Enzymatic hydrolysis of starchy waste for bio-ethanol production

A

DISSERTATION REPORT

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR

THE

AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

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Submitted by

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled "Enzymatic hydrolysis of starchy waste for bio-ethanol production", in partial fulfillment of the requirement for the award of the degree of Master of Technology, Department of Biotechnology , Thapar University, Patiala, is an authentic record of my own work during the period of eleven months from July 2007 to June 2008, under the supervision of Dr. Dinesh Goyal, Associate Professor, Head of Department of Biotechnology, Department of Biotechnology, Thapar University. The thesis report has not been submitted for the award of any other degree or certificate in this or any other University.

Place: Patiala

Date: 15th July, 2015

(Sandeep Kaur Saggi)

CERTIFICATE

This is to certify that the Thesis report entitled to "Enzymatic hydrolysis of starchy waste for bio-ethanol production", submitted by Sandeep Kaur Saggi in the partial fulfillment of the requirement for the award of degree of the Master of Technology to the Thapar University, Patiala, is a record of Student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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Sandeep Kaur Saggi

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LIST OF ABBREVIATIONS

OPW	Orange peel waste
PPW	Potato peel waste
BPW	Banana peel waste
DNS	3,5-dinitrosalicylic acid
et al	And others
etc	And other things
rpm	Rotation per minute
viz	As follows
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
K_2HPO_4	Di potassium hydrogen phosphate
$MgSO_4.5H_2O$	Magnesium sulphate pentahydrate
$PbCl_2$	Lead chloride
$(NH_4)_2SO_4$	Ammonium sulphate
OD	Optical density
g	Gram
g/L	Gram Per liter
h	Hour
L	Litre
M	Molar
mg	Milligram
min	Minute
mL	Millileter
mm	Millimeter
mM	Millimolar
µg	Microgram
ANOVA	Analysis of variance

CO_2	Carbon dioxide
FTIR	Fourier transfer infrared spectroscopy
XRD	X-ray diffraction
SEM	Scanning electron microscope

SYMBOLS

β	Beta
α	Alpha
%	Percentage
°C	Celsius
µ	Micro
®	Registered
™	Trade mark
±	Plus-minus

ABSTRACT

Amylase activity in the cell free supernatant of starch degrading bacteria *Bacillus licheniformis* NA11 was studied using 1% starch. The protein precipitated up to 20-80% saturation with ammonium sulphate and further partially purified was found stable between pH 5-7 and temperature between 40-60° C with optimal activity at pH 7.0 and temperature 60°C. The Km value of the partially purified enzyme for starch substrate was 0.621 mg/ml and Vmax=0.0354 µmole/min/ml. Amylase activity of partially purified enzyme from *Bacillus licheniformis* showed maximum activity of 1.12 U/ml/min, with production of 0.467 mg/ml reducing sugar when 1% starch was used as substrate. When starchy wastes were used as substrate the enzyme activity in 160 min at 60°C was 1.048 U/ml/min for orange peel waste (OPW) with production of 0.435 mg/ml of reducing sugar, 0.99 U/ml/min for banana peel waste (BPW) with production of 0.411 mg/ml of reducing sugar and 0.959 U/ml/min for potato peel waste (PPW) with production of 0.398 mg/ml of reducing sugar. Iodine assay in test tube and micro-plates, showed blue colour in the presence of starch- iodine and at 60-70°C, all blue color disappeared after 60 min. Statistical optimization using response surface methodology predicted amylase activity of 0.47 U/mL, which coincided with the experimental amylase activity of 0.46 U/mL using starch (10 g/L), yeast extract (6 g/L), and $MnCl_2.4H_2O$ (1.0 g/L).

Starchy waste such as Potato peel waste (PPW), orange peel waste (OPW), banana peel waste were (BPW) biomass were washed to remove adhering debris, dried and powdered (0.3- 0.5 mm) to investigate its physico-chemical properties. Potato peel waste (PPW) was found to contain 6.64% moisture, 5.48% ash and 75.79 % volatile matter, similarly orange peel waste (OPW) was found to contain 5.451 % moisture, 9.717 % ash, 84.52 % volatile matter and banana peel waste (BPW) was found to contain 5.01 % moisture, 7.35 % ash, 81.40 % volatile matter.

