

“Enzymatic versus chemical conversion of Soybean hull to useful products”

A Dissertation submitted in partial fulfilment of the
requirement for the award of the degree of

MASTERS OF SCIENCE

IN

BIOCHEMISTRY

By

TARANVIR KAUR

Roll no.: 301507010

Under the supervision of

Dr. Jyoti Rani



School of Chemistry and Biochemistry

THAPAR UNIVERSITY

PATIALA-147004

July 2017

CERTIFICATE

This is to certify that the thesis entitled “**Enzymatic Vs Chemical conversion of Soybean hull to useful products**” is an authentic record of my work carried out as requirements for the award of the degree of **Master of Science in Biochemistry** at **Thapar University, Patiala** under the supervision of **Dr. Jyoti Rani**, Assistant Professor (FT), Department of Biotechnology, Thapar University, Patiala during July, January to July, 2017. No part of the matter embodied in this report has been submitted to any other university or institute for the award of any degree.

Place: Patiala

Date: 12/9/17

It is certified that the above statement made by the student is correct to the best of my/our knowledge and belief.

 12/9/17

Dr. Jyoti Rani,

Assistant Professor (Project supervisor),

Department of Biotechnology,

Thapar University, Patiala-147004.

Candidate's Declaration

I hereby declare that the work being presented in the dissertation entitled "**Enzymatic vs Chemical conversion of Soybean hull to useful products**" in the partial fulfilment of the requirements for the award of the degree of Masters in Biochemistry and being submitted to School of Chemistry and Biochemistry, Thapar University, Patiala, is my own work during the period of January to July 2017, under the supervision of **Dr. Jyoti Rani, Assistant Professor (FT), Department of Biotechnology, Thapar University, Patiala**. I have not submitted the contents embodied in this dissertation for the award of any other degree.

Place: Patiala

Date: 12 Sept. 2017


Taranvir Kaur

ACKNOWLEDGEMENT

To make a project successful, there are many helping hands. I would like to express my sincere gratitude and appreciation to many people who helped keep me on track toward the completion of my project.

Firstly, I express my sincere thanks to **Dr. Jyoti Rani**, Assistant Professor (FT), Department of biotechnology, Thapar university, Patiala for providing exemplary guidance all necessary facilities, full co-operation, monitoring and constant encouragement throughout the course of this project work. The blessing, help and guidance given by him time to time shall carry me a long way in the journey of life on which I am about to embark.

I am obliged to **Dr. Anil Kumar Dutta**, Director of TIFAC-CORE, **Dr. Dinesh Goyal**, Professor, DBT for using his lab at STEP. I am grateful to all laboratory staff for their cooperation during the period of my assignment, which helped me in completing this task through various stages. In my daily work I have been blessed with a friendly and cheerful group of research scholars Ms. Ishpreet Kaur and Ms. Simarpreet Kaur and Ms. Fatima Bhadra for assistance in learning statistics.

I owe the biggest thanks and sense of gratitude to **Dr. Moushumi Ghosh**, Head, Department of Biotechnology; **Dr. Amjad Ali**, Head (SCBC), and other faculties of DBT for guiding me and providing me support whenever I needed.

I wish to express my gratitude to my parents and friends for their encouragement and blessings which supported me throughout this, without which this project would not be possible.



Taranvir Kaur

INDEX

List of Contents

Chapter1: Introduction	1-2
Chapter2: Review of literature	3-12
2.1. Introduction to Soybean and Soy hull	3
2.1.1. Soybean	3
2.1.2. Soybean Hull	3-4
2.1.3. Soybean hull as dietary fiber	4
2.1.4. Soybean hull pre-treatments	4-5
2.1.5. Mineral absorption by soybean hull	5
2.2. Various useful applications of soybean hull	5
2.2.1. Animal feed	5-6
2.2.2. For waste water treatment	6-7
2.2.3. As source of dietary fibre	7-8
2.2.4. As medicine	8
2.3. Various products obtained after conversion of soybean hull	8
2.3.1. Ethanol	9-10
2.3.2. Bio-fuels	10
2.3.3. Polysaccharides	10
2.3.4. Microfibrils	11
2.3.5. Peroxidases	11
2.3.6. Oligopeptides	11-12
Chapter3: Materials and Methods	13-20
3.1. Pre-requisites required for the study	13
3.1.1. Obtaining hulls from soybean	13
3.1.2. Pectinase enzyme	13
3.1.3. Cellulase enzyme	13
3.1.4. Sodium hydroxide	13
3.1.5. Water bath	13
3.1.6. Autoclave	13
3.1.7. Whatman paper	14
3.1.8. Refrigerators and deep freezers	14
3.1.9. Spectrophotometer	14
3.1.10. SEM	14
3.2. Experimental planning	14
3.2.1. Processing and treatment of soybean hull	14-15
3.2.2. Treatment process of hull by chemical and enzymatic methods	16
3.2.2.1. Chemical method	16
3.2.2.2. Enzymatic treatment methods	16
3.2.2.2.1. Pectinase treatment	16
3.2.2.2.2. Cellulase treatment	16
3.3. Isolation of <i>Lactobacillus casei</i>	17
3.4. Gram staining	17
3.5. Enumeration of <i>Lactobacillus casei</i> (both isolated and directly from yakult)	17-18

on different experimental samples of soybean hull	
3.6. Enumeration of Yeast (<i>Saccharomyces cerevisiae</i>) on different experimental samples of soybean hull	18
3.7. Enumeration of <i>Aspergillus niger</i> (fungal mould) on different experimental samples of Soybean hull	18
3.8. Chemical analysis of soybean hull of treated soybean hull	18
3.8.1. Moisture content of soybean hull	19
3.8.2. Sugar content.	19
3.8.2.1. Benedict's test	19
3.8.2.2. DNS assay	19
3.8.2.2.1. Preparation of standard curve	19
3.8.2.2.2. Preparation of samples	19
3.9. SEM analysis	20
Chapter4: Results and discussions	21-34
4.1. Chemical analysis of raw material	21
4.1.1. Physical characteristics of raw material	21
4.1.2. Moisture content analysis	21-22
4.1.3. Gram staining	23
4.1.4. Soy hull treatment	22-23
4.1.5. Sugar content of hull	23
4.1.5.1. Benedict's test	23
4.1.5.2. DNS assay	23-25
4.1.6. SEM analysis of the hulls	25
4.2. Growth of micro-organisms on the treated hull samples	25
4.2.1. Isolation of <i>Lactobacillus casei</i> (probiotic micro-organism)	25-26
4.2.2. Utilization of treated soybean hull samples as growth medium	26
4.2.2.1. For <i>Lactobacillus casei</i>	26-28
4.2.2.2. For Yeast (<i>Saccharomyces cerevisiae</i>)	29-31
4.2.3. For <i>Aspergillus niger</i> (fungus)	31-32
Chapter5: Conclusion	33
References	34-39

ABSTRACT

Existing study was focused on the utilization of soybean by-product waste that is soy hull conversion into useful products for human use. Commercially, the soybean hulls were used for animal feedstock and production of various useful industrial products. An initiative has been done to utilize soybean hull as source of growth for gut probiotic bacteria that are useful for humans, so that the all the major by-product produced by the soybean processing industries can be utilized and the waste results into another useful consumable product. The chemical and enzyme assisted (pectinase and cellulase) methods have been implemented to observe the superficial changes in the soy hull through SEM. All the three samples (two treated with enzymes such as cellulase and pectinase and one with NaOH) were analyzed for growth of micro-organisms like *Lactobacillus casei*, *Saccharomyces cerevisiae* and *Aspergillus niger* showing that soy hull can be used as a substrate for its utilization and substitution as a growth medium for the growth of useful microorganisms.

CHAPTER-1

INTRODUCTION

Soybean (*Glycine max*) is a native legume of East Asia but is most widely grown edible bean all over the world these days due to its high protein content. It is one of the most important crop with various uses in foods (used for consumption by both humans as well as animals), pharmaceuticals, nutritional and industrial uses. It's production has been increased in recent years due to its increasing demand in vegetable protein and oil and also other constituents. (Harman *et al.*, 2011).

The Soybean seed consists of about 90 per cent of cotyledons while 8 per cent is its hull or seed coat and 2 per cent is germ. After dehulling, the main constituents of soybean reported were 40 per cent protein content, 35 per cent carbohydrates, 20 per cent oil and 5 per cent minerals on a dry matter (DM) basis (USDA, 2009).

The carbohydrates predominantly present in soybean meal are non-starch polysaccharides (NSP) and free sugars, such as mono-saccharides, di-saccharides and oligosaccharides (sucrose, stachyose and xylanose) (Choct, 1997). Nearly less than 1 per cent of starch is present (Choct, 1997). NSP are classified into three main groups namely cellulose, non-cellulosic polymers and pectic polysaccharides (pectin and cellulose).

Raw soybean reported toxic to humans due to naturally occurring trypsin inhibitors. These chemicals can be destroyed upon heating, so soybean is usually heated with moisture before human consumption (i.e. either steamed, boiled or pouched). Besides this, (Rebello, Greenway, & Finley, 2014).

Soybean had remained as staple food in various different parts of world for years, allowing humans to produce various different products produced using soybean. Some of the common products produced using soy sauce, tempeh, tofu, vegetable oil, soy milk / soy-drink, soy flour.

Soybean hull is primarily the outer covering of the soybean seed obtained from the raw soybean after its processing for oil by various different methods in the mill. The soybean were cleaned by removing foreign particles and then broken into flakes or small portions so as to separate hull part from the beans. The hull fractions were then passed to sifter separating them into three categories: large hulls or meat, small hulls or meats and the fines. The fines were returned to the primary soybean stream. The large and small soybean hull and meat

fractions were sent for secondary dehulling process. During this process, the hulls were removed from the soybean meats and the hulls are passed into the toaster to destroy urease activity. Following toasting, the remaining hull fraction is ground to the desired particle size. The hull can either be pelleted or kept in bulking for selling to markets. Pelleted soybean hulls helps in reducing transportation cost significantly. However, many commercial feed companies prefer the bulk unpelleted form for inclusion of hulls into their products(Proctor, 1997).

Soybean hulls consists of 67 per cent NDF and 12.2 per cent moderate crude protein whereas cellulose and hemicellulose are main components of fibre consisting of about 47 per cent and 20 per cent of DM. These hulls reported were highly digestible by ruminants due to low lignin component (Garleb *et al.*, 1987) and relatively high energy value.

Soybean hulls chemical composition varies with efficiency of their dehulling process and may contain variable amount of cellulose (21-51 per cent), hemicelluloses (10-25 per cent), lignin (1- 4 per cent), pectins (4-8 per cent) and proteins (11-15 per cent) and also some minor extractives (Mielenz *et al.*, 2009 ; Yoo *et al.*, 2011). These are primarily lignocellulosic material.

With the fact that only 8 per cent of soybean seed contributed by soy hull it should not be considered as waste. As shown above it has hemicelluloses, lignin, pectins and proteins as well in appropriate amount. Beside these qualities it is very difficult to degrade because of lignocellulosic material and it becomes tough. An attempt to degrade it chemically and through enzymes has been performed in the present study to make it available as dietary fibre for human end use.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Introduction to Soybean and Soy Hull

2.1.1 Soybean:

Soybean also known as ‘The Miracle Crop’ is a green, bushy legume related to alfalfa, peas and peanuts. It is one of the world’s most versatile crop that can be grown in wide variety of soil and climatic conditions. Soybeans due to its high protein and oil content and wide uses were included in one of the worthiest crop in the world (Small, 1999). Soybean protein has been used for feeding livestock and aquaculture feeds for its highly digestibility, along with many other foods consumed by humans. Soybean oil is used as a food and feed ingredient as well as in biodiesel production and cosmetics. It can produce both edible as well as non-edible oil used for economic purposes. In past few years, scientists have been able to manufacture a wide variety of soybean by-products that are beneficial in animal feed as well as human feed and also used for various industrial uses.

According to a study (Jonas Laukkonen Ravn *et al.*, 2015) the soybean and soybean meal was investigated for the degradation that happened to the soybean fibre after being fermented by *Aspergillus aculeatus* to obtain a commercial multi-enzyme product. The analysis was done using viscosity measurements, dietary fibre component analysis and different microscopic techniques using different histochemical dyes and antibody labelling. The results demonstrated reducing effect of the enzyme preparation on soluble polysaccharides and notable solubilisation in all polysaccharide components of non-starch polysaccharide. The degradation was visualized using Corihosphine O and alcian blue dyes. These dyes used were successful in following the pectin solubilisation after enzyme treatment. The combination of these techniques helped in observing visual and quantitative degradation of hemicelluloses pectin of soybean cell wall component of undesirable anti-nutrients in the feed of animals.

2.1.2 Soybean hull:

Soybean hulls accounts for the largest quantity of by-products in the industries of soybean processing as they account for 7-8 per cent of total mass of soybean. Contradictory to oils and proteins produced in soybean processing industry for economic use, the “hull” is totally a waste product produced along with the useful products. It has been estimated that

the world's total production of soybean will be about 371.3 million tons by around 2030 from which 29.7-37.1 million tons of soybean hulls will be produced (Salunkhe, 1992).

