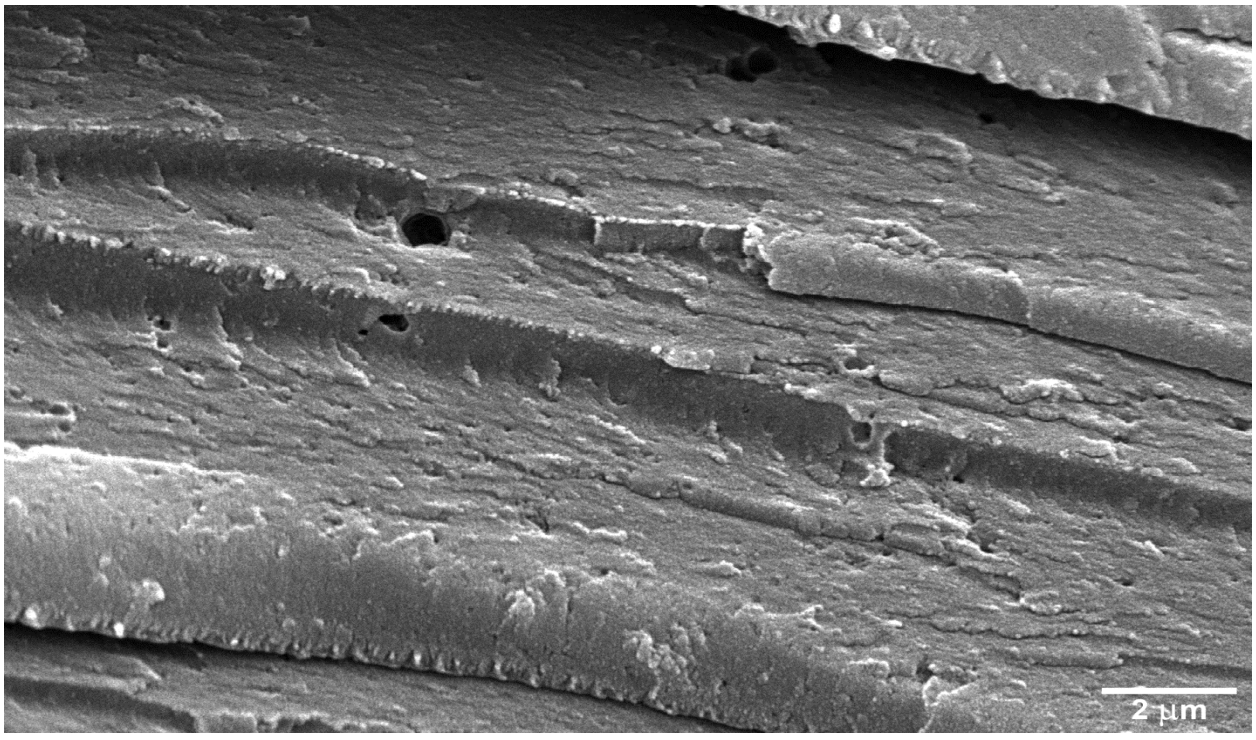


Figure 4.13. Scanning electron microscopy images for continuous dissolution of SBM

Continuous dissolution studies are important to characterize the stability of the materials when subjected to continuous acidic and alkaline environments of stomach and intestine respectively. In our study, the continuous dissolution was high for SBP (70.2%) compared to SBM (20.3%). Previous studies (Dimantov et al., 2004a; Dimantov et al., 2004b) have suggested that the materials for targeted delivery to colon can undergo approximately 40% of continuous dissolution. The degree of dissolution from our study suggests that SBM could be an ideal material for targeted colon delivery due to the lower solubility (20.3%) while SBP could not be used for targeted colon specific delivery. However, the SBP could be designed for specified delivery in the upper gastro intestinal tract. Thus, considering the individual dissolutions under simulated stomach and intestine conditions (**Table 4.2 and 4.3**) with continuous dissolution, it could be concluded that SBM is suitable for targeted delivery in the lower GI tract while SBP is potential for delivery to the upper GI tract.

4.2.3. Enzymatic digestion of SBP and SBM under simulated physiological condition

The resistance of SBP and SBM to pancreatic enzymatic digestion was studied under simulated physiological condition. The degree of digestion for SBP and SBM was 34.1% and 14.3%. The analysis of variance showed that there was significant difference in the degree of digestions at $p<0.05$. The degree of digestion was higher for SBP compared to SBM. The digestion studies were further evidenced by SEM images. **Figure 4.14** shows the digestion of pancreatic enzyme treated and untreated SBP. The SBP that was subjected to pancreatic enzymatic digestion did not show the presence of tubular structure, which is seen in the untreated SBP. The disappearance of the tubular structures in the enzymatically treated SBP is likely a result of enzymatic activity. According to **Figure 4.15** the enzymatic digestion of SBM shows

the formation of holes on the surface in contrast to the surface seen in the enzymatically untreated SBM. The holes are most likely to be formed by the enzyme activity. Overall, in both SBP and SBM there is a clear change in the morphology due to enzymatic digestion, which is quantitatively and qualitatively characterized by dissolution tests.

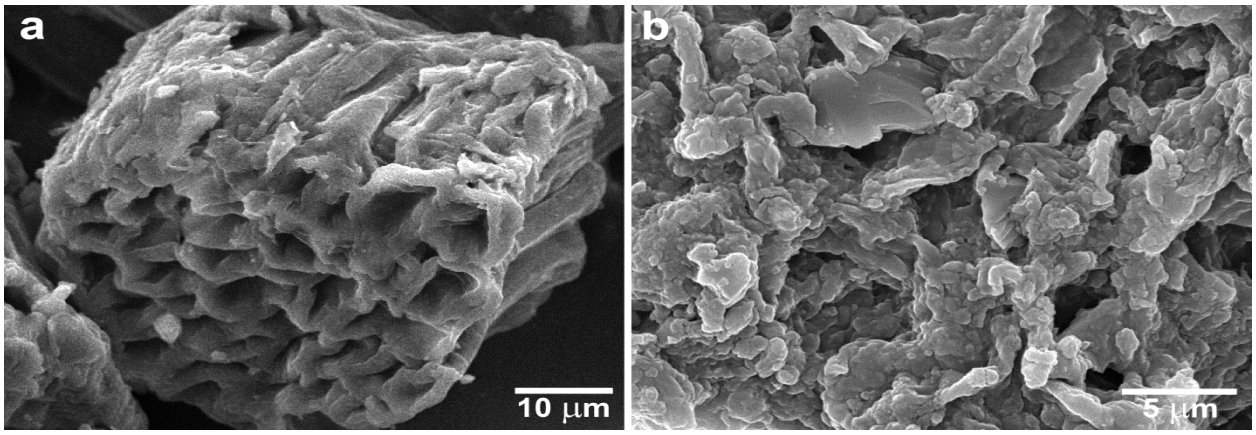


Figure 4.14. Scanning electron microscopy images for pancreatic enzymatic digestion of SBP(a) before enzymatic treatment; (b) after enzymatic treatment