Pre-treatment of starchy waste using dilute acid-alkali and enzymatic hydrolysis were done to enhance the yield of reducing sugar for ethanol production. Enzymatic pre-treatment was found to be the best pre-treatment strategy with increase in amylose subunits, while decrease in starch content. Yield of reducing sugar was enhanced from 0.195mg/ml to 0.467 mg/ml.

Scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier transform infrared analysis (FTIR) analysis of native and enzyme pre-treated biomass was done to study physical and chemical changes.

Enzymatically pre-treated starchy wastes (PPW, OPW and BPW) were used as substrate for ethanol production using cell free supernatant from *Bacillus licheniformis* NA11 followed by

ethanol fermentation using *Saccharomyces cerevisiae* (NCIM 3215) via SSF. Ethanol production using starch as substrate was 2.51%, with orange peel waste it was 1.33%, with banana peel waste it was 1.03% and with potato peel waste it was 0.89%. The high catalytic activity and its stability to temperature, pH, indicated that the amylase enzyme from *Bacillus licheniformis* NA11 is a good candidate for hydrolysis of starchy waste into bio-refinery processes.

CHAPTER 1
INTRODUCTION

During the recent years, demand of energy due to transportation, heating and industrial processing has been increased drastically (Marcos et al., 2007). Major countries in the world are facing serious challenges to manage such issues associated with conventional energy sources (Koppram *et al.*, 2014). In such scenario, alternative and renewable sources of energy gained huge attention from governments of many countries across the world to protect the environment as well as to supply energy needs while reducing dependency on fossil fuels (Ghosal *et al*., 2013). In accordance to current energy and environmental status, bio-energy sources like bio-ethanol has emerged as a viable, economically alternative and renewable energy source with high efficiency and low environmental impact (Hahn et al., 2008).

Worldwide production of ethanol has been estimated around 51,000 million liters (Duhan et al., 2013). Among that production, fuel covers 73% of produced ethanol, while beverage and industrial ethanol constitute 17 and 10%, respectively (Sanchez et al., 2008). As a fuel enhancer, ethanol is well known for some added advantages. As a fuel, ethanol combustion emits low CO as well as low unburned hydrocarbon and SO_2 free exhaust. Along with this, ethanol is an octane enhancer with is rich in octane number of 120 as compared to 87- 98 in case of gasoline (Purohit et al., 2012). Another significant reason behind use of ethanol as bio-fuel is its zero CO_2 emission. As ethanol burns, it emits CO_2 which is assimilated by plant in next for carbohydrate production during photosynthesis. In addition, well known application of ethanol found in alcoholic beverages where it also served as an antioxidant. Bio-ethanol is mainly produced by fermentation process from starchy materials. Carbohydrate is the main precursor for ethanol, in fermentation process. Due to such advantages, ethanol is blended in gasoline in a range of 5-20 % (Purohit et al., 2012). Various raw materials can be utilized for ethanol fermentation. The most commonly used types of feedstock for ethanol production are mainly associated with sugar crops (sugar cane), starch-containing plants (corn, wheat, potato) and lignoellulosic biomass (P.R. et al., 2009). Sugarcane, sugar beets and molasses are feasible for ethanol fermentation and have been used extensively; however, these carbon sources are valuable products in terms of food sources (Nalley et al., 2003). Being an industrial waste, molasses contain high amount of impurity which necessitates a long pretreatment steps. In order to select the cost effective process, lingo-cellulosic biomass is another option for ethanol fermentation. But lignocellulosic

biomass (Dweikat et al., 2012) (wood, straw) has to undergo the most complicated pre-treatment process prior to fermentation which includes removal of lignin followed by the hydrolysis of cellulose (D suza *et al.,* 2014). The latter process is far more difficult than the saccharification of starch. However, research effort to reduce the production cost of this bio-ethanol was successful when starchy raw materials were introduced with improved hydrolysis efficiency.