Depending upon the efficiency of soybean dehulling the, its chemical composition may vary. The dehulled soybean hulls may constitute varying degree of these components such as cellulose (29–51 per cent), hemicelluloses (10–25 per cent), lignin (1–4 per cent), pectins (4–8 per cent), proteins (11–15 per cent), and minor extractives. But soybean hulls are more easily degradable than the lignocellulosic materials like switchgrass or hardwood.(Mood *et al.*, 2013)

2.1.3 Soybean hull as Dietary Fibre:

The hulls obtained from soybean can be utilized to expand the fibre potential for human use with the increasing value of the crop. To the best of our knowledge, this may be the first study to develop dietary fibre from yellow soybean hulls using both traditional methods AOAC (991.43) and newly developed AOAC (2011.25) methods. It may be also the first rheological evaluation of the application of soybean hull fibres in dough. Fibres from yellow soybean hulls could have great potential in various food applications due to their functional properties. Further investigations were needed to establish the precise functions of fibre components on human health and nutrition.(Yang, Xiao, & Wang, 2014)

Unlike soybean hulls, the acid base hydrolysis was given to oats hull by conditioning the hulls to gain 32 per cent moisture, oats hulls were dipped in NaOH to attain pH of 11.5 and equilibrated in refrigerated for 24 hrs. Then sprinkled with 7 per cent H₂O₂ and again refrigerated for 1 hr. Then the hulls were extruded and dried at 50°C to attain 13 per cent moisture and stored in sealed polythene bags at room temperature, as described by Galdeano and Grossmann (2005) for their use in snacks (cookies) with a wide acceptance of texture and taste. The oats cookies were accepted because of the increased fibre content which helps in preventing constipation symptoms and diverticular disease (Silva *et al.*, 1998).

2.1.4. Soybean hull pre-treatments:

Soybean hulls were given both enzymatic and chemical treatments so that the total dietary fibre content of the hulls can be increased for their in snacks as the acid-base hydrolysis increases the TDF (total dietary fibre) content up to 50 per cent which is good for use in diet as according to researchers 3- 45 g of insoluble dietary fibre should be included in our daily diet consumption in order to prevent gastric disorders.(Committee, 2000)

Also the enzymes cellulase and pectinase were used to degrade the hemicelluloses and pectins present in the cell wall of hulls to increase the cellulose conversion to glucose by removal of the hemicelluloses. These types of treatments were to increase the glucose uptake in animal feedstock. Also in industries these treatments helped in increased production of fruit beverages. The combination of cellulase and pectinase enzyme helped industries in converting lignocellulosic components to bio-fuels for the increased production of the bio-fuels.(R. Kumar, Singh, & Singh, 2008)

2.1.5. Mineral absorption by soybean hull:

The relation between bioavailability of minerals and dietary fibre vary depending on the source of fibre. The bran of different cereals was investigated for mineral binding capacity under the mimicked gastrointestinal conditions created *in-vitro*. Soybean hull has 10 to 20 times higher calcium and iron content than the corn bran but their bioavailability is limited in gastrointestinal tract due to high insoluble dietary fibre (Laszlo, 1989).

Amongst Mn, Zn, Cu and Fe mineral binding capacity of rice and wheat bran studied under the physiological gastrointestinal conditions mimicked in the laboratory conditions, rice bran shows the highest mineral binding capacity for Mn, Zn, Cu in small intestine except for Fe. But still further investigations are required to understand the mechanism of mineral absorption by different fibres under the gastrointestinal conditions which could be useful for consumers (Ghodrat, Yaghobfar, Ebrahimnezhad, Aghdam Shahryar, & Ghorbani, 2017).

2.2. Various Useful Applications of Soybean Hull

Some of the various useful applications of soybean hull for both animal and humans are discussed as below:

2.2.1. Animal feed:

Soybean hulls are the by-products produced from soybean processing industries have become an alternative dietary source to corn grains in ruminants due to high cost of corn grains for their increasing demand in ethanol industry (Ferreira, Pires *et al.* 2011). Also, the soybean seeds when de-hulled leaves behind a more digestible, fibrous feed (Bittner, Erickson, Mader, & Johnson, 2013). Many studies have revealed that soybean hulls act as an advantageous energy source for ruminants in place of corns (Kabe *et al.*, 2016). According to some studies done, soybean hull has led to decrease in starch level without any fall in levels of caloric density of feed in the equine diet (Geor, 2007) whereas earlier in equine diet the

high level of starch used in their diets leads to amylolytic bacterial growth in large intestine due to fermentation of the material ingested. This fermentation results in production of high level of lactic acid and short chain fatty acids that can cause intestinal disorders like laminitis (De Fombelle, Julliand, Drogoul, & Jacotot, 2001). It has been shown that equine diet containing 28percent of soybean hull has no effect on digestibility (Kabe *et al.*, 2016). Soybean hull can also be used as dietary source to maintain sheep meat production, improve lactation period in goats and increases storage time of chicken nuggets (Módenes, Espinoza-Quiñones, Colombo, Geraldi, & Trigueros, 2015).

2.2.2. For waste water treatment:

Fresh water present as a natural resource for sustainability of aquatic as well as for human growth is becoming a limited source in today's era. Due to the growing population of world with high increase in the industrial development which requires a huge amount of water consumption and then releasing the waste polluted water back into the water bodies has led to a great effect on decreasing the quality of available fresh water (Módenes *et al.*, 2015). The various methods that are currently used to treat the polluted waste water are chemical precipitation, oxidation and chemical reduction, filtration, electrochemical treatment, ion exchange, reverse osmosis, evaporation, and adsorption (Bueno, Torem *et al.* 2011); (Fu & Wang, 2011). The most efficient and economically important technique amongst all of these is adsorption which is very simple operating and flexible and uses regenerative adsorbents for highly efficient removal of both organic and inorganic pollutants (Fu and Wang 2011).

Now-a-days bio-sorption technique has gained popularity as a bio-sorption technique. Bio-sorption involves binding of radionuclides and different metal ions containing functional groups and ligands on the cellular structure of biological material. This kind of biological material used for bio-sorption is called bio-sorbents. Different materials used as bio-sorbents are lignocellulosic i.e. materials containing cellulose, hemicellulose, lignin and highly adsorptive property for ion exchange capabilities (Krishnani, Meng, Christodoulatos, & Boddu, 2008). The major advantage of using bio-sorbents is that they can be recovered for reuse after being regenerated, there is no great input energy required and sludge that is produced is non-toxic to environment [(Rizzuti, Ellis, & Cosme, 2015); (Volesky 2007)]. One of the novel bio-sorbent used as a good metal binder is soybean hull without Soluble dietary fibre (SDF) (Jia, Enzan, Haijia, & Tianwei, 2011). The preparation of soybean hull involves steps such as pre-treatment,

drying, modifications, activation and many more to make this process economically feasible [(Módenes, Espinoza-Quiñones *et al.* 2015);(Marshall & Wartelle, 2003); (Marshall, Wartelle, Boler, Johns, & Toles, 1999)]. For removal of Cd²⁺ and Pb²⁺ various modification have been done which showed up to 20 per cent more absorption of Pb²⁺ than compared to unmodified hull. But there was rarely increase in the absorption capacity of Cd²⁺ ions when the hull was modified after treatment of hull with strong base (0.1M – 1.0M NaOH) (Módenes *et al.*, 2015). Studies revealed that soybean hull efficiently removed dyes in textile industry from waste water sources. Also, Safranin T and Direct Violet 51 dyes can be removed from the solution using soybean as well as rice hull. The removal of Remazol Brilliant Blue R dye was done brilliantly by soybean hull than the rice hulls (Rizzuti & Lancaster, 2013).

2.2.3. As source of Dietary fibre:

Dietary fibre has taken an important place in our diet plans due to its beneficial role in human health and nutrition. It is important in normal gastrointestinal and physiological functions such as carbohydrate and lipid metabolism, reduction in coronary heart diseases, diabetes, obesity, and some cancers (Mann & Cummings, 2009).

Dietary fibre can be defined as “the part of plant which is edible or similar carbohydrates that are completely or partially fermented in large intestine but resistant to digestion and absorption in the human small intestine” (Wanders *et al.*, 2011). Dietary fibre is a natural complex carbohydrate polymer consisting of variety of non-starch polysaccharides such as hemicellulose, cellulose, lignin and pectin (Abirami, Nagarani, & Siddhuraju, 2014). Dietary fibre is of two types: i.e. soluble dietary fibre and insoluble dietary fibre (IDF). SDF mainly contains pectins and some hemicelluloses whereas Cellulose, lignin, and some hemicelluloses are all examples of dietary fibre classified as IDF. The majority of fibres present in soybean hull were of IDF in high level. Soybean when subjected to acid-base hydrolysis and autoclaving show significant effect on SDF, IDF and total DF distribution.

According to a report by (V. Kumar, Kumar Biswas, Kumar Chatli, & Sahoo, 2011) and (Goldmon & Brown, 1992) addition of 4 per cent of soy hull flours showed no impact on the cooking yield and texture of chicken nuggets and pork patties, respectively. According to a study done by (V. Kumar, Biswas, Sahoo, Chatli, & Sivakumar, 2013) the emulsion stability and water-holding capacity of chicken nuggets can be improved by addition of 3-5 per cent of soybean hull flour. (Kim, Lee, & Kim,

2015) showed the positive effects on reduction in cooking loss and high level of hardness in meat without effecting colour alteration, cohesiveness and springiness. Some studies revealed that that the total dietary fibre content of soybean hull can be increased by acid-base hydrolysis and autoclaving process showing its great influence in various food applications due to the functional properties (Yang *et al.*, 2014). In a study by Marshal *et al.* in 1999 reported that the cell wall of the soy hull can be a source of pectin that was extracted using 0.05N hydrochloric acid and then precipitated with alcohol.

2.2.4. Soy hull as Medicine:

Different varieties of soybean such as black, yellow, brown and green pods have anti-oxidant capacity which differ in colour due to different phenolic levels and difference in anthocyanins, phenolic groups (chlorogenic and caffeic acids), isoflavones, and pro-anthocyanidins composition [(Slavin, Kenworthy, & Yu, 2009); (Xu & Chang, 2008); (Todd & Vodkin, 1993)]. In most of the cases black soybean has been used as an herbal medicine to treat oedema and jaundice. Also enuresis has been treated by soybean while affecting the spleen and kidney functions. Headaches, vertigo, detoxification and diuresis has been treated using hull of black soybean (Fukuda *et al.*, 2011). An investigation revealed that anthocyanins were present in only black soybean whereas proanthocyanins were found in both brown and black soybean (Xu & Chang, 2008); (Todd & Vodkin, 1993)]. Black soybean showed highest amount of anti-oxidant activity as compared to other colored soybeans. This anti-oxidant activity was due to the phenolic pigments present in the seed coats. The polysaccharide from black soybean induced differentiation and inhibits proliferation in human leukemic U937 cells in vitro anti-cancer reports. Anthocyanins that were obtained by isolation from black soybean hulls shows inhibitory growth effects and induction of strong apoptosis effect against human leukaemia Molt 4B cells. The intake of extracts from black soybean hulls significantly increases memory and learning ability in rats. The pigments and nutraceuticals that were used as dietary ingredients had been obtained from the black soybean extract. (H.-M. Liu & Li, 2017)

2.3. Various Products Obtained After Conversion of Soybean Hull

Some of the various useful products that were obtained from the soybean hull after being processed are discussed as below:

2.3.1. Ethanol:

According to *The National Biofuels Action Plan, October 2008*, it states that the annual biofuels production would increase to 36 billion gallons by 2022 would be a key component in America's movement toward clean, affordable, and secure energy sources. Basically ethanol production takes place from sugarcane and corn (in Brazil and USA respectively) contributing to about 66percent of world's total ethanol production. During the past decade, the interest for ethanol production from renewable resources such as cellulosic biomass has increased because of abundant biomass feedstock which is inexpensive and made of high cellulosic content. In order to release fermentable sugars, lignocellulosic biomass needs to be degraded to its monomers either by dilute acid hydrolysis or by using enzymes. Before breaking down the rigidity of the biomass, pre-treatment to biomass prior to enzymatic treatment is a necessary step. Due to low lignin proportion (1.4-2 per cent) of the soybean hull as compared to other biomass, soybean hulls acts as a valuable source of fermentable sugars for ethanol production from cellulosic mass. Thus, pre-treatment by various technologies is a crucial prerequisite to break down the rigidity of the biomass prior to enzyme hydrolysis process. Soybean hulls are an agricultural residue produced during the processing of soybeans, and the lignocellulosic material contains a small proportion of lignin (1.4–2 per cent) when compared to other biomass. Therefore, soybean hulls are an attractive source of fermentable sugars for cellulosic ethanol. The hydrolysis of soybean hull was a two-step process that involves sulphuric acid-enzymatic pre-treatment that resulted in about 72 per cent saccharification. There was 38-47 per cent yield of bioconversion of soybean hull into ethanol.