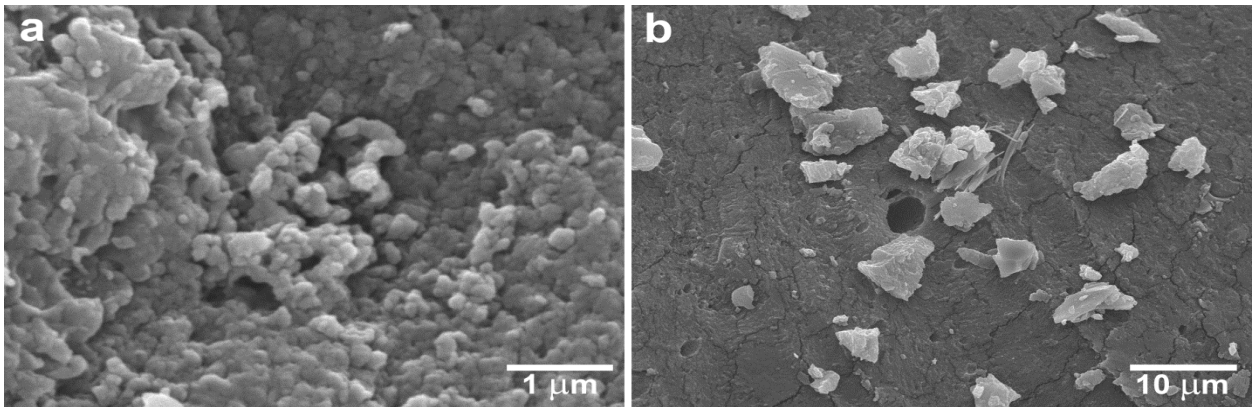


Figure 4.15. Scanning electron microscopy images for pancreatic enzymatic digestion of SBM (a) before enzymatic treatment; (b) after enzymatic treatment

The resistance of SBP and SBM to pancreatic enzymatic digestion is an important parameter to be considered in designing coating materials for specified delivery. The SBP and

SBM used in this study are composed of enzyme resistant carbohydrates, NSP and RS respectively. However, the increased dissolution of SBP compared to SBM indicates that the SBP is susceptible to pancreatic enzymatic digestion. This is further proven by the XRD pattern observed in study 1 (**Table 4.4**). The predominant form of A-type crystalline pattern in SBP is an indication of susceptibility of the material to pancreatic α-amylase. In contrast, the presence of mixture of A- and B- type pattern in SBM suggests that the presence of B-type pattern makes the SBM to be more enzyme resistance. The degree of digestion under simulated physiological condition with pancreatic enzymes is low for SBM compared to previous studies, which showed approximately 20-25% digestion (Macleod et al., 1997; Dimantov et al., 2004b). Thus, the observation of pancreatic digestion tests with SBP and SBM suggest that SBM could remain more intact than SBP in the GI tract, which could be designed for specified delivery in the upper and lower GI tract.

4.2.4. Thermal stability of SBP and SBM under physiological temperature

Differential scanning calorimetry (DSC) was used in studying the thermal properties of the materials. The DSC diagram for SBP is shown in **Figure 4.16**. The SBP shows high exothermic peaks at 133°C and 148°C. However, there is a distinct endothermic peak at 60°C. In contrast, the DSC of SBM shows an endothermic peak at 115°C in **Figure 4.16**. There are no exothermic peaks observed in SBM. The exothermic and endothermic temperatures are important in predicting the thermal stability of the materials.

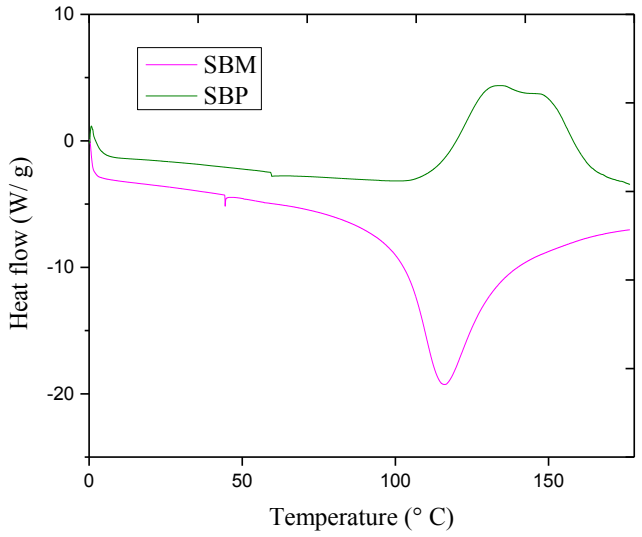


Figure 4.16. Differential scanning calorimetry for SBP and SBM

The DSC analysis was done in this study to predict the thermal stability of the materials; SBP and SBM under physiological temperature (37°C). Both SBP and SBM show stability under physiological temperature. However, the distinct endothermic peak at 60°C for SBP shows that the material will start to melt at 60°C, which is in accordance with previous studies (Mohamad et al., 2007). The Tg of SBM at 115°C indicates that SBM will melt at 115°C. The DSC of SBP and SBM indicate that the materials are stable at physiological temperature. The stability of the materials under physiological conditions suggests that SBP and SBM could be used in targeted delivery as they will not degrade at physiological temperature.

4.3. Study 3

In-vitro fermentation of soybean meal resistant starch nano particle by *Bifidobacterium brevis*

Resistant starch (RS) is a fraction of starch that escapes digestion in the upper GI tract. The RS can subsequently be fermented by the predominant gut micro-flora such as *Lactobacillus* and *Bifidobacterium* found in the lower GI tract (Cummings et al., 2004; Murphy et al., 2008). The fermentation of RS yields short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate. The SCFAs in turn provide the host with substantial health benefits by reducing cholesterol levels. In addition, the fermentation provides sufficient energy necessary for the metabolism of the epithelial cells of colon, which in turn maintains the gut health (Wolever et al., 1996). The fermentation can also lead to a reduced pH level in the lower GI tract, which facilitates the absorption of minerals such as Calcium (Ca), Magnesium (Mg), Iron (Fe), and Zinc (Zn) (Murphy et al., 2008). Therefore, in the current study nano scale RS derived from soybean meal was used as the substrate for in vitro fermentation by *Bifidobacterium brevis*. The potential of the *B. brevis* to grow and produce SCFAs in the presence of soybean meal RS was characterized under in vitro conditions.