Starchy materials require hydrolysis to break down the starch into fermentable simple sugars (saccharification). Acid hydrolysis is normally carried out at high temperature and considered as an energy intensive process. To convert starch into the fermentable sugars, another hydrolysis technique is enzymatic hydrolysis (M.A. *et al.,* 2007). The conventional process of enzyme hydrolysis of starch to produce fermentable sugars includes following steps: gelatinisation, liquefaction with thermo stable a-amylase, and saccharification (Wang et al., 2005). Enzyme hydrolysis process has some own advantages as well as disadvantages to use. Enzyme hydrolysis yields high conversion efficiency of glucose to ethanol. Mainly the enzyme Amylases (α-amylase, β-amylase and glucoamylase) are employed for hydrolysis of starchy materials. In that process α-amylase hydrolyses the 1, 4-α-D-glucosidic linkages in the linear amylase chain while amyloglucosidase cleaves the 1,6-α-linkages at the branching points of amylopectin as well as 1,4-α-linkages. The energy consumption of such enzymatic processes usually amounts about 30–40% of total energy required for the overall ethanol production and hence considered as expensive process (Singhanian *et al.,* 2013).

In recent years, two new production methods, namely separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) has been introduced successfully to overcome those problems associated with chemical and enzymatic process (Lu et al., 2005). The separate hydrolysis and fermentation (SHF) process involved with the two distinct process of starch hydrolysis and glucose fermentation. The principal advantage of such process is that starch hydrolysis and sugar fermentation can be treated separately and hence minimizing the interactions between these steps. Basically in this process, starch molecule is initially hydrolyzed by the action of amylolytic enzymes: α-amylase (for liquefaction) and glucoamylase (for complete saccharification) (Liebmann et al., 2009). After complete hydrolysis, the fermentation is generally conducted in a single stage separately. Such two-step process by enzymatic hydrolysis and fermentation using *Saccharomyces cerevisiae* was successfully reported by (Mojovic et al. 2006). However the main difficulty associated with this process is α-amylases are often inhibited by the accumulation of sugars which hinders to achieve reasonable ethanol concentrations at high rates and with high yields

(Borzani et al., 1998). To achieve total conversion of simple sugars from starch, instead of using direct expensive enzymes, submerged cultures of amylases producing bacteria or fungi can be effectively used in the first stage of this production process. To construct the overall simultaneous process, a novel concept was introduced to make both hydrolysis and fermentation of starch in a single vessel maintaining a single phase which is referred as (SSF) simultaneous saccharification and fermentation (Neves et al., 2007). In this process, the saccharification of sugars released during starch hydrolysis is conducted simultaneously with fermentation (Goldemberg *et al.,* 1993). The presence of yeast or bacteria along with enzymes minimizes the sugar build up in the vessel. The higher rates, yields and concentrations of ethanol can be obtained using SSF rather than SHF as because the sugar produced during starch breakdown slows down α-amylase action. The process of simultaneous saccharification and fermentation (SSF) is an all together energy and water-saving process as it results in higher ethanol productivity by avoiding the loss of fermenting sugars which may occur during heating of fermentation broth (Josefin et al 2011).

The objectives of the present investigation work are:

1. Screening and characterization of bacterial isolates for amylolytic activity.
2. Effect of different parameters on amylase activity from *Bacillus licheniformis* NA11 using starch as substrate and its optimization.
3. Bioconversion of starch and other starchy wastes by *Bacillus licheniformis* NA11 for the of ethanol production by simultaneous saccharification fermentation (SSF).

CHAPTER 2
REVIEW OF LITERATURE

One day the world will run out of fossil fuels as the main source of energy, where as bio-fuels from plants represents a sustainable approach. The term bio-fuel is attributed to any alternative fuel that is derived from organic material, such as **Energy crops** (corn, wheat, sugar cane, sugar beet, cassava, among others), (Agarwal *et al,* 1998) , sweet sorghum (Bulawayo *et al,* 1996), Ethanol is fuel which is produced from the plant source like corn, maize (USA) , sugarcane (Brazil) and sugar beet (Europe). Higher content for production of ethanol source is mainly sugarcane than low sugar content corn (Othman *et al,* 1992). **Crop residues** such as rice straw, rice husk, corn Stover and corn cobs or **waste biomass** for instance, food waste, livestock waste, paper waste and construction-derived wood residues (Sun *et al*, 2002).