Rojas *et al.*, (2014) discovered the recovery of proteins from soybean hulls, mainly as oligopeptides, and the production of ethanol from the remaining lignocellulosic fraction. Also Zhang and Hu (2012) found a new application of soybean hulls. They converted soybean hull into fungal lipids for the production of biodiesel through solid-state fermentation. The total final lipid achieved from a 1g soybean hull after the conversion was about 47.9 mg, which was 3.3 fold times higher as compared to the initial lipid reserve in the hulls. Because of low-energy expenditure, its low capital cost, less expensive downstream processing, high volumetric productivity, low wastewater output, and less fermentation space needed,

the solid-state fermentation process proves to be more cost-effective process(Hölker, Höfer, & Lenz, 2004).

2.3.2. Bio-fuels:

A complex liquid mixture of water and organic chemicals such as alcohols, aldehydes, acids, ketones, esters, heterocyclic derivatives, and phenolic compounds is called as bio-oil (Yaman, 2004). There are typically two thermochemical processes to produce high yield of bio-oil are pyrolysis and liquefaction. In pyrolysis process, lignocellulosic biomass is heated at a quiet high temperature of about (400-1000°C) leading to formation of bio-oil and gaseous products. Biomass conversion to liquid fuels in solvents such as acetone, water, ethanol, or their mixtures by liquefaction. The analyses of different components of bio-fuels were done by GC/MS. The results indicated that bio-fuels formation from soybean hulls by pyrolysis was fast (Oliveria *et al.*, 2015). Also, studies revealed that soybean hull bio-fuel highly added value to the sources of chemical products.

2.3.3. Polysaccharides:

These are species of macromolecules substances that occur widely in living organisms. Plant polysaccharides and even their derivatives have strong antioxidant activities that can lead to discovery novel potential antioxidants. Mostly these polysaccharides are targets for exploring effective and non-toxic medicines against strong free scavenging and antioxidant activities. Soybean hull insoluble carbohydrate fraction mainly comprises of 50percent hemicelluloses, 30 per cent pectin, 20 per cent celluloses (H. Liu, Guo, Li, Zhu, & Li, 2013). Therefore, soybean hull can be a potent commercial source of polysaccharides. According to Liu.*et.al* (2016) extraction of polysaccharides from soybean hull can be done by hot compressed water in a batch system. Nagata *et. al.*, (2001) showed the effects of polysaccharides from soybean hull on serum immunoglobulin concentration and Nitric oxide (NO) production and interleukin 1 β from peritoneal macrophages. Soybean hull polysaccharides with molecular weight of 5,00,000 were reported to comprised of arabinose, galactose, xylose, glucose and rhamnose. Soybean hull polysaccharides increased humoral immunity, activated macrophages which lead to augmentation of immune responses in rats.

2.3.4. Microfibrils:

In early 1980's, Turbak and co-authors produced micro fibrillated cellulose for first time through mechanical treatments such as refining and high pressure homogenization. The bio-based micro-fibrillated cellulose has intrinsic properties which has many applications. According to some reports, microfibrillar cellulose from soybean hull was short, rod-shaped fibres having 83.79 per cent of cellulose content and 70 per cent crystallinity index (Merci, Urbano, Grossmann, Tischer, & Mali, 2015).

2.3.5. Peroxidases:

The enzymes obtained from agro-industrial residues can be an important alternate to reduce cost in bio-catalysis production. Soybean hull Peroxidase (SHP, E.C.1.11.1.7) is a glycoprotein belonging to plant peroxidase superfamily that includes HRP, peanut and barely peroxidises (Kamal & Behere, 2002). As compared to HRP, SHP observed to be a promising biocatalyst for industries due to its high thermostability, broad pH stability, and cheap production from soybean hull. Previously, SHP was used for removal of aqueous phenols from waste water in stirred membrane reactor, a bromination catalyst, for luminal oxidation, synthesis of polyaniline. In comparison to free enzymes, immobilized enzymes have more advantages of increased stability against several denaturing conditions, easier products, enzyme recovery, and higher catalytic activity. Charas *et.al.*,(2015)obtained peroxidase enzyme from soybean hull and immobilized the enzyme on cross linked beads of chitosan with glutaraldehyde.

2.3.6. Oligopeptides:

Soybean oligopeptides which are widely used in food industry are produced using techniques such as proteolysis or microbial fermentation followed by purification. Soybean hull comprised of 11-15 per cent proteins which can be transferred into the oligopeptides using various techniques. Mostly batch hydrolysis depending on several different factors such as protein denaturation, hydrolysis temperature and protease activity are used for commercial production of oligopeptides. The protein hydrolysate is a complex mixture of various different lengths of peptides. Rojas *et.al.*, (2014) found that the proteins can be recovered by hydrolysis from soybean hull indicating that soybean hull is a promising feedstock for

production of high value protein hydrolysate composed mainly of low molecular weight.

As the literature reviewed it can be pondered that soy hull has amenable uses in various industries. This study has been deliberately executed on the above cited review of literature to convert the soy hull to consumable form for the human consumption.

OBJECTIVES

1. Comparing the surface characteristics degradation using chemical versus enzymes (Cellulase and pectinase).
2. Samples obtained from the treatments as a substrate for the growth of various useful micro-organisms.

CHAPTER-3

MATERIALS AND METHODS

3.1. Pre-Requisites Required for the Study

3.1.1. Obtaining the hull from the soybean seeds:

The soy hull or seed coat of soybean was obtained by pre-soaking (overnight) method. Soybeans were measured according to the amount required for the experiments. Soak the whole soybeans in triple the amount of fresh water overnight for about 10-12 hours. Make sure to add enough water and use a large enough pot for soaking as the beans can become triple to its original size. When properly soaked, hulls will start to float on surface of water or hands can be used to dehull the soybean. Repeat the whole process until majority of the beans are split and hulls are removed.

3.1.2. Pectinase enzyme:

Pectinase enzyme was used to treat the soy hulls so as to check the hydrolysis in hull. The pectinase used was of Hi-Media brand and has enzyme activity of about 8000-12000U/g. It was provided by the Department of biotechnology, TU, Patiala.

3.1.3. Cellulase enzyme:

Cellulase was used to hydrolyse the hull to check if there is any change in surface characteristics or any change taken place in the structure of hull. Cellulose used was of Hi-Media brand. It was made available by the Department of Biotechnology, TU, Patiala.

3.1.4. Sodium hydroxide:

It was used to break the bonding between cellulose and hemicelluloses fibres in the soy hull and make it free from lignin or other components.

3.1.5. Water bath:

The Serological water bath was used at 50°C to treat the hull with both pectinase and cellulase to activate the enzymatic activity.

3.1.6. Autoclave:

It was used to treat the hull at high temperature and high pressure of 121°C and 15 lb respectively.

3.1.7. Whatman filter paper:

Whatman filter paper was used to dry the treated hull samples. So that extra moisture can be removed from the hull.

3.1.8. Refrigerators and deep freezers:

The LG refrigerator was used to store the samples so as to protect them from degradation by bacteria.

3.1.9. Spectrophotometer:

Hitachi spectrophotometer was used to measure the UV-Visible wavelength absorbance of different samples. The amount of glucose released from different treated samples was measured with the help of spectrophotometer.

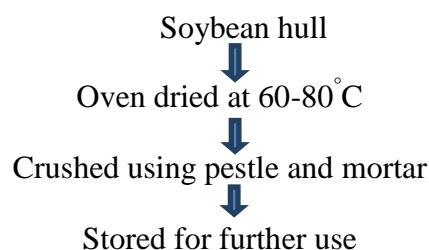
3.1.10. SEM (Scanning Electron microscope):

A typical Scanning Electron Microscope (SEM) uses a focused beam of high energy electrons to generate a variety of signals at the surface of the solid samples to produce high resolution images of that sample. The SEM analysis of the sample was done from the SAI LAB, Thapar University, Patiala. The SEM was used to observe the structural changes occurring in the soy hull after enzymatic treatment.

3.2 Experimental Planning

3.2.1. Processing and treatment of soybean hull:

Firstly the hulls were removed from the soybean manually after overnight soaking of the beans. Then, the hulls were oven dried at 60 -80°C for about four to five hours. Precautions should be taken so as to prevent the charring of hulls. The hulls were immediately crushed into coarse particles when hot otherwise they cannot be crushed once cooled. Then the crushed hulls can be stored for further use in experiments.



Conversion of soybean hull into crushed form:

It has been seen that the effective media for the growth of *Lactobacillus casei* bacteria is MRS broth as it contains all the nutrients required for the growth of the bacteria. But during the research it has been seen that the bacteria was able to grow on the crushed treated samples of the soybean hull. The hull was removed from the soybean seed and dried properly to form a crushed sample. Then these crushed samples were taken in three different flasks and treated with 0.5 per cent of cellulase and pectinase enzymes respectively in two flasks for 4 to 5 hours in water bath at 50° C and in one flask given alkali treatment (NaOH treatment) by dipping the hull in NaOH to attain pH of 11.5 for 24 hours in deep refrigerator and then sprinkling the hull sample with 7 percent H₂O₂ for 1 hour and then autoclaving the sample for 20 minutes at 15 lb pressure. All the three different samples prepared were then stored in deep refrigerator to avoid contamination till further use. Then 1g of these different samples were taken as growth medium for *Lactobacillus casei*, *Saccharomyces cerevisiae* and *Aspergillus niger*.



Fig 1: Showing wet soybean hull



Fig 2: Hot air oven dried soybean hull.

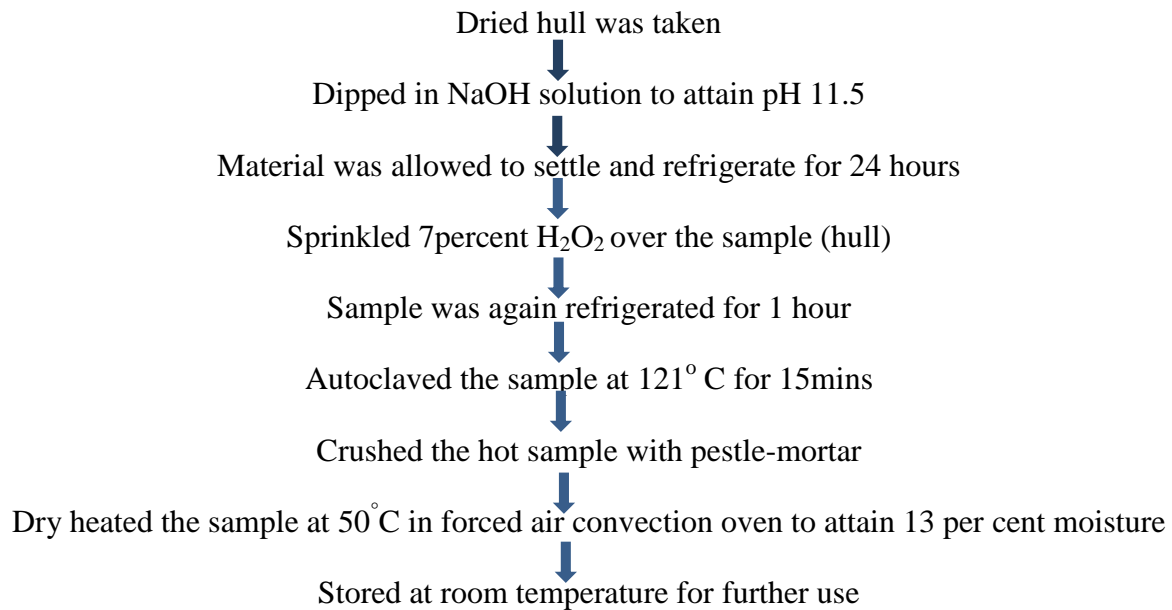


Fig 3: Dried crushed soybean hull.

3.2.2. Treatment process of hull by chemical and enzymatic methods:

3.2.2.1. Chemical method:

In this method sodium hydroxide (NaOH) and Hydrogen peroxide (H₂O₂) were used to chemically treat the hull as following:



3.2.2.2. Enzymatic treatment method:

3.2.2.2.1. Pectinase treatment:

Pectinase enzyme was taken, weighed and dissolved in approximately 7-10 ml distilled water for every 1-2 g of sample. Then this solution was added to hull. The hull was then kept at 50° C in water bath for enzyme activation for four hours. Then deactivate the enzyme by heating to 100° C followed by immediate cooling. Then freeze stored the sample for future use.