4.3.1. In vitro utilization of soybean meal resistant starch by *Bifidobacterium brevis*

Bifidobacterium brevis is one of the most abundant *Bifidobacterium* species found in the human gastro intestinal (GI) tract. The *B. brevis* has the potential to ferment resistant starch (RS). Fermentation of RS provides health benefits to hosts through increased richness of the *B. brevis* population and the production of short chain fatty acids (SCFA) (Kumar et al., 2012). Therefore, in this study both the growth and the SCFA production of *B. brevis* were evaluated through the fermentation of soybean meal resistant starch. **Figure 4.17** shows the optical density (OD) of the *B. brevis* measured at 600 nm (OD_{600}) at every one hour interval. According to **Figure 4.17** the OD_{600} shows an increase from the 0^{th} hour to the 16^{th} hour in the presence of soybean meal RS.

In contrast, the OD_{600} of the blank, which did not contain RS as the carbon source, did not show a specific increase. The OD_{600} of the RS derived from soybean meal and the blank indicates that the growth of *B. brevis* was greater in the presence of the RS when compared to the blank.

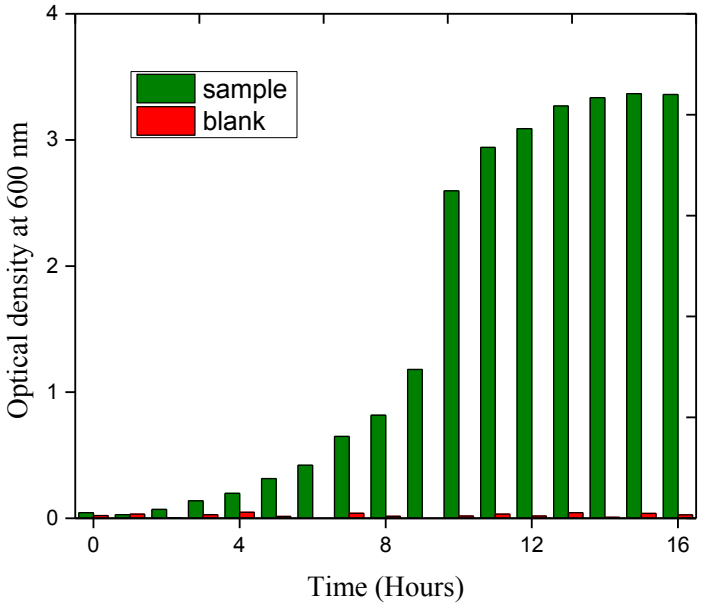


Figure 4.17. The maximum OD_{600} obtained by *Bifidobacterium brevis* for 16 hours (data were mean values of duplicates)

The growth of the *B. brevis* was further quantified by the plate counts in terms of colony forming unit per mL (CFU/mL). **Figure 4.18** depicts the growth of the bacterium at every four hour interval. The total count started to increase after 4 hours, while a steep increase in growth was seen from the 4^{th} to the 8^{th} hour. The slow growth in the first 4 hours could be attributed to the time it took the bacteria to adapt to the growth media (Lesmes et al., 2008). The increased growth from the 4^{th} to the 8^{th} hour could have arisen from the use of soybean meal RS in addition to the *bifidobacterium* specific growth media **(Figure 4.18)**. Furthermore, the increased growth

was not observed for the blank, which contained only *bifidobacterium* specific growth media. The results of this study clearly show that the increased population of the *B. brevis* is mainly due to the use of RS derived from SBM as the substrate for fermentation in addition to the *bifidobacterium* specific growth media.

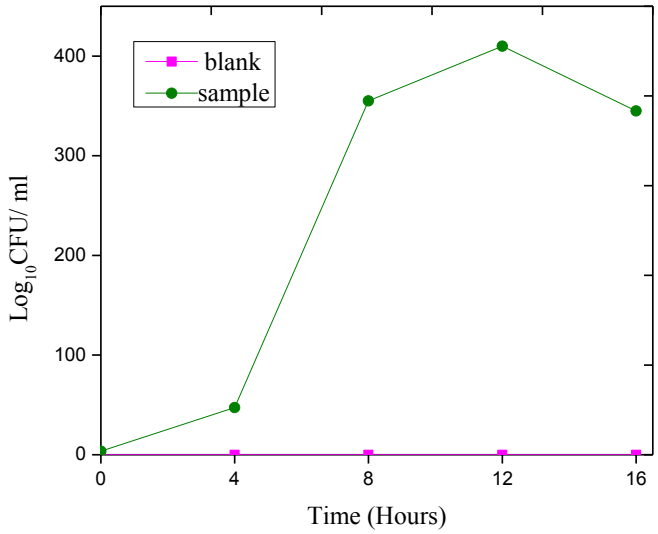


Figure 4.18. Growth of *Bifidobacterium brevis* at every four hour interval for 16 hours (data were mean value of the duplicates)

Bifidobacterium is one of the most important probiotics recommended for human applications. *Bifidobacterium* species provide several nutritional benefits such as enhancing the bioavailability of essential elements and the synthesis of vitamin B complexes. Furthermore, it is essential to maintain the richness of *bifidobacterium* throughout human life as the level of *bifidobacterium* decline with age. The current study clearly shows that RS derived from soybean meal could be used as a good substrate for the proliferation of *bifidobacterium* in the GI tract. Utilization of RS derived from SBM for various human applications in both food and

nutraceutical sectors could provide dual benefits by delivering the essential food and nutraceutical components while increasing the richness of *bifidobacterium* species.

4.3.2. Short chain fatty acid analysis of soybean meal RS by *Bifidobacterium brevis*

Bifidobacteria in the gut ferments RS anaerobically to produce SCFAs, which in turn provides various health benefits to the host. The SCFA was quantified at every four hour interval for *B. brevis*. As shown in **Figure 4.19** the total SCFA increased with time. The SCFA included acetate, propionate, butyrate, and valerate with trace amounts of branched chain fatty acids such as isobutyrate and isovalerate. The quantification of most important SCFAs, acetate, propionate, and butyrate, are shown in **Table 4.6**. **Table 4.6** shows an increase in acetate from the 0^{th} hour to the 16^{th} hour, while propionate and butyrate decreased during 16 hour incubation. The decrease of propionate with time can be attributed to the conversion of propionate to acetate through methylmalonyl CoA pathway (Lesmes et al., 2008). In the case of butyrate previous studies have reported that bifidobacteria does not produce butyrate (Zampa et al., 2003; Gibson & Roberfroid 1994). In contrast, current study shows the presence of butyrates. The production of butyrates by *B. brevis*, could be due to the possible pathway of butyrate production through butyryl COA/ acetyl COA mediated by free acetate (Lesmes et al., 2008). Overall, this study clearly shows that the soybean meal RS can be a potential substrate for gut micro-flora such as Bifidobacteria.