STARCH

Starch is the major contributor of carbohydrates in our diet. It is present in large amount in plants; stored in the seeds, roots and fibres as food reserve. Starch has a great potential as a renewable energy source for future production of high-yielding ethanol. With an annual global production of more than 1.1×10^9 per year, starch can replace fossil fuel based ethanol production as a renewable energy source. Starch is a polymer of glucose (Monosaccharide's) found in plants. Their intact granules are insoluble in cold water but can hydrolysis them using hot water. The principal sources of starch are cassava, cereals, potatoes, corn and rice. Starch occurs naturally as small globules 1 to 100 µm (Phillips and Williams 2000) enclosed in cellular tissue. Its density is 1.5 gm/cm^3. These are soaked in water and crushed. Dissolving starch in warm water gives wheat paste, which can be used as a thickening, stiffening or gluing agent.

Carbohydrate is major source of energy from plants and animal (tetrose (C4); pentose (C5); hexose (C6); heptose (C7)). Starch and celluloses are large high molecular weight biopolymers which are constructed by joining of large units of monosaccharide (glucose) together by glycosidic bond. Starch consists of two fraction Amylose one has mol wt =105 to 106 g/mol and another is Amylopectin has mol wt= 107 to 109 g/mol. Amylose is 20% water soluble material. It consist molecule of linear chain of several monosaccharides' units of glucose molecule joined by alpha C-1 to C-2 glycosidic bond (Fig. 1). Majority of another material found in starch is amylopectin (70-80%) consisting of short linear and

branched network of several monosaccharide's of glucose from C-1 to C-4 and C-1 to C-6 glycosidic linkage and is normally insoluble in water (Sanchez et al., 2008).

STARCH DEGRADING ENZYMES

1) **FIRST ENZYME**

1. **ALPHA AMYLASE (α):** Alpha amylase is the first enzyme that acts randomly along the starch chain hydrolyzing the alpha (1-4) glycosidic bonds to produce a combination of maltotriose, maltose and dextrin pH 6.7 or 7.0 (V.D *et al.*, 2002).
2. **BETA AMYLASE (β):** which works from the non reducing end of the polymer hydrolyze the second alpha(1-4) glycosidic bond to produce two- glucose sugar maltose
3. **GAMMA AMYLASE:** also work on non-reducing end of amylase to cleave alpha (1-4) glycosidic bonds to produce glucose, and also cleaves alpha (1-6) linkage found in pectin.

2) **SECOND ENZYME**

1. **GLUCOAMYLASE:** Produced by fungi is an Exo-amylase, it hydrolyses the dextrin and maltose from the non-reducing end of molecule. It hydrolyses both alpha (1-6) and alpha (1-4) bond to completely degrade dextrin to glucose at pH 3.5-4.5.