3.2.2.2.2. Cellulase treatment:

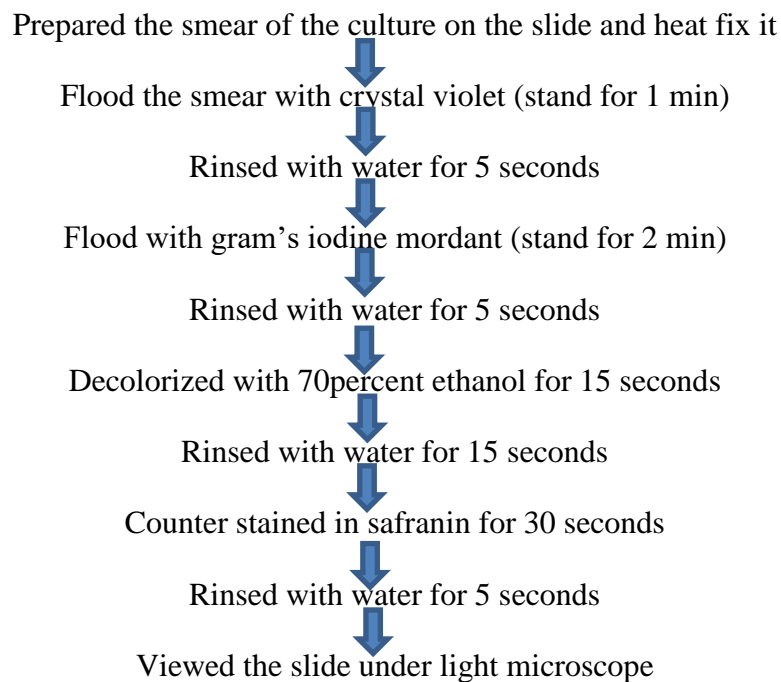
The cellulase enzyme was weighed as 0.5 percent according to weight of soybean hull taken and dissolved in distilled water about triple times the amount of soybean hull. Added this solution to the hull and kept it at 50° C for four hours in water bath to make the enzyme functional. After the enzyme treatment of the hull, the enzyme was made inactive by heating the enzyme treated hull up to 100° C in the water-bath for 15 minutes.

3.3 Isolation of the *Lactobacillus casei*

The *Lactobacillus casei* was isolated from the Yakult (liquid) a probiotic drink commercially available in Indian market. The Yakult solution was directly streaked on MRS selective media for selective growth of *Lactobacillus casei* (acetified with glacial acetic acid to pH 5.7). After that the plates were incubated at 37°C for 48 hours to check the selective growth of *Lactobacillus casei*. Selected colonies were used to produce a pure culture of *Lactobacillus casei* in MRS liquid broth by incubating at 37°C for 48 hours and used for further tests.

3.4 Gram Staining

Differential staining was done to confirm whether the bacteria etiology was in pure form or a mixture of components. Gram staining has been the best method to distinguish different types of bacteria. The gram stain is the most useful and widely employed differential stain in bacteriology. It divides bacteria into two main groups: Gram Positive and Gram Negative. The whole procedure is as follows:



3.5 Enumeration of *Lactobacillus casei* (both isolated from yakult and yakult) on the different experimental samples of soybean hull

A set of four test tubes were prepared for each treated sample of soybean hull (cellulose treated hull, pectinase treated hull and NaOH treated hull) along with the non-treated soybean hull. After sterilization and cooling, all the test tubes were inoculated with freshly prepared culture of *Lactobacillus casei* solution which has been isolated from the MRS plates inoculated

with yakult. To observe the growth of bacteria on the hull the samples were incubated at 37°C in incubator shaker and maintained at the pH of about 5.5. The growth was observed after every 24 hours using spectrophotometer at 760 nm wavelength. After that the growth curve was plotted to see the growth of *Lactobacillus casei* on the samples.

3.6 Enumeration of Yeast (*Saccharomyces cerevisiae*) on different experimental samples of soybean hull

A set of four test tubes were prepared for each treated sample of soybean hull (cellulase treated hull, pectinase treated hull and NaOH treated hull) along with the non-treated soybean hull. After sterilization and cooling, all the test tubes were inoculated with freshly prepared culture of yeast (1g in 100ml of distilled water). The test tubes were incubated at 37°C in incubator shaker. To observe the growth pattern of yeast on different samples of soybean hull the growth medium was maintained at the pH of about 4.5 to 5 and observed after every 24 hours in spectrophotometer at 600nm wavelength.

3.7 Enumeration of *Aspergillus niger* (fungal mould) on different treated samples of soybean hull

A set of four test tubes were prepared for each treated sample of soybean hull (cellulose treated hull, pectinase treated hull and NaOH treated hull) along with the non-treated soybean hull. After sterilization and cooling, all the test tubes were inoculated with *Aspergillus niger* (grown on the nutrient plates as a pure isolated form) and kept at 25°C under continuous shaking conditions with the pH ranging between 3.5 to 4.5. The growth of *Aspergillus niger* was observed by direct visibility of the black spores of the fungus on the soybean hull samples after 48 and 72 hours.

3.8 Chemical analysis of the soybean hull

The parametric tests done for the chemical analysis of the soybean hull was done as below:

3.8.1. Moisture content of soybean hulls:

The moisture content of soybean hulls was estimated by hot air oven drying method (according to AOAC, 2005). To check the moisture content in the hulls, hulls were isolated from the soybean. Pre-weighed and dried aluminium jars was placed on the weighing balance, noted the weight of the sample. Place the aluminium jar along with the sample in the hot oven at 70±

80°C to dry until the weight comes to be constant (usually 5 hours is sufficient). After the samples were dried, cool them to room temperature and again weighed the sample.

Calculation of Moisture Content on wet basis (percent):

$$\text{Moisture Percentage} = \frac{w_i - w_f}{w_i} \times 100$$

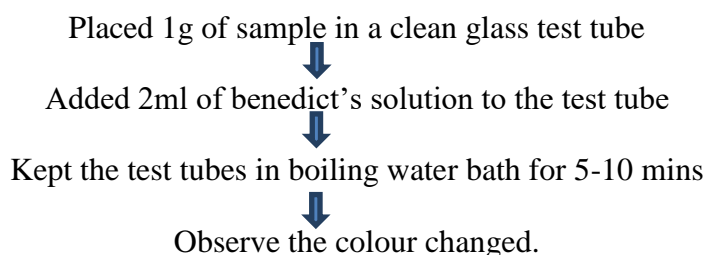
W_i = initial weight of the soybean hull sample without aluminium jar

W_f = final weight of the soybean hull sample without aluminium jar

3.8.2. Sugar Content of the treated soybean hull samples:

3.8.2.1: Benedict's Test:

Benedict's test is used to test the presence of total sugars present in the sample. It generally identifies the reducing sugar's (some monosaccharides and disaccharides) have free ketone or aldehyde group. It was done to check whether the enzyme was breaking the soy hull composition or substrate with the production of reducing sugars. The whole procedure is given below:



3.8.2.2. DNS Assay (3-amino, 5-Dinitrosalicylic acid):

DNS method was used to estimate the sugar content of food samples in food industries where quality control is very important. The procedure used to estimate the sugar level in the soybean hull sample is as follows:

3.8.2.2.1. Preparation of standard curve:

The standard glucose curve was prepared from the standard glucose solution (100mg/100ml) by taking 0,0.2,0.3, 0.4, 0.5, 0.6, 0.7,0.8, 0.9,1.0 ml of working solution and the volume was made up to 1ml using distilled water. To this 3ml of DNS reagent was added and the mixture was heated for 5-10 minutes in a boiling water bath. After the colour was developed, absorbance was recorded at a wavelength of 540nm in the spectrophotometer.

3.8.2.2.2. Preparation of sample:

Taken 0.4 g of sample and mixed with 0.6 ml of distilled water to make 1ml of solution. Then 3ml of DNS reagent was added to it and heated in a boiling water bath at 100°C for 5 to 10 min. Then, the absorbance was recorded at 540 nm using spectrophotometer.

3.9 SEM Analysis:

SEM analysis of the soybean hull was done to check if any change occurs to the anatomical structure of the soybean.

CHAPTER IV

RESULTS AND DISCUSSION

To reveal the results from chemical and enzymatic treatment on soy hull was the main component of this study. An attempt has been done to observe the change in surface characteristics of soybean hull after being treated by both chemical and enzyme assisted methods. During the processing of soybean in food industries for various purposes, the soybean hull was left as a waste material but here a trial has been attempted to use this hull for the growth of *Lactobacillus casei* bacteria as a substitutes for gut colonizer for human use. The growth of *Saccharomyces cerevisiae* and fungus *Aspergillus niger* was observed to analyse whether it can be used for agricultural purpose. The results of selected experiments have been discussed with tables, growth curves under the following headings and sub-headings.

4.1. Chemical Analysis of the Raw Material

4.1.1. Physical characteristics of the raw material:

The soybean hulls were light tan to golden in colour and have nut like smell. The soybean hulls were oven dried, crushed and stored in refrigerator. The colour of the hulls changed to slightly brown when treated with pectinase, cellulase or NaOH. All these samples were carefully stored in frozen conditions to save from contamination till use for further application.

4.1.2. Moisture content analysis:

Initially the soybean seeds were dipped in water to remove the hulls. The hulls obtained were then oven dried till they become dried and can be easily crushed. The moisture content was checked and recorded to calculate the amount of water required for addition to hulls for their optimum activity when treated with the enzymes. The moisture content of the hulls tends to increase as with the drying of the hull. Their size was reduced.

Table1: Moisture content of the soy hull

SAMPLE	MOISTURE CONTENT (%) (after 5 hrs drying in hot air oven at 70°C to 80°C)
SOY HULL	66.81
	74.23
	69.64
	70.45
Average value	70.28

On an average the moisture content of the hull came out to be 70.2 percent.

4.1.3. Gram staining

The slides of lactobacillus when viewed under the microscope show the bacteria as gram positive as it takes up the staining, rod shaped, non-sporing and non-motile bacteria. The bacterium seen under microscope was like shown as below:

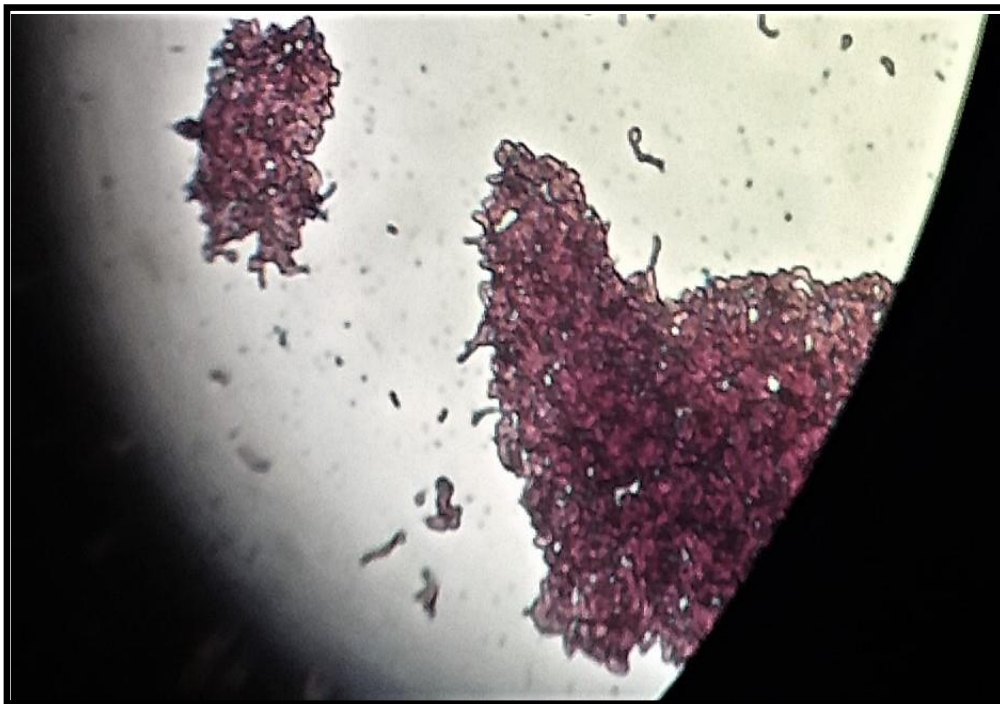


Fig4: Showing the *Lactobacillus casei* bacteria under microscope isolated from the yakult

4.1.4. Soybean hull treatment:

The hull was removed from the soybean seed and dried properly whole as a sample. Then, these crushed were taken in three different flasks and treated with 0.5 per cent of cellulase and pectinase enzymes respectively in two flasks for 4 to 5 hours in water bath at 50°C and in one

flask given alkali treatment (NaOH treatment) by dipping the hull in NaOH to attain pH of 11.5 for 24 hours in deep refrigerator and then sprinkling the hull sample with 7 per cent H₂O₂ for 1 hour and then autoclaving the sample for 20 minutes at 15 lb pressure. All the three different samples prepared were then stored in deep refrigerator to avoid contamination till further use

4.1.5. Sugar content of the hulls:

The sugar content of the hulls was recorded to calculate the level of enzymes required to degrade the hull surface to make it useful for human purposes as earlier it was seen that due to more lignocellulosic content it was not utilized by humans. So the following results had been recorded using:

4.1.5.1. Benedict's Test:

Observation as change in the colour of the solution recorded that predicted that whether the sugar released was appropriate or negotiable. In the hulls treated with enzymes the colour that was recorded was brick – red indicating an appropriate amount of sugar was present in the samples indicating the pectinase enzyme is degrading to some extent the pectin content in the soybean hull.