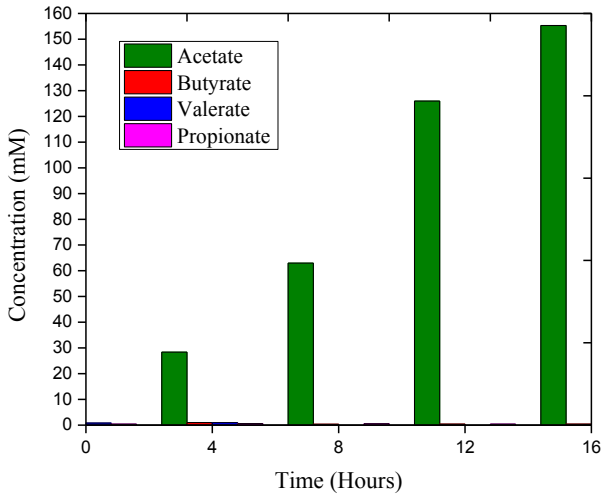


Figure 4.19. Total SCFA production by *Bifidobacterium brevis* during in vitro fermentation for 16 hours (data were mean values of duplicates)

Table 4.6. Short chain fatty acid (SCFA) profile

Time (hr)	Acetate (%)	Propionate (%)	Butyrate(%)
0	64.1	3.1	7.8
4	83.2	1.7	3.0
8	94.2	0.8	0.5
12	97.7	0.3	0.3
16	98.3	0.3	0.3

*Each value is a mean of duplicate

The SCFAs play an important role in human gut health. Acetate, propionate, and butyrate are the main SCFAs produced by the fermentation of gut micro flora. This study clearly showed that the concentration of acetate increased with time while the concentration of propionate and butyrate decreased after the 4th hour of incubation. Lesmes et al., 2008 study performed with RS derived from high amylose corn starch, showed an increase in the concentration of acetate, while propionate and the butyrate were detected only after the 10th and the 24th hour of incubation

respectively. However, the values reported in our study are higher compared to Lesmes et al., 2008 study. Higher values reported in this study could be mainly due to the use of different substrate at nano scale. The increased production of SCFA in our study strongly suggests that the RS derived from soybean meal at nano scale could be a good substrate for the fermentation of *bifidobacterium* species found in the human GI tract.

The SCFAs are important because they benefit health at multiple levels. For instance, acetate inhibits fatty acid oxidation while reducing serum cholesterol levels (Wolever et al., 1996; Soral- Smietana et al., 2004). In addition propionate also decreases the serum cholesterol levels (Wolever et al., 1996; Soral- Smietana et al., 2004). Furthermore, the butyrate acts as an important substrate for ATP production, which provides 60-70% of the energy requirements for the colonic epithelial cells (De-Fillipo et al., 2010). Moreover, health benefits of SCFAs could be addressed by considering the ratios of acetate: propionate and acetate: butyrate. The recommended general ratios of acetate: propionate and acetate: butyrate is 60:25 and 60: 15 respectively (Puneeth et al., 2012). In our study the acetate: propionate and acetate: butyrate ratios are higher compared to recommended general ratios (**Table 4.6**). The higher ratios can be attributed to the increased production of acetate compared to propionate and butyrate. The accumulation of acetate could induce the formation of butyrate through the formation of acetyl Co-A (Lesmes et al., 2008). However, our study cannot confirm the route from which butyrate could be produced. Overall, our observations on SCFA production by *B. brevis*, suggests that RS derived from SBM at nano scale could be a potential substrate for *bifidobacterium* species in the GI tract.

4.4. Study 4

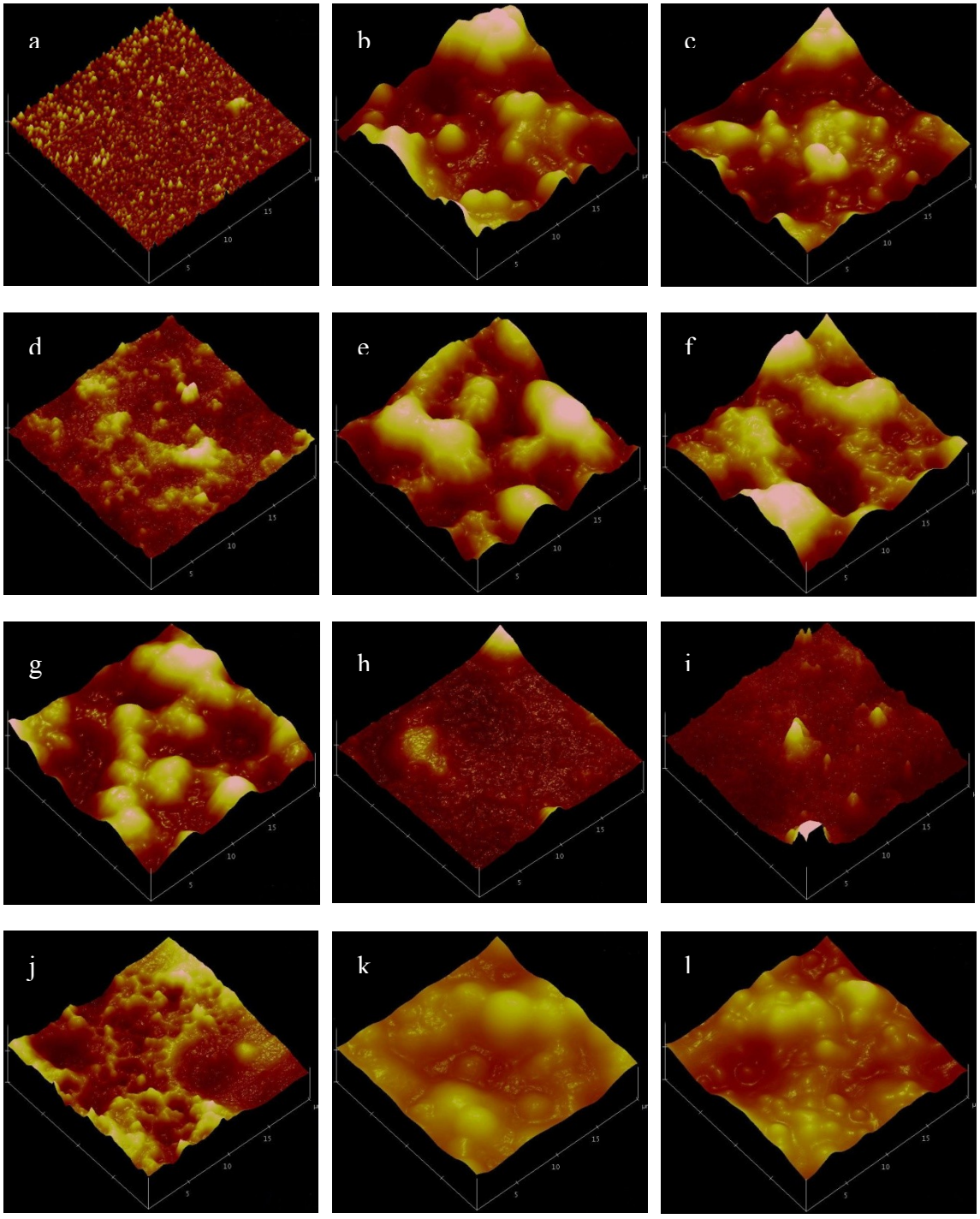
Semi-synthetic thin film formation using soybean meal RS nano particles, carboxymethylcellulose and pectin.