STRUCTURE OF STARCH

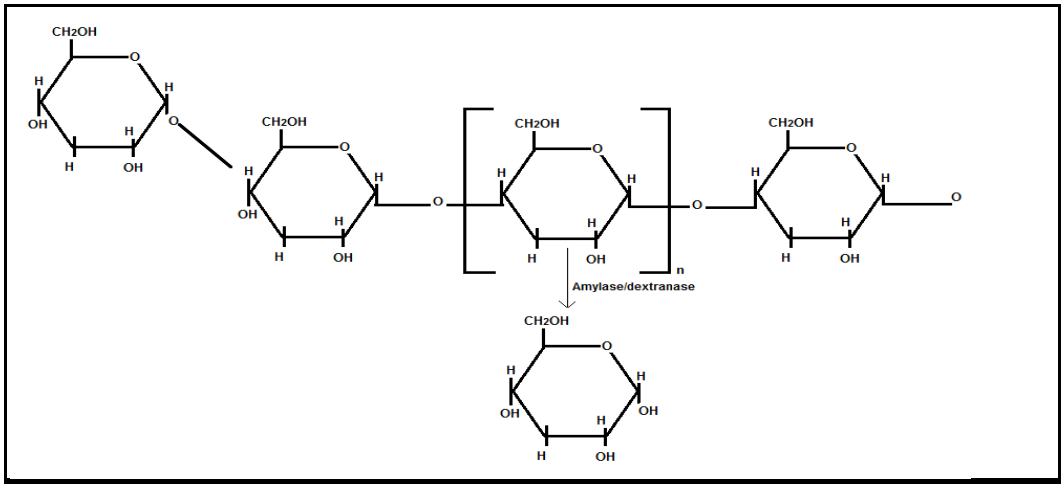


Fig. 1 Ethanol fermentation metabolism chart (Sanchez et al., 2008)

It is actually a mixture of two structurally different polysaccharides, amylose (20-30%) and amylopectin (75-80 %) (Neves *et al.*, 2007). When it is heated with hot water, it can be separated into these components. The part which is soluble in water is amylose and remaining

fraction is amylopectin. Both these amylose and amylopectin consists of D- glucose units. Amylose is a linear component mostly comprised of α-1, 4-linkages (linkages between C-1 of one glucose and C-4 of the next glucose unit), with an average degree of polymerization (DP) up to 6,000 and molecular mass of 105 to 106 g/mol (Phillips et al., 2000). The number of D-glucose units in amylase ranges from 300 to 3000. The structural formula of amylose is represented in (Fig.2).

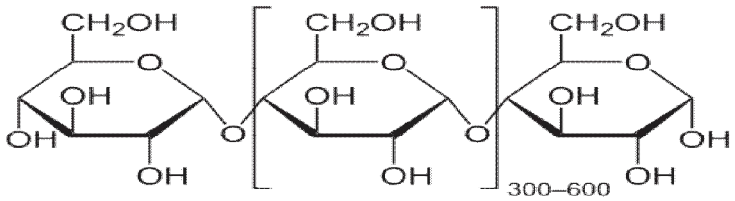


Fig. 2 Structural formula of amylose **(**Sanchez et al., 2008)

Amylopectin a linear glucose polymer consisting of α (1→4) glycosidic bonds. Branching takes place with α (1→6) bonds occurring every 24 to 30 glucose units, resulting in a soluble molecule which can be readily degraded as it has many end points for enzymes to attach onto. In contrast, amylose contains very few α (1→6) bonds or even none at all. This results in its slow hydrolysis. The structural formula of Amylopectin is shown in (Fig.3).

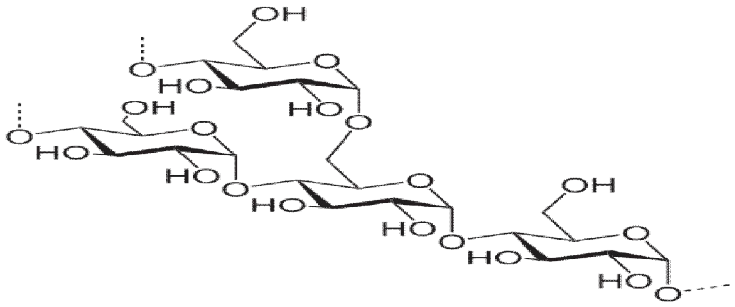


Fig. 3 Structural formula of Amylopectin **(**Sanchez et al., 2008)

STARCH HYDROLYSIS

Process to produce the sugars molecules from starch molecules involves sequence of steps beginning with hydrolysis where starch is pretreated with acids or alpha-amylases in order to produce glucose by saccharification enzymes used in this process are produced by microorganism's bacteria such as *Bacillus licheniformis:* α- and β-amylases (liquefaction), glucoamylases (saccharification) and glucanases/ cellulases (Verma *et al.,* 2012) /xylanases viscosity reduction (Purohit *et al.,* 2012). For hydrolysis of starch, suspension should used is heated up to 50–120°C in order to breakdown the starch kernels to optimize the reaction

yields for bio-ethanol production (Alonso *et al.,* 2010).Combination of α-amylases and glucoamylases are used to produce fermentable sugar at low temperature (30–48 °C) (Purohit *et al.,* 2012) (Fig. 4).