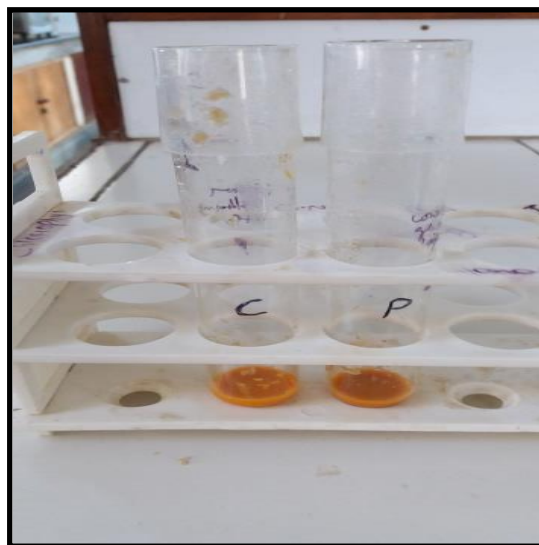


Fig 5: Depicting the colour change in the cellulase and pectinase treated hull by benedict's test.

4.1.5.2. DNS Assay:

The sugar content of all the treated samples was assessed with the help of DNS method. The concentration of the sugars in the samples was calculated from the graph. The maximum of sugars were present in the sample treated with pectinase enzyme (8.39 mg/dl) than the other methods.

Table 2: Absorbance and concentration of sugars released by hull samples by DNS method

Sr.No.	SAMPLES	Incubated at 5-10min in boiling water bath	ABSORBANCE (540nm)	CONCENTRATION (mg/dl)
1	Cellulase treated		3.1595	8.27
2	Pectinase treated		3.177	8.39
3	NaOH treated		1.373	3.68



Fig 6: Intensity of colour of sugars released by the soy hulls.

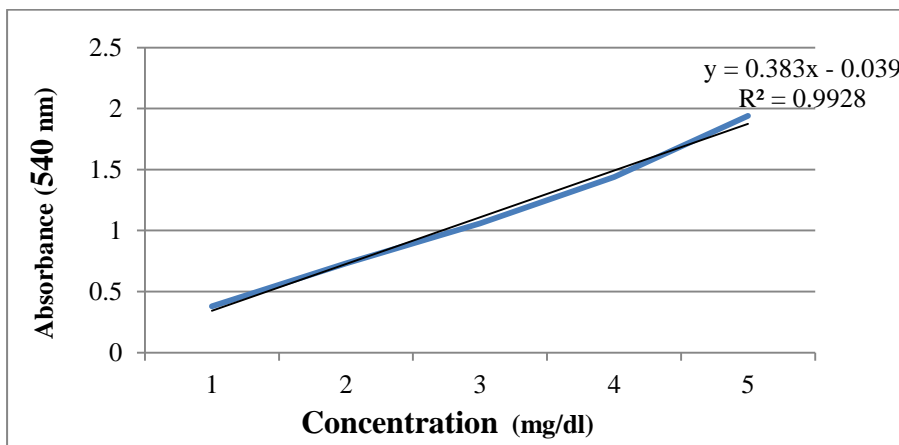


Fig 7: Absorbance of different treated soy hulls at 540 nm by DNS method.

Comparing the results observed in treatment of soy hull as depicted in table 1. It has been observed that NaOH (pH-11.5) treated soy hull for 24 hours used as sample even on vigorous

heating in the water bath for 5 to 10 mins didn't not caused release of sugars from cellulose and hemicelluloses in the hull. Pectinase treated soy hull and cellulase treated soy hull has shown values of 8.39 mg/dl and 8.27 mg/dl respectively. The values in two enzyme assisted extraction was observed close with a slight variation while the NaOH treated soy hull observed with 3.68 mg/dl value for sugars that can be seen in the fig.1. Hence, from the graph it can be concluded that the enzyme assisted conversion method has been successful but need to be standardized on the basis of pH, incubation time and other factors.

4.1.6. SEM Analysis of the Hulls:

SEM analysis of the soybean hulls was done to observe the change in surface characteristics that occurred to anatomical structure of the hull indicating that enzymes used has successfully degraded the hull surface area, so that the lignocellulosic part of the hull is now degraded. The degradation due to enzymatic treatment has been best seen in the samples treated with the pectinase and cellulase enzymes than other soybean hull treated samples. The degradation was not much but a combination of enzymes can be explained.

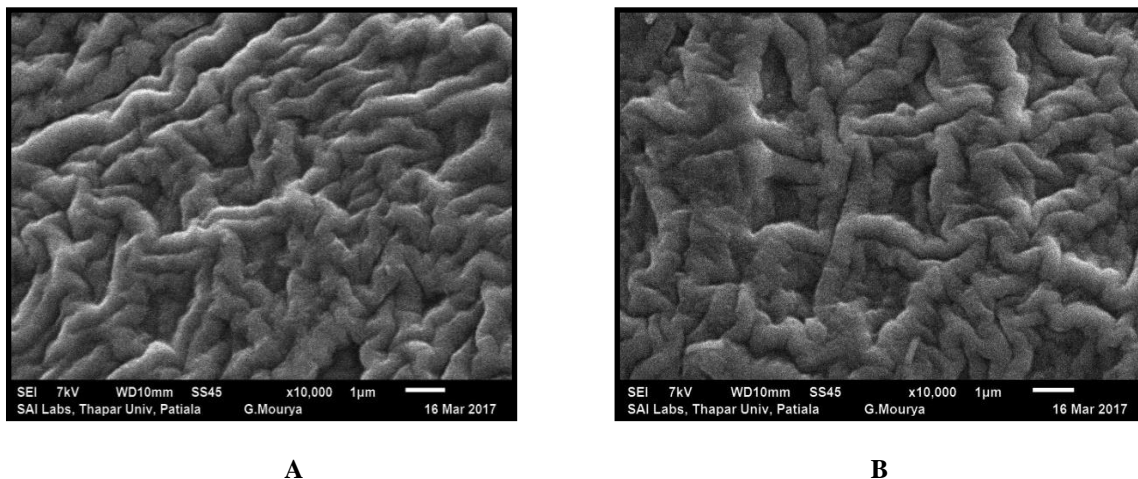


Fig 8: A. Pectinase treated hull SEM picture; B. Cellulase treated hull SEM picture

4.2. Growth of Micro-Organisms on the Treated Soy hull Samples

4.2.1. Isolation of *Lactobacillus casei* (Probiotic micro-organism):

The *Lactobacillus casei* was isolated from Yakult which is used as a probiotic drink by both adults and children. The procedure for isolation has been described in the materials and methods. The colonies of *Lactobacillus casei* were observed on the MRS plates. The selected colonies were further inoculated to MRS broth media for 24-48 hours for obtaining liquid culture of *Lactobacillus casei*. The growth observed has been shown in the figure given below:



Fig 9: Isolation of *Lactobacillus casei* on MRS plate

4.2.2. Utilization of Treated Soy hull Samples as Growth Medium

4.2.2.1 For *Lactobacillus casei*:

The *Lactobacillus casei* has shown its growth in the all the treated samples of soybean hull along with the non-treated crushed soybean hull. The optical density (OD) was measured at 760nm after every 24 hours and the observations were recorded up to 72 hours as shown the table. The growth of the bacteria seems to increase in all the samples after 48 hours of incubation. To compare the growth of isolated bacteria, all the samples were inoculated with the yakult directly and the growth of bacteria in yakult also seems to increase as shown in the following graphs (12-15). The growth of *Lactobacillus casei* in all the samples inoculated with MRS broth was nearly stable but the growth rate increases in all the samples inoculated with yakult as being observed according to the table 2.

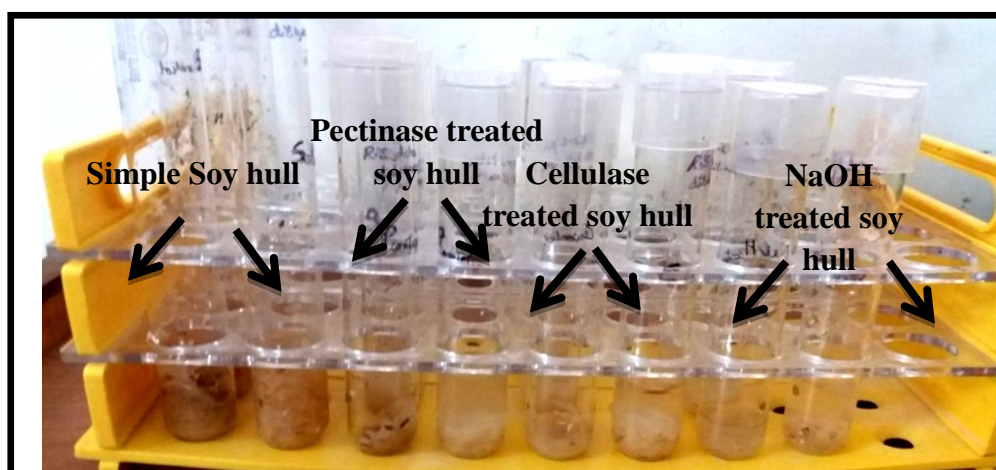


Fig 10: Showing growth of *Lactobacillus casei* in different treated hulls.

Table 3: Growth of *Lactobacillus casei* on the treated soybean hull samples.

Sr. No.	SAMPLES	<i>Lactobacillus casei</i> OD at 760 nm							
		0 hr		24 hr		48 hr		72 hr	
		Broth	Yakult	Broth	Yakult	Broth	Yakult	Broth	Yakult
1	Simple hull	0.211	1.274	0.167	1.653	0.577	2.017	0.462	1.689
2	Cellulase treated hull	0.346	0.602	0.080	1.159	0.465	1.724	0.680	1.217
3	Pectinase treated hull	0.332	1.439	0.084	1.358	0.664	1.406	0.254	1.111
4	Alkali treated hull	0.320	0.402	0.216	1.066	0.227	1.303	0.291	1.498

The results obtained for the growth of micro-organisms as shown in the table 2. It was observed that the simple hull, NaOH, cellulase and pectinase treated soy hull don't showed any growth in the MRS broth but have shown growth in yakult sample but quite ambiguous results as in yakult itself the lactose sugar was less but the sucrose and dextrose amounts were also less. That indicated that the probiotic drink can be substituted with fibre from enzyme treated soy hull. As the result in alkali treated were not changed even after 72hrs of research in broth while in yakult it has also shown reasonably accountable growth.

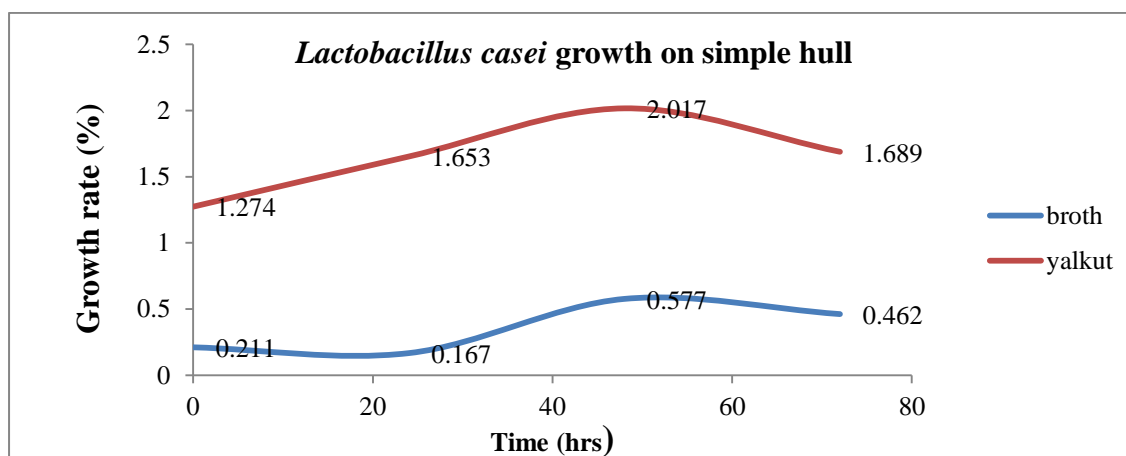


Fig 11: Growth of *Lactobacillus casei* (isolated and in yakult) on soy hull.