Bio-based packaging materials are widely used in food and biomedical sectors for the encapsulation of vitamins, minerals, probiotics, and prebiotics (Sozer & Kokini, 2009). Bioactive packaging can tailor the target and controlled release of bioactive components in the human gastro intestinal (GI) tract. The most widely used packaging materials are composed of modified starch such as high amylose corn starch. However, starch is susceptible to enzymatic digestion in the oral cavity, which in turn does not become effective in targeted or controlled delivery in the GI tract. Thus, a combination of starch with other polymers such as pectin, cellulose, ethylcellulose, and carboxymethylcellulose have been used in previous studies for targeted release of bioactive compounds (Mishra et al., 2012; Macleod et al., 1997; Wakerly et al., 1997; Dimantov et al., 2004b). To date, there are no studies reported with the use of resistant starch (RS) as a component for bioactive packaging although there have been suggestions on potential use of RS for bioactive packaging (Fathi et al., 2012). Thus, in study 4 we examined the physical properties of thin films formed with different combinations of soybean meal RS, pectin, and carboxymethylcellulose. The film formed solely by the soybean meal RS was used as the control to compare the physical properties of the other films. The surface characterization of the films was carried out by atomic force microscopy (AFM). In addition, the thermal stability of the films was quantified by differential scanning calorimetry (DSC). The AFM and DSC were used derive the physical properties of the films for potential food and biomedical applications.

4.4.1. Surface topography

4.4.1.1. Three-Dimensional topographic imaging

Three-Dimensional (3-D) topographic imaging of AFM is important for qualitative analysis of the surface. **Figure 4.20** displays the 3-D images of the thin films. **Figure 4.20 a**, represents the topography of the thin films produced from soybean meal RS nano particles, which was used as the control. The surface is almost flat and smooth with a few minor bumps. The smooth surface suggests that the material has effective barrier properties towards moisture and gases (Garcia- Ayuso et al., 1996). **Figure 4.20 b- h**, represents thin films formed with different proportions of soybean meal RS, pectin, and carboxymethylcellulose. **Figure 4.20 b- c, e- g** show large bumps with few depressions on the surface compared to the control (**Figure 4.20 a**). The presence of larger bumps could be attributed to the presence of dust particles on the surface. **Figure 4.20 d** has very few larger bumps compared to **Figure 4.20 b- c, e- g**. However, the surface of **Figure 4.20 d** is rougher than the control. In contrast the **Figure 4.20 h**, shows similar surface compared to the control. Thus, the overall 3-D topographical image with soybean meal RS, pectin, and carboxymethyl cellulose with different proportions provides preliminary results on the surface roughness, in prediction of the barrier properties.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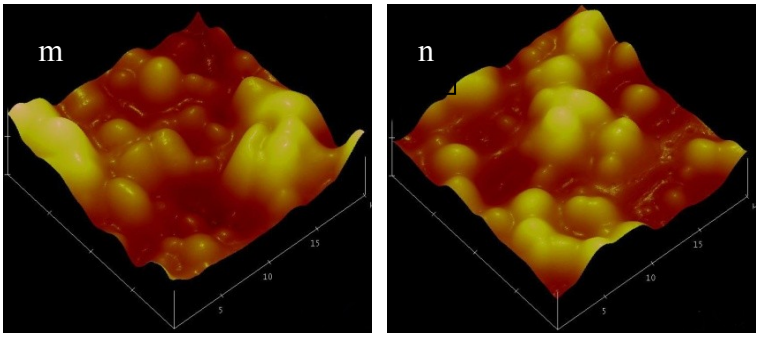


Figure 4.20. 3-D topographical images of the surface of the thin films (a) control- soybean meal RS nanoparticles; (b) Soybean meal RS: pectin: carboxymethylcellulose (1:1:1); (c) Soybean meal RS: pectin: carboxymethylcellulose (1:1:3); (d) Soybean meal RS: pectin: carboxymethylcellulose (1:3:1); (e) Soybean meal RS: pectin: carboxymethylcellulose (3:1:1); (f) Soybean meal RS: pectin: carboxymethylcellulose (3:3:1); (g) Soybean meal RS: pectin: carboxymethylcellulose (3:1:1); (h) Soybean meal RS: pectin: carboxymethylcellulose (1:3:3); (i) Soybean meal RS: pectin (1:3); (j) Soybean meal RS: pectin (3:1); (k) Soybean meal RS: pectin (1:1); (l)Soybean meal RS: carboxymethylcellulose (1:3); (m) Soybean meal RS: carboxymethylcellulose (3:1); (n) Soybean meal RS: carboxymethylcellulose (1:1)

Furthermore, the films discussed above were also prepared with different proportions of soybean meal RS: pectin and soybean meal RS: carboxymethylcellulose. **Figure 4.20 i - k**, represents the films prepared by different combinations of soybean meal RS: pectin. Compared to the control (**Figure 4.20 a**), the surface of the films prepared with higher proportion of pectin showed a smoother surface (**Figure 4.20 i**). The smoother surface of such films suggests that the film can have more effective barrier properties than the control, which is composed solely of RS derived from soybean meal (**Figure 4.20 j and k**). Furthermore, **Figure 4.20 l–n**, shows different proportions of soybean meal RS: carboxymethylcellulose. The surface of all three different combinations with soybean meal RS: carboxymethylcellulose (**Figure 4.20 l-n**) shows that the surface is characterized by larger bumps, which in turn could be due to the adhesion of

dust particles. Overall, the 3-D topographic analysis qualitatively characterizes the surface of the thin films produced with different combinations.