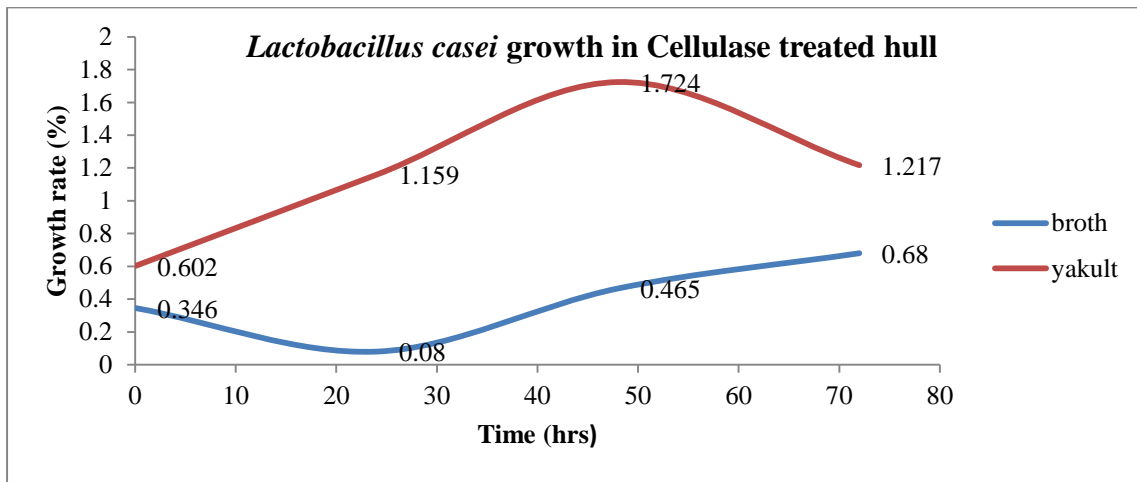


Fig 12: Growth of *Lactobacillus casei* (isolated and in yakult) on Cellulase treated soy hull.

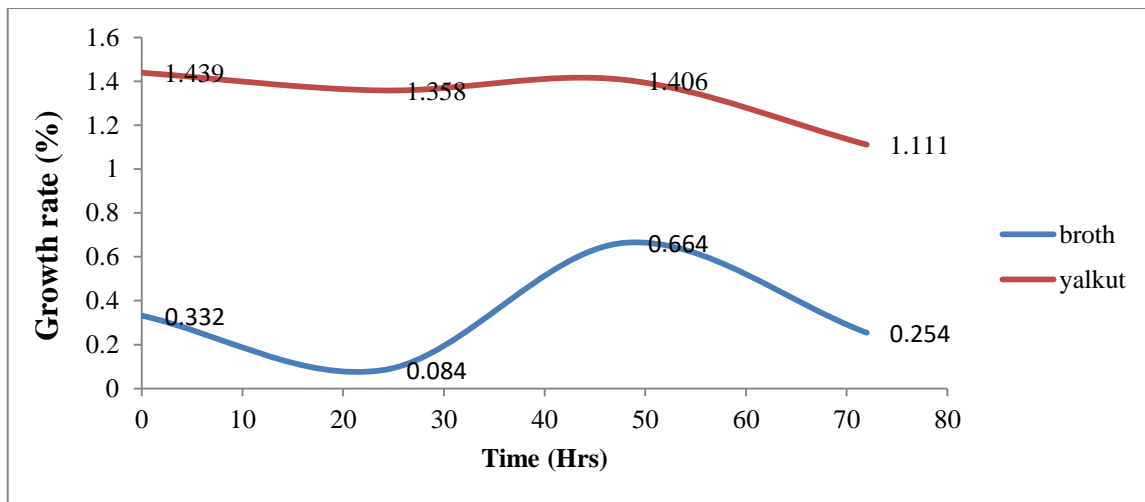


Fig 13: Growth of *Lactobacillus casei* (isolated and in yakult) on Pectinase treated soy hull.

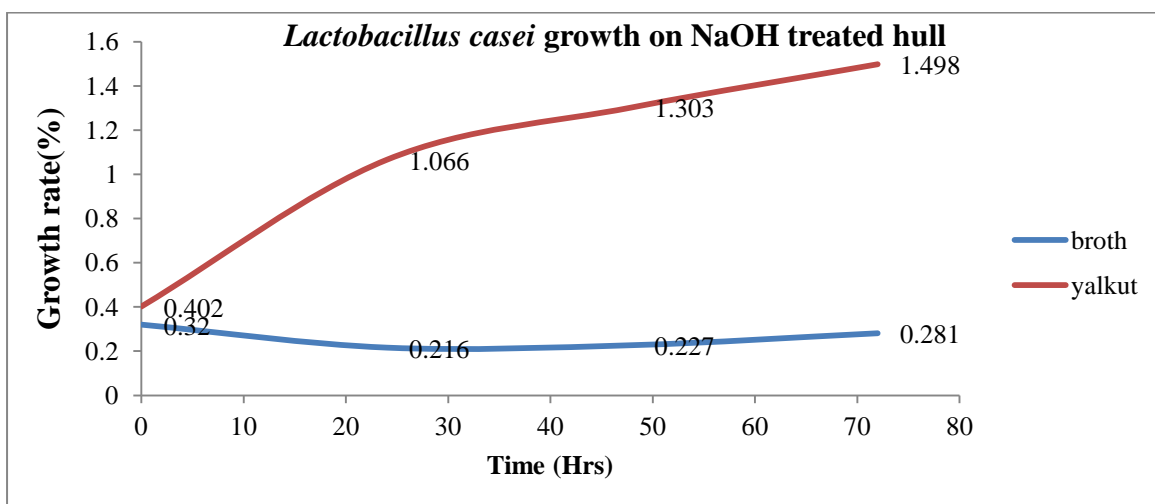


Fig14: Growth of *Lactobacillus casei* (isolated and in yakult) on NaOH treated soy hull.

4.2.2.2. For Yeast (*Saccharomyces cerevisiae*):

The growth of yeast was observed in all the different samples of soybean hull incubated at a temperature of 37° C with a maintained pH of about 5. The Optical Density of yeast was measured at 600 nm after every 24 hours up to 72 hours to observe the growth rate of the yeast in all the samples. The growth curve for yeast in different samples is shown in the table 4. The yeast (*Saccharomyces cerevisiae*) tends to increase in all the samples as shown in the figures.

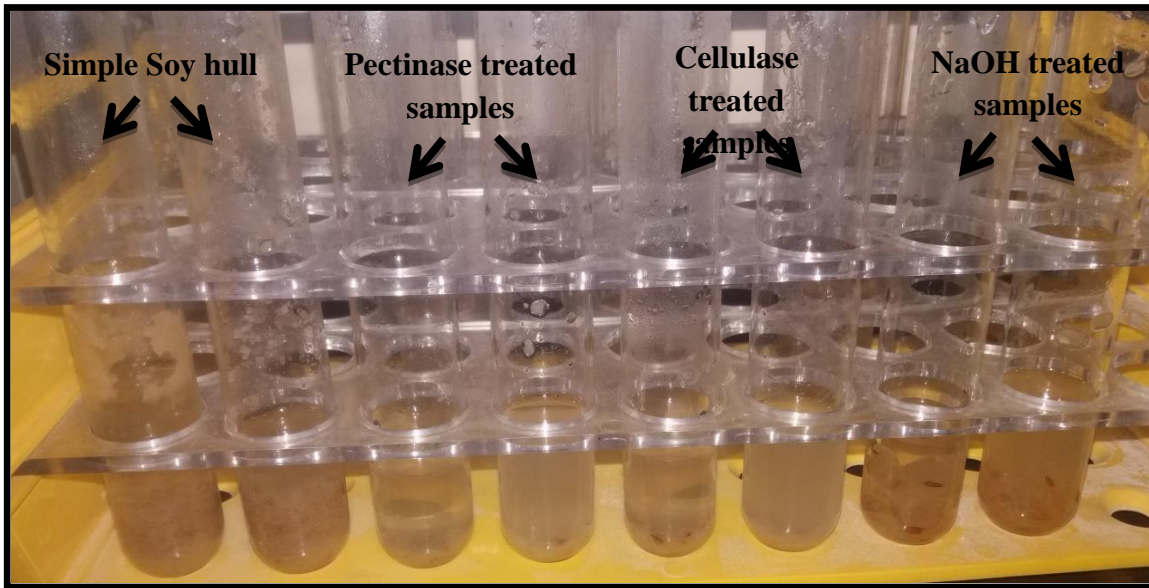


Fig 15: Depicting the growth of yeast on the treated hull samples.

Table 4: Growth of Yeast (*Saccharomyces cerevisiae*) on soy hull samples at O.D. 600 nm.

Sr. No.	SAMPLES	ABSORBANCE AT 600 nm			
		0 hr	24 hr	48 hr	72 hr
1	Simple hull	1.475	1.716	0.968	1.316
2	Cellulase treated hull	0.824	1.105	1.242	1.548
3	Pectinase treated hull	0.752	0.814	1.129	0.983
4	Alkali treated hull	0.581	0.526	0.517	0.748

The results obtained for the growth of *Saccharomyces cerevisiae* were as shown in the table 3. It was observed that the simple hull showed a limited growth but slow growth. In case of cellulase and pectinase treated soy hull showed substantial growth of yeast on them but still limited. At the end the alkali treated soy hull also showed negligible to slow growth. The reason

for slow growth may be because the starch converted to reducing sugars and available for its growth in the case of enzyme treated soy hull. While simple and untreated soy hull showed very slugging rate for the growth of yeast. Hence, it has been proved that enzyme causes degradation to the soy hull and made the possible for the growth of yeast which grow on free C.

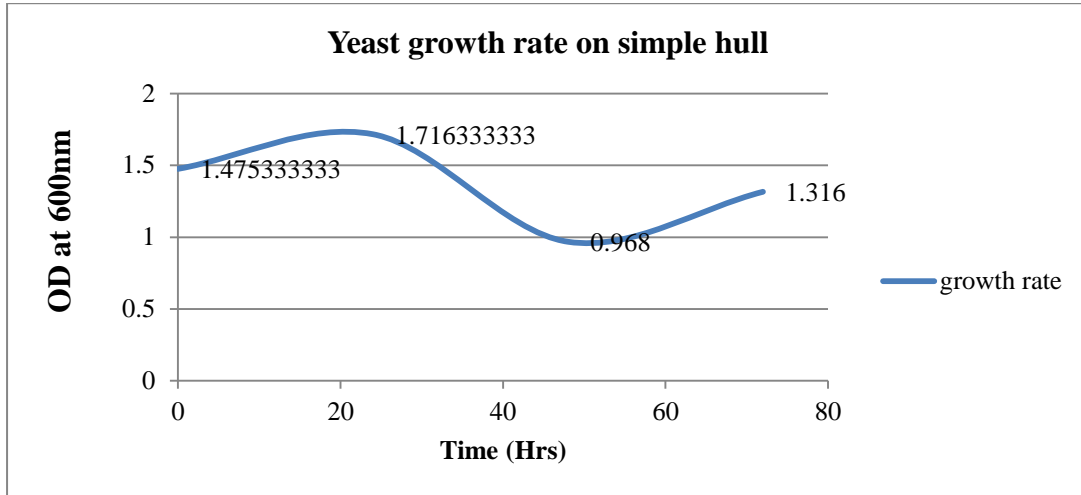


Fig 16: Growth of Yeast (*Saccharomyces cerevisiae*) on soy hull.

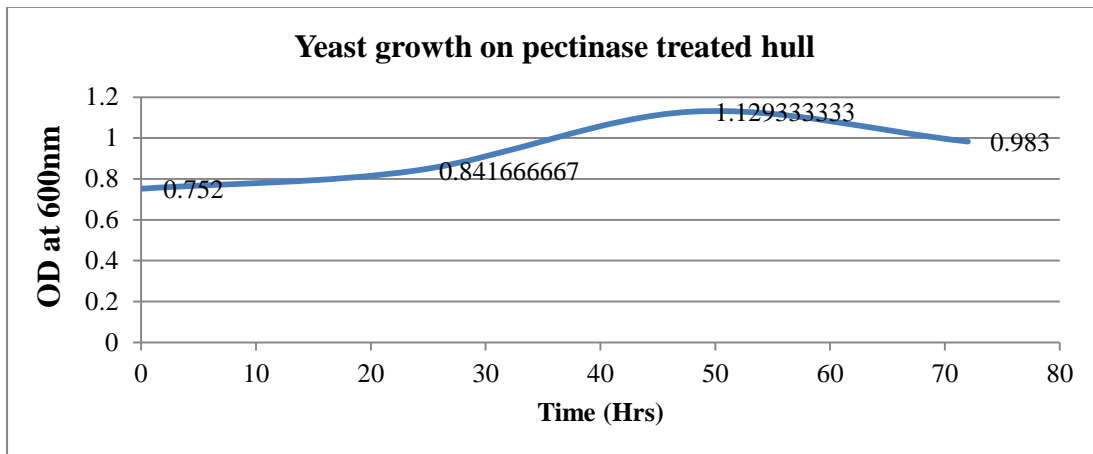


Fig 17: Growth of Yeast (*Saccharomyces cerevisiae*) on Pectinase treated soy hull.

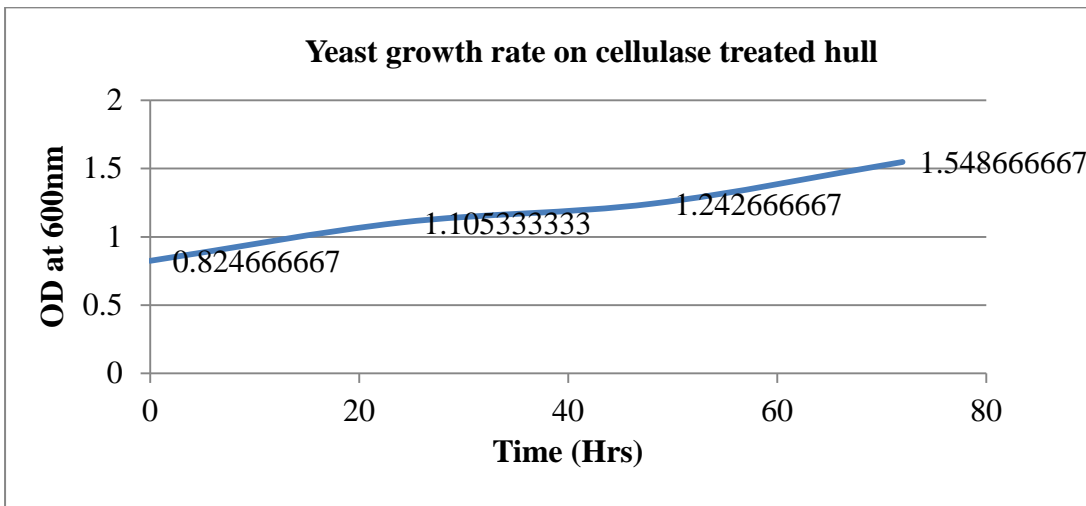


Fig 18: Growth of Yeast (*Saccharomyces cerevisiae*) on Cellulase treated soy hull.

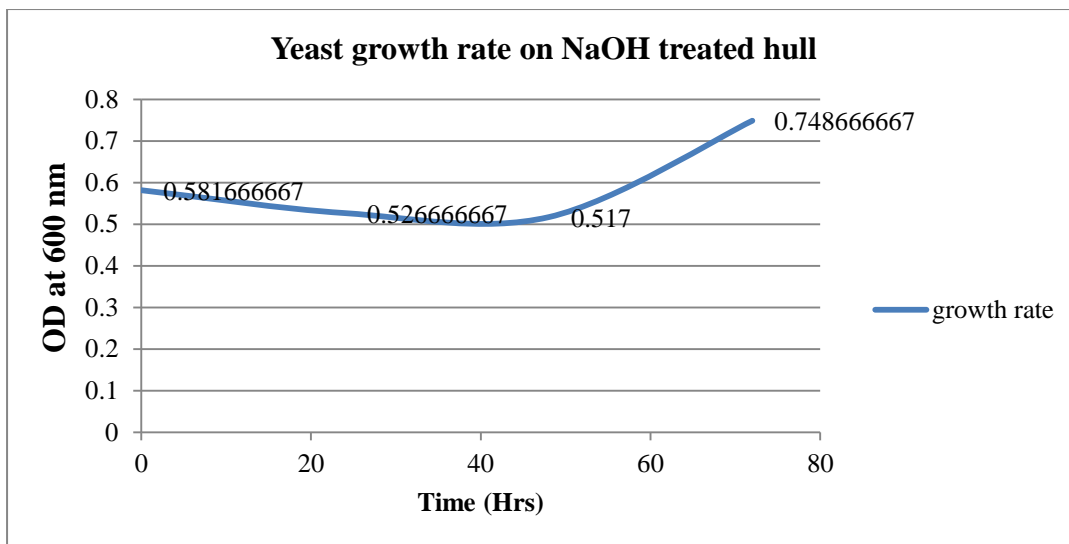


Fig 19: Growth of Yeast (*Saccharomyces cerevisiae*) on NaOH treated soy hull.

4.2.2.3. For *Aspergillus niger* (fungus):

The growth rate of *Aspergillus niger* on different treated hull samples was observed after every 24 hours up to 72 hours by maintain their ph. between 3.5 to 4.5. The growth of fungus was observed visibly as the fungal growth was quiet high in all the samples of hulls but it was more significant in simple hull than in cellulase and pectinase treated hulls the reason may be that the *A. niger* grow most commonly on fructose and sucrose were found to be suitable sources of carbon for a fungal isolates, whereas glucose and maltose proved good carbon source to have a higher affinity. Starch was a poor source of carbon for the growth as postulated by O. HAMAD *et al.*, 2014. But was less in NaOH treated hull.

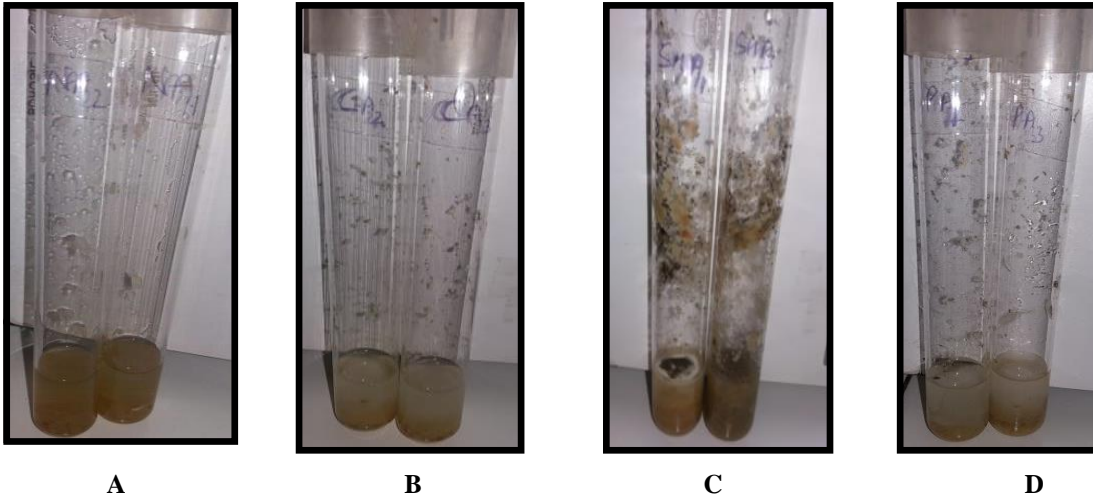


Fig20: A: Growth of *Aspergillus niger* on NaOH treated soy hull.

B: Growth of *Aspergillus niger* on Cellulase treated soy hull.

C: Growth of *Aspergillus niger* on soy hull.

D: Growth of *Aspergillus niger* on Pectinase treated hull.

The growth results of *Aspergillus niger* on the soy hulls were as shown in figure20. When observing the results it was found that untreated soy hull was recorded with *Aspergillus niger* growth followed by pectinase treated soyhull and near about to nil found in cellulase treated soyhull. Hence it was found that soyhull can be originally found best for the growth of *Aspergillus niger* and once it was treated with NaOH and enzymes its growth was reduced drastically that means it can be preserved once it treated and as such can be further utilized as dietary fibre substitution.

CHAPTER-5

CONCLUSION

The investigation was carried out to study the process of enzymatic as well as chemical treatment to the soybean hull.

It was concluded that enzymatic treatments given to the hull are more effective in degrading the lignocellulosic part of the hull rather than chemical treatment. It was seen that the pectinase enzyme was most efficient in degrading the non-digestive lignocellulosic part of the hull.

The soybean hulls were used for the growth of *Lactobacillus casei* so that if it can be used in humans for colonising agent of gut microflora. Interesting observations were seen that the bacteria in the yakult also show similar pattern of growth.

Also, *Saccharomyces cerevisiae* (yeast) was grown on the different samples of soybean hull. And it was seen that the growth was observed in all the samples but it was best seen in pectinase treated hull and more or less in cellulase treated hull but it was least in chemical treated hull.

Also growth of *Aspergillus niger* was best observed in the simple hull, were not in pectinase as well as cellulase treated hull may be because of absence of free C.

Thus, from all these investigations done it can be concluded that the soybean hull which is a major waste product produced from the soybean processing unit can be used in pharmaceutical and food industries for making useful products for human use.

REFERENCES

1. Abirami, A., Nagarani, G., & Siddhuraju, P. (2014). Measurement of functional properties and health promoting aspects-glucose retardation index of peel, pulp and peel fiber from *Citrus hystrix* and *Citrus maxima*. *Bioactive Carbohydrates and Dietary Fibre*, 4(1), 16-26.
2. Alpeeva, I. S., & Sakharov, I. Y. (2005). Soybean peroxidase-catalyzed oxidation of luminol by hydrogen peroxide. *Journal of agricultural and food chemistry*, 53(14), 5784-5788.
3. Ashraf, H., & Husain, Q. (2010). Stabilization of DEAE cellulose adsorbed and glutaraldehyde crosslinked white radish (*Raphanus sativus*) peroxidase.
4. Bittner, C. J., Erickson, G. E., Mader, T. L., & Johnson, L. J. (2013). Utilization of soybean hulls when fed in combination with mdgs in finishing diets.
5. Bueno, B. Y. M., Torem, M. L., De Carvalho, R. J., Pino, G. A. H., & De Mesquita, L. M. S. (2011). Fundamental aspects of biosorption of lead (II) ions onto a *Rhodococcus opacus* strain for environmental applications. *Minerals Engineering*, 24(14), 1619-1624.
6. Chagas, P. M. B., Torres, J. A., Silva, M. C., & Corrêa, A. D. (2015). Immobilized soybean hull peroxidase for the oxidation of phenolic compounds in coffee processing wastewater. *International journal of biological macromolecules*, 81, 568-575.
7. Choct, M., Dersjant-Li, Y., McLeish, J., & Peisker, M. (2010). Soy oligosaccharides and soluble non-starch polysaccharides: a review of digestion, nutritive and anti-nutritive effects in pigs and poultry. *Asian-Australasian Journal of Animal Sciences*, 23(10), 1386-1398. Boyles, S. BEEF COW NUTRITION.
8. Cruz-Silva, R., Romero-García, J., Angulo-Sánchez, J. L., Ledezma-Pérez, A., Arias-Marín, E., Moggio, I., & Flores-Loyola, E. (2005). Template-free enzymatic synthesis of electrically conducting polyaniline using soybean peroxidase. *European polymer journal*, 41(5), 1129-1135.
9. De Fombelle, A., et al. (2001). "Feeding and microbial disorders in horses: 1-Effects of an abrupt incorporation of two levels of barley in a hay diet on microbial profile and activities." *Journal of Equine Veterinary Science* 21(9): 439-445.
10. Erickson, D. R. (Ed.). (2015). *Practical handbook of soybean processing and utilization*. Elsevier.
11. Ferreira, E. M., Pires, A. V., Susin, I., Mendes, C. Q., Gentil, R. S., Araujo, R. C., ... & Loerch, S. C. (2011). Growth, feed intake, carcass characteristics, and eating behaviour of

- feedlot lambs fed high-concentrate diets containing soybean hulls. *Journal of animal science*, 89(12), 4120-4126.
12. Flock, C., Bassi, A., & Gijzen, M. (1999). Removal of aqueous phenol and 2-chlorophenol with purified soybean peroxidase and raw soybean hulls. *Journal of Chemical Technology and Biotechnology*, 74(4), 303-309.
 13. Franěk, F., & Fussenegger, M. (2005). Survival Factor-Like Activity of Small Peptides in Hybridoma and CHO Cells Cultures. *Biotechnology progress*, 21(1), 96-98.
 14. Fu, F., & Wang, Q. (2011). Removal of heavy metal ions from wastewaters: a review. *Journal of environmental management*, 92(3), 407-418.
 15. Fukuda, I., Tsutsui, M., Yoshida, T., Toda, T., Tsuda, T., & Ashida, H. (2011). Oral toxicological studies of black soybean (*Glycine max*) hull extract: Acute studies in rats and mice, and chronic studies in mice. *Food and chemical toxicology*, 49(12), 3272-3278.
 16. Galdeano, M. C., & Grossmann, M. V. E. (2005). Effect of treatment with alkaline hydrogen peroxide associated with extrusion on color and hydration properties of oat hulls. *Brazilian Archives of Biology and Technology*, 48(1), 63-72.
 17. Geor, R. J. (2007, March). Equine carbohydrate nutrition: implications for feeding management and disease avoidance. In *Proceedings of the 5th Mid-atlantic Nutrition conference* (pp. 154-160).
 18. Goldmon, D. C. and N. E. Brown (1992). "Effects of fat level and addition of soy fiber on sensory and other properties of ground pork patties." *Journal of Foodservice* 7(1): 1-13.
 19. Guide, F. Soybean.
 20. Hartman, G. L., West, E. D., & Herman, T. K. (2011). Crops that feed the World 2. Soybean—worldwide production, use, and constraints caused by pathogens and pests. *Food Security*, 3(1), 5-17.
 21. Jia, L., Enzan, C. H. E. N., Haijia, S. U., & Tianwei, T. A. N. (2011). Biosorption of Pb²⁺ with modified soybean hulls as absorbent. *Chinese Journal of Chemical Engineering*, 19(2), 334-339.
 22. Jiang, Y. H., Jiang, X. L., Wang, P., & Hu, X. K. (2005). In -vitro antioxidant activities of water-soluble polysaccharides extracted from *Isaria Farinosa* B05. *Journal Of Food Biochemistry*, 29(3), 323-335.
 23. Kabe, A. M. G., de Souza, A. D., de Moro Sousa, R. L., da Silva Bueno, I. C., Mota, T. P., Crandell, K., ... & Brandi, R. A. (2016). Soybean hulls in equine feed concentrates: Apparent nutrient digestibility, physicochemical and microbial characteristics of equine feces. *Journal of Equine Veterinary Science*, 36, 77-82.