4.4.1.2. Surface roughness analysis

The surface roughness of the thin films can be quantitatively characterized by various parameters such as root-mean square roughness (R_q), peak- to- valley height (R_z), surface skewness (R_{sk}), and surface kurtosis (R_{ku}). **Table 4.7** shows the quantitative analysis of surface roughness in terms of all of the parameters mentioned above. According to **Table 4.7**, the root mean square roughness showed lower values for films with a combination of RS from soybean meal: pectin 3:1 and RS from soybean meal: pectin: carboxymethylcellulose 1:3:1 compared to the control. Lower values for root-mean square roughness indicate that the polymer chain entanglements are induced to a coiled structure leading to a decreased surface area by reducing the distance between peak and valley height (Stawikowska & Livingston, 2013). The lower root-mean square roughness further suggests that the films with lower root-mean square roughness will have better barrier properties for moisture and gases (Garcia-Ayuso et al., 1996; Zoubi, 2005). Thus, the films with lower root-mean square roughness compared to the control (**Table 4.7)** provide more effective barrier properties.

In addition to root-mean square roughness and peak-to valley height, parameters such as surface skewness and surface kurtosis is important in further characterizing the surface of the films. Our study shows a negative value for the skewness of the control film (**Table 4.7**). A negative value of skewness indicates that the surface has more valleys compared to peaks, which leads to a more porous surface. In contrast, a positive value for surface skewness indicates that the peaks are dominant (Stawikowska & Livingston, 2013). In our study, the positive values are

seen only for four films (**Table 4.7**). Furthermore, the greater positive values for skewness indicate that polymer chains tend to organize themselves into higher compliance resulting in mitigated depression or porosity. Thus, the films with higher positive value for surface skewness (**Table 4.7**) have potential applications in food and biomedical sectors, for encapsulating and packaging vitamins, minerals, probiotics, and prebiotics, due to the rigidity of the film.

Table 4.7. Quantitative analysis (summary) of surface roughness analysis

Film type	R_q(nm)	R_z(nm)	R_{sk}	R_{ku}
F_a	84.62	840.65	-12.81	239.79
F_b	283.68	1585.00	0.06	0.20
F_c	174.21	1034.00	0.21	0.97
F_d	79.37	618.20	-0.05	0.14
F_e	229.31	1259.00	-0.13	0.52
F_f	218.75	1231.00	-1.34	11.81
F_g	307.54	1602.00	0.17	0.75
F_h	131.02	1232.00	-0.29	1.55
F_i	86.02	889.03	-5.22	72.50
F_j	56.94	388.42	-5.26	73.11
F_k	206.51	999.51	0.01	0.02
F_l	231.84	1310.00	-0.03	0.08
F_m	459.83	2354.00	-0.004	0.01
F_n	319.23	1538.00	-0.001	0.001

*Each value is the mean of duplicated

In addition to the skewness, the kurtosis further characterizes the surface of the films. Kurtosis indicates the sharpness of the film height. Kurtosis values less than 3 indicate that the film surface is flat and repetitive. In contrast, kurtosis values greater than 3 indicate that the film surface has sharper heights, which leads to less porous surface (Stawikowska & Livingston, 2013). Our study indicates that the kurtosis is highest for the control compared to the other films (**Table 4.7**). However, the kurtosis is greater than 3 for three films other than the control (**Table 4.7**). Overall, the quantitative analysis of thin films in terms of lower root-mean square roughness, lower peak-to- valley height, higher positive surface skewness, and surface kurtosis

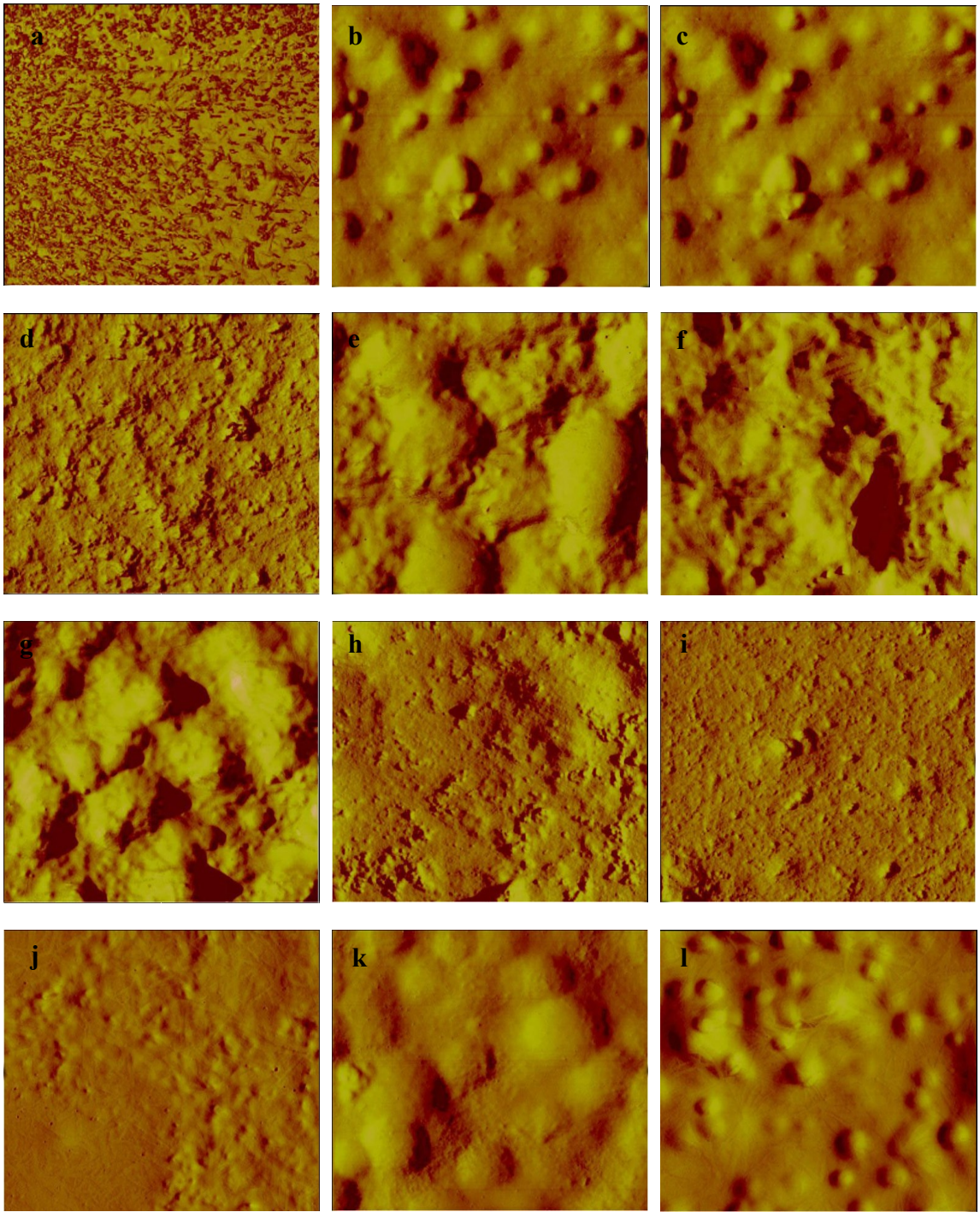
with greater than 3 can be regarded as an indication of potential film for bioactive packaging in food and biomedical sectors. Moreover, phase image analysis reported further supports this conclusion.

4.4.1.3. Analysis of phase image

Phase images (phase lags) depicts phase contrast at higher resolution. Phase lag is important to monitor the viscoelastic variations of the film surface, which results from entanglement of polymer chain. **Figure 4.21a** represents the phase lag of the control. The phase lag of the control shows the presence of "islands" on the surface. The presence of such a structure can be related to the compactness of the packaging of the films. Phase lag with islands have been reported in a previous study by Stawikowska & Livingston, 2013. However, in our study the appearance of "islands" is observed only for the control film (**Figure 4.21 a**). The phase lags for the films formed with different combinations of soybean meal RS, pectin, and carboxymethylcellulose are shown in **Figure 4.21 b-n**. The surface of the phase lag is smoother for the film that has the combination of soybean meal RS: pectin 3:1 ratio (**Figure 4.21 j**). In addition, the surface of the films is also characterized by light and dark regions (**Figure 4.21 a-n**). Darker regions represent the presence of pores while lighter regions represent heights. **Figure 4.21 e- g** clearly shows the presence of darker regions, which represents the presence of pores on the surface lead to less compact films. Thus, the presence of lighter regions compared to darker regions lead to a compact film, which in turn can possess several benefits towards constructing bioactive packaging materials.

Quantitative analysis of phase lag is important to draw a definite characterization of the film surface. The phase lag analysis can be associated with dense packaging of the film. The

lower root-mean square value for phase lag suggests that the surface of the film is less porous with a dense packing of polymer chain. The dense packing leads to a stiffer surface and in turn gives a low viscoelasticity. Lower viscoelatic property is an important characteristic of the surface phenomenon to maintain the rigidity of the surface. In our study, the lower viscoelastic film is represented by the film formed with soybean meal RS: pectin 3:1. In contrast, the highest viscoelastic film is formed in the control, which is made up of RS derived from soybean meal. The observations with phase lag suggest that the film formed by the combination of soybean meal RS: pectin 3:1 possesses densely packed film with lower viscoelastic property, which in turn could maintain a rigid surface. Rigid films are important for the encapsulation of the bioactive components. Moreover, in order to fully understand the films for applications in food and biomedical sectors it is necessary to analyze the thermal stability of the films.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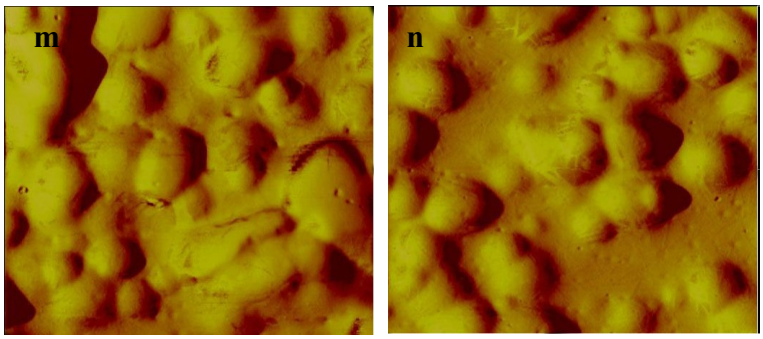

Figure 4.21. 3-D topographical images of the surface of the thin films (a) control- soybean meal RS nanoparticles; (b) Soybean meal RS: pectin: carboxymethylcellulose (1:1:1); (c) Soybean meal RS: pectin: carboxymethylcellulose (1:1:3); (d) Soybean meal RS: pectin: carboxymethylcellulose (1:3:1); (e) Soybean meal RS: pectin: carboxymethylcellulose (3:1:1); (f) Soybean meal RS: pectin: carboxymethylcellulose (3:3:1); (g) Soybean meal RS: pectin: carboxymethylcellulose (3:1:1); (h) Soybean meal RS: pectin: carboxymethylcellulose (1:3:3); (i) Soybean meal RS: pectin (1:3); (j) Soybean meal RS: pectin (3:1); (k) Soybean meal RS: pectin (1:1); (l)Soybean meal RS: carboxymethylcellulose (1:3); (m) Soybean meal RS: carboxymethylcellulose (3:1); (n) Soybean meal RS: carboxymethylcellulose (1:1)

Table 4.8. Quantitative surface phase analysis

Film type	P_q (°)	P_z (°)
F_a	19.17	138.03
F_b	12.58	76.59
F_c	11.13	75.20
F_d	12.08	90.45
F_e	15.89	88.79
F_f	14.55	77.15
F_g	16.63	89.51
F_h	10.57	66.41
F_i	8.11	71.27
F_j	4.25	70.80
F_k	9.12	71.83
F_l	9.31	89.66
F_m	14.80	107.63
F_n	13.86	82.31

*Each value is the mean of duplicate

4.4.2. Thermal stability of the film

The thermal stabilities of the films were analyzed using differential scanning calorimetry (DSC). **Figure 4.22** clearly shows that all the films have an endothermic peak at around 110-120°C, suggesting that the films undergo thermal degradation at the specified temperature range. However, the films formed with a combination of soybean meal RS: pectin: carboxymethyl cellulose 1:1:3 (**Figure 4.22 DSC 2**) showed a distinct exothermic peak at around 105°C. The distinct exothermic peak indicates that the film undergoes a phase transition from solid to liquid (melting) before it starts degrading completely. Furthermore, there is no significant difference in the stability of the films except for the film that was composed of soybean meal RS: pectin: carboxymethylcellulose 3:1:1 (**Figure 4.22 DSC 6**). The significant difference of such film can be attributed to the greater amount of heat that is given out of the film during melting, which in turn indicates the greater stability of the bonds formed within the polymers in the film. Overall, the DSC thermogram suggests that the films are thermally stable up to a temperature of 110-120°C, which can be processed for the encapsulation of the bioactive components in the food and biomedical sectors.