24. Kamal, J. A., & Behere, D. V. (2002). Thermal and conformational stability of seed coat soybean peroxidase. *Biochemistry*, *41*(29), 9034-9042.
25. Karr-Lilienthal, L. K., Kadzere, C. T., Grieshop, C. M., & Fahey, G. C. (2005). Chemical and nutritional properties of soybean carbohydrates as related to non-ruminants: A review. *Livestock Production Science*, *97*(1), 1-12
26. Kim, H. W., Lee, Y. J., & Kim, Y. H. B. (2015). Efficacy of pectin and insoluble fiber extracted from soy hulls as a functional non-meat ingredient. *LWT-Food Science and Technology*, *64*(2), 1071-1077.
27. Krishnani, K. K., Meng, X., Christodoulatos, C., & Boddu, V. M. (2008). Biosorption mechanism of nine different heavy metals onto biomatrix from rice husk. *Journal of Hazardous Materials*, *153*(3), 1222-1234.
28. Kulshrestha Y, Husain Q. Decolorization and degradation of acid dyes mediated by salt fractionated turnip (*Brassica rapa*) peroxidases. *Toxicological & Environmental Chemistry*. 2007;*89*(89):255–267. DOI:10.1080/02772240601081692
29. Kumar, V., Biswas, A. K., Sahoo, J., Chatli, M. K., & Sivakumar, S. (2013). Quality and storability of chicken nuggets formulated with green banana and soybean hulls flours. *Journal of food science and technology*, *50*(6), 1058-1068.
30. Kumar, V., Kumar Biswas, A., Kumar Chatli, M., & Sahoo, J. (2011). Effect of banana and soybean hull flours on vacuum-packaged chicken nuggets during refrigeration storage. *International journal of food science & technology*, *46*(1), 122-129.
31. Li, X. M., Li, X. L., & Zhou, A. G. (2007). Evaluation of antioxidant activity of the polysaccharides extracted from *Lycium barbarum* fruits in vitro. *European Polymer Journal*, *43*(2), 488-497.
32. Liao, H. F., Chou, C. J., Wu, S. H., Khoo, K. H., Chen, C. F., & Wang, S. Y. (2001). Isolation and characterization of an active compound from black soybean [*Glycine max* (L.) Merr.] and its effect on proliferation and differentiation of human leukemic U937 cells. *Anti-cancer drugs*, *12*(10), 841-846.
33. Liu, H. M., & Li, H. Y. (2017). Application and Conversion of Soybean Hulls. In *Soybean-The Basis of Yield, Biomass and Productivity*. InTech.
34. Liu, H., Guo, X., Li, J., Zhu, D., & Li, J. (2013). The effects of MgSO₄, d-glucono- δ -lactone (GDL), sucrose, and urea on gelation properties of pectic polysaccharide from soy hull. *Food hydrocolloids*, *31*(2), 137-145.

35. Liu, J., Liu, H., Zhang, Y., Qiu, L., Su, F., Li, F., ... & Li, J. (2007). A simple preparation method of crystals of soybean hull peroxidase. *Applied microbiology and biotechnology*, 74(1), 249-255.
36. Liu, K. (2012). *Soybeans: chemistry, technology, and utilization*. Springer.
37. Mann, J. and J. Cummings (2009). "Possible implications for health of the different definitions of dietary fibre." *Nutrition, Metabolism and Cardiovascular Diseases* 19(3): 226-229.
38. Marshall, W. E. and L. H. Wartelle (2003). "Acid recycling to optimize citric acid-modified soybean hull production." *Industrial crops and products* 18(2): 177-182.
39. Marshall, W. E., et al. (1999). "Enhanced metal adsorption by soybean hulls modified with citric acid." *Bioresource Technology* 69(3): 263-268.
40. Merci, A., Urbano, A., Grossmann, M. V. E., Tischer, C. A., & Mali, S. (2015). Properties of microcrystalline cellulose extracted from soybean hulls by reactive extrusion. *Food Research International*, 73, 38-43.
41. Módenes, A. N., Espinoza-Quiñones, F. R., Colombo, A., Geraldí, C. L., & Trigueros, D. E. (2015). Inhibitory effect on the uptake and diffusion of cd^{2+} onto soybean hull sorbent in cd - pb binary sorption systems. *Journal of environmental management*, 154, 22-32.
42. Oliveira, T. J. P., Cardoso, C. R., & Ataíde, C. H. (2015). Fast pyrolysis of soybean hulls: analysis of bio-oil produced in a fluidized bed reactor and of vapor obtained in analytical pyrolysis. *Journal of Thermal Analysis and Calorimetry*, 120(1), 427-438.
43. Otlés, S., & Ozyurt, V. H. (2015). Classical Wet Chemistry Methods. *Handbook of Food Chemistry*, 133-149.
44. Prokopijevic, M., Prodanovic, O., Spasojevic, D., Stojanovic, Z., Radotic, K., & Prodanovic, R. (2014). Soybean hull peroxidase immobilization on macroporous glycidyl methacrylates with different surface characteristics. *Bioprocess and biosystems engineering*, 37(5), 799-804.
45. Ranathunga, S. D. (2008). *Replacement of starch from corn with non-forage fiber from distillers grains in diets of lactating dairy cows*. South Dakota State University.
46. Rizzuti, A. M., Ellis, F. L., & Cosme, L. W. (2015). Biosorption of mercury from dilute aqueous solutions using soybean hulls and rice hulls. *Waste and Biomass Valorization*, 6(4), 561-568.
47. Rizzuti, A.M., & Lancaster, D. J. (2013). Utilizing soybean hulls and rice hulls to remove textile dyes from contaminated water. *Waste and Biomass Valorization*, 4(3), 647-653.

48. Rojas, M. J., Siqueira, P. F., Miranda, L. C., Tardioli, P. W., & Giordano, R. L. (2014). Sequential proteolysis and cellulolytic hydrolysis of soybean hulls for oligopeptides and ethanol production. *Industrial Crops and Products*, *61*, 202-210.
49. Silivia, M. R., Silva, M. A. A. P., & CHANG, Y. K. (1998). Utilização da farinha de jatobá (*Hymenaea stigonocarpa* Mart.) na elaboração de biscoitos tipo cookie e avaliação de aceitação por testes sensoriais afetivos univariados e multivariados. *Ciência e Tecnologia de Alimentos*, *18*(1), 25-34.
50. Slavin, M., Kenworthy, W., & Yu, L. (2009). Antioxidant properties, phytochemical composition, and antiproliferative activity of Maryland-grown soybeans with colored seed coats. *Journal of agricultural and food chemistry*, *57*(23), 11174-11185.
51. Sonomoto, K., & Okamoto, Y. (1995). An integrated bioreactor system for biologically active peptides from isolated soybean protein. *Annals of the New York Academy of Sciences*, *750*(1), 435-440.
52. Todd, J. J., & Vodkin, L. O. (1993). Pigmented soybean (*Glycine max*) seed coats accumulate proanthocyanidins during development. *Plant Physiology*, *102*(2), 663-670.
53. Turbak, A. F., Snyder, F. W., & Sandberg, K. R. (1983, January). Microfibrillated cellulose, a new cellulose product: properties, uses, and commercial potential. In *J. Appl. Polym. Sci.: Appl. Polym. Symp.:(United States)* (Vol. 37, No. CONF-8205234-Vol. 2). ITT Rayonier Inc., Shelton, WA.
54. Volesky, B. (2007). Biosorption and me. *Water research*, *41*(18), 4017-4029.
55. Wanders, A. J., van den Borne, J. J., de Graaf, C., Hulshof, T., Jonathan, M. C., Kristensen, M., ... & Feskens, E. J. (2011). Effects of dietary fibre on subjective appetite, energy intake and body weight: a systematic review of randomized controlled trials. *Obesity Reviews*, *12*(9), 724-739.
56. Wenwu, L., Changwei, H. U., Yu, Y., Liangfang, Z. H. U., & Dongmei, T. O. N. G. (2010). Effect of the interference instant of zeolite HY catalyst on the pyrolysis of pubescens. *Chinese Journal of Chemical Engineering*, *18*(2), 351-354.
57. Xu, B., & Chang, S. K. (2008). Antioxidant capacity of seed coat, dehulled bean, and whole black soybeans in relation to their distributions of total phenolics, phenolic acids, anthocyanins, and isoflavones. *Journal of agricultural and food chemistry*, *56*(18), 8365-8373.
58. Yaman, S. (2004). "Pyrolysis of biomass to produce fuels and chemical feedstocks." *Energy conversion and management* **45**(5): 651-671.

59. Yang, J., Xiao, A., & Wang, C. (2014). Novel development and characterisation of dietary fibre from yellow soybean hulls. *Food chemistry*, *161*, 367-375.

Annexure-1

List of tables

Table no.	Table name	Page no.
1.	Moisture content of the soy hull	22
2.	Absorbance and concentration of sugars released by hulls by DNS method	24
3.	Growth of <i>Lactobacillus casei</i> on the treated soybean hull	27
4.	Growth of Yeast (<i>Saccharomyces cerevisiae</i>) on soy hull	29

Annexure-II

List of figures

Fig no.	Fig. Name	Page no.
1.	Showing wet whole soybean hull	15
2.	Showing hot air oven dried whole soybean hull	15
3.	Dried crushed soy hull	15
4.	Showing <i>Lactobacillus casei</i> bacteria under microscope isolated from yakult	23
5.	Depicting the color change in the cellulase and pectinase treated hulls by benedict's test	24
6.	Intensity of color of sugars released by soy hulls	25
7.	Absorbance of different treated soy hulls at 540 nm by DNS method.	25
8.	A. Pectinase treated hull SEM pictures B. Cellulase treated hull SEM pictures	26
9.	Isolation of <i>Lactobacillus casei</i> on MRS plate	27
10.	Showing growth of <i>Lactobacillus casei</i> in different treated hulls	27
11.	Growth of <i>Lactobacillus casei</i> (isolated and in yakult) on Soy Hull	28
12.	Growth of <i>Lactobacillus casei</i> (isolated and in yakult) on Cellulase treated soy hull	29
13.	Growth of <i>Lactobacillus casei</i> (isolated and in yakult) on Pectinase treated soy hull	29
14.	Growth of <i>Lactobacillus casei</i> (isolated and in yakult) on NaOH treated soy hull	29
15.	Depicting the growth of Yeast (<i>Saccharomyces cerevisiae</i>) on the treated hull samples	30
16.	Growth of Yeast (<i>Saccharomyces cerevisiae</i>) on soy hull	31
17.	Growth of Yeast (<i>Saccharomyces cerevisiae</i>) on Pectinase treated soy hull	31
18.	Growth of Yeast (<i>Saccharomyces cerevisiae</i>) on Cellulase treated soy hull	32
19.	Growth of Yeast (<i>Saccharomyces cerevisiae</i>) on NaOH treated soy	32
20.	A. Growth of <i>Aspergillus niger</i> on NaOH treated soy hull B. Growth of <i>Aspergillus niger</i> on Cellulase treated soy hull C. Growth of <i>Aspergillus niger</i> on Pectinase treated soy hull. D. Growth of <i>Aspergillus niger</i> on soy hull	33

Annexure-III

List of Abbreviations

Abbreviation	Name
AOAC	Association of analytical communities
°C	Degree Celsius
DF	Dietary fibre
G	Gram
GC-MS	Gas chromatography-mass spectrometry
Hr	Hour
HRP	Horseradish peroxidase
IDF	Insoluble dietary fibre
M	Molar
Mins	Minutes
Mg/dl	Milligram/deciliter
NaOH	Sodium hydroxide
Nm	Nanometre
NO	Nitric oxide
O.D.	Optical Density
pH	Potential of Hydrogen
SDF	Soluble dietary fibre
SEM	Scanning electron microscope
SHP	Soybean Hull Peroxidase

TU Thapar University

U/g Unit/gram

Soy

ORIGINALITY REPORT

% 13	% 11	% 5	% 2
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.intechopen.com Internet Source	% 7
2	www.ksre.ksu.edu Internet Source	% 1
3	Yang, Jun, Anhong Xiao, and Chunwei Wang. "Novel development and characterisation of dietary fibre from yellow soybean hulls", Food Chemistry, 2014. Publication	% 1
4	dergi.ksu.edu.tr Internet Source	<% 1
5	www.preparedfoods.com Internet Source	<% 1
6	spotidoc.com Internet Source	<% 1
7	Ravn, Jonas Laukkonen, Helle Juel Martens, Dan Pettersson, and Ninfa Rangel Pedersen. "Enzymatic Solubilisation and Degradation of Soybean Fibre Demonstrated by Viscosity,	<% 1