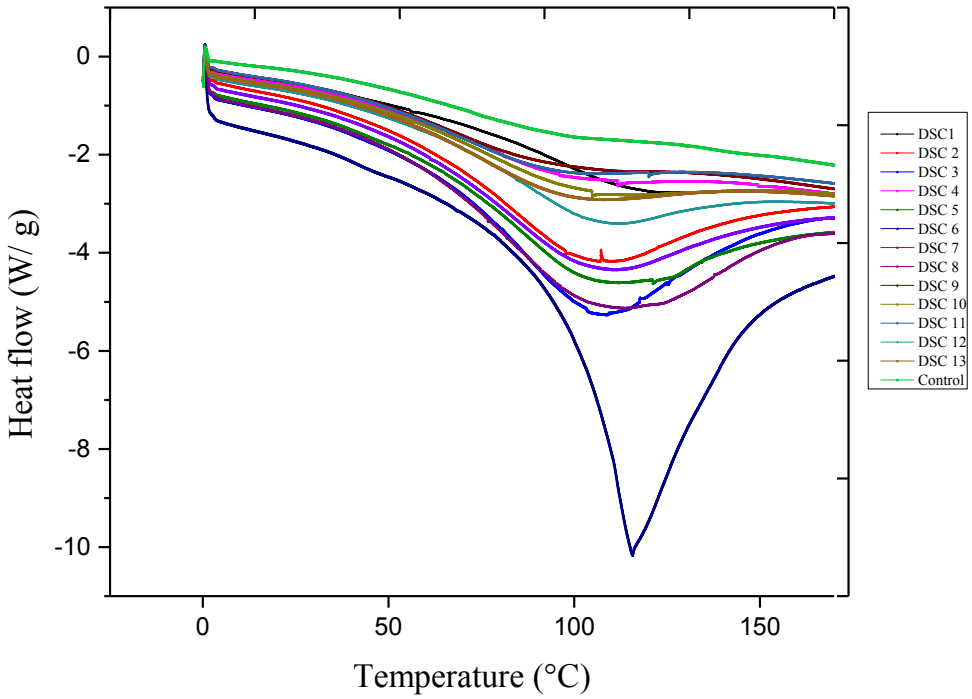

Figure 4.22. DSC thermograms of the films; DSC 1- R: P: C (1:1:1); DSC 2- R: P: C(1:1:3); DSC 3- R: P: C (1:3:1); DSC 4- R: P: C (3:1:1); DSC 5- R: P: C (3:3:1); DSC 6- R: P: C (3:1:3); DSC 7- R: P: C (1:3:3); DSC 8- R: P (1:3); DSC 9- R: P (3:1); DSC 10- R: P (1:1); DSC 11- R: C (1:3); DSC 12- R: C (3:1); DSC 13- R: C (1:1)

4.4.3. Potential films for bioactive packaging in food and biomedical applications

The films used for bioactive packaging in food and biomedical sectors should be physically stable for effective delivery of the bioactive components. Our study characterized the physical and thermal properties of several combinations of films formed using soybean meal RS, pectin, and carboxymethylcellulose. Overall characterization of the thin films suggests that the films formed with soybean meal RS: pectin 1:3 and 3:1 are potential for bioactive packaging. Furthermore, previous studies have shown that due to the higher solubility of pectin, it should be

used in combination with other polysaccharides such as ethylcellulose (Wakerly et al., 1997; Macleod et al., 1997; Mishra et al., 2012). However extensive surface characterizations of such combinations have not been reported to date. In contrast an extensive analysis of the surface characterization and thermal properties in our studies clearly show that the pectin used in combination with soybean meal RS is physically and thermally stable. The stability of the soybean meal RS and pectin suggests that the films composed of these two polymers can be used as bioactive packaging material for controlled and targeted release of bioactive components in the GI tract.

5. CONCLUSION

Study 1, which was to prepare micro and nano scale materials clearly showed that the three methods; sulfuric acid hydrolysis, complex formation to ethanol, and buffer extraction, can be used to produce micro and nano scale materials from agricultural by-products such as SBM, DDG, and SBP. The morphological features suggest that the unique tubular structure of SBP produced by sulfuric acid hydrolysis at micro scale could be a potent encapsulating material in food and other applications. Furthermore, the RS derived from soybean meal at nano scale through buffer extraction suggest that well resolved nature of the nano particles could be used as nano particulate delivery system for controlled and targeted release of bioactive compounds in the GI tract.

Study 2 involved examining the stability of micro scale SBP produced by sulfuric acid hydrolysis and nano scale SBM produced by buffer extraction. This study showed the stability of both the micro scale tubular structure of SBP and nano scale RS derived from SBM under simulated stomach and intestine conditions. The degrees of dissolution and digestion were greater for SBP compared to SBM. However, both materials were stable under physiological temperature (37- 40°C). The dissolution and digestion studies clearly show that the SBP could be designed for targeted delivery in the upper GI tract, where as SBM could used for targeted delivery in the lower GI tract. Therefore, the different degrees of stability under simulated physiological conditions could be used in designing the encapsulating and/ or packaging materials for food and other applications.

Study 3 characterized the in vitro fermentation of nano scale RS derived from soybean meal by *Bifidobacterium brevis*. The results clearly showed that the *B. brevis* was able to grow

well in the presence of RS derived from soybean meal. Furthermore, the *B. brevis* also showed an increased production of SCFAs such as acetate, propionate, and butyrate. However, the production of acetate was higher compared to propionate and butyrate. Both the growth and SCFA production suggests that the RS derived from soybean meal at nano scale could be a good substrate for gut micro-flora such as *Bifidobacterium* species. Therefore, encapsulation of vitamins and minerals by nano scale RS derived from soybean meal not only can deliver the components at the targeted site but also can stimulate the prebiotic effect.

In study 4 the RS derived from soybean meal was used in combination with pectin and carboxymethylcellulose to produce semi-synthetic thin films for encapsulation and food packaging applications. The extensive surface characterization showed that the films formed with soybean meal RS: pectin 1:3 and 3:1 were stable in terms of rigidity, barrier property, and thermal stability compared to other films. However, the present study was a baseline to identify the best combination of soybean meal RS nano materials with other compounds for potential applications. The results suggest that future studies with different combinations of soybean meal RS nano particles and pectin can be done further to draw strong conclusions for the applications in food and biomedical sectors.

6. FUTURE DIRECTION

The results of study 1 clearly showed DDG is a good starting material to produce nano particles with higher yields. Therefore, the DDG could be further studied for nano scale based food and pharmaceutical applications.

In study 3 the in vitro fermentation of soybean meal resistant nano particles was carried out with only Bifidobacterium species. However, Lactobacillus is one of the most abundant gut micro-flora. Therefore, the in vitro fermentation of soybean meal resistant starch nano particles by Lactobacillus species can be a future study to determine the ability of Lactobacillus species to utilize soybean meal resistant starch nanoparticles.

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