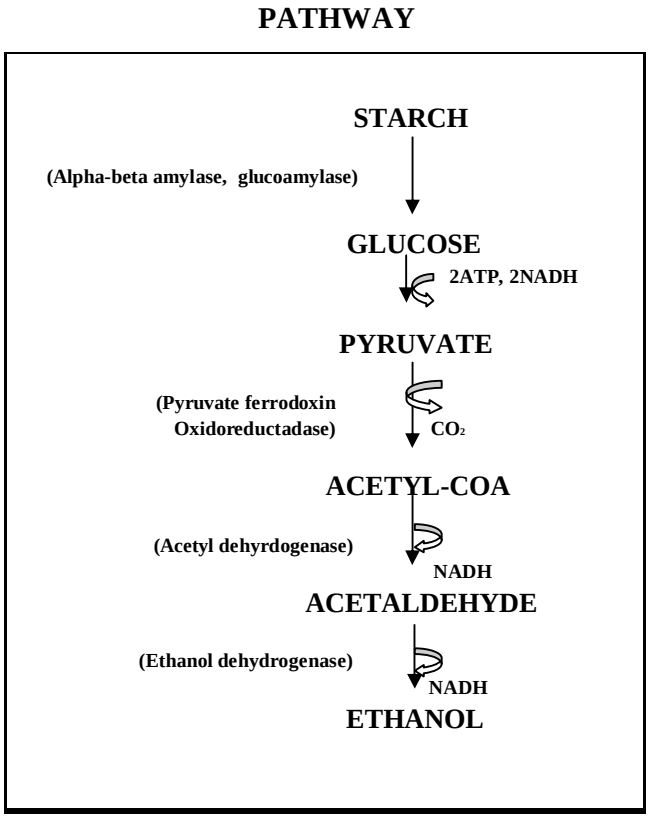


Fig. 4 Ethanolic fermentation metabolism chart **(**Duhan *et al* 2013)

The conventional energy intensive method of starch hydrolysis using acid in the initial stage of starch based ethanol production, has been already replaced by processes using starch saccharifying enzymes. Basically, the starch molecule is initially hydrolyzed by the action of amylolytic enzymes: α-amylase (for liquefaction) and glucoamylase (for saccharification). There are numerous bacteria and fungi from which amylases can be isolated but the most thoroughly studied species are *Bacillus* and *Aspergillus spp*. Industrially important enzymes including amylases have traditionally been obtained from submerged cultures due to ease of handling and greater control of environmental factors such as temperature and pH. But the direct use of commercial amylases for saccharification process may not be cost-effective. Submerged cultures of those specified species which can produce amylases using starchy food waste will not be the only alternative way for the production of amylases but at the same time it will be helpful for the production of simple sugars from starch. *Saccharomyces*

cerevisiae is well known for its conversion efficiency of simple sugars like glucose and fructose into ethanol. By incorporating both type of microbial cultures, simultaneous saccharification and fermentation (SSF) will definitely open a simple, efficient, cost effective and environment-friendly route for bio-ethanol production (Sanchez et al., 2008).

SUBSTRATE FOR BIO-ETHANOL PRODUCTION

Present status of bioconversion of carbohydrates for the production of bio-ethanol falls into using three different types of substrates such as cellulose, starch and sugar (Table 1; Fig. 5).

Table 1 Sources of Ligno-cellulose, starch and sugar

SOURCES	LIGNO-CELLULOSIC	STARCHY	SUGARY
1.	Leaf litter biomass, rice straw, corn stalks, corn cobs, bagasse, cane filter cake, kenaf stalk, animal manure, pineapple pulp, wheat brain (Rehman *et al.*, 2013).	Cassava meal, Cassava effluent, Potato, Rice, Corn, orange, banana (Meenakshi *et al.*, 2014), (Liimatainen *et al.*, 2010).	Mollases, Corn steep liquor, Pulp water liquor, Coconut water (Dias et al., 2010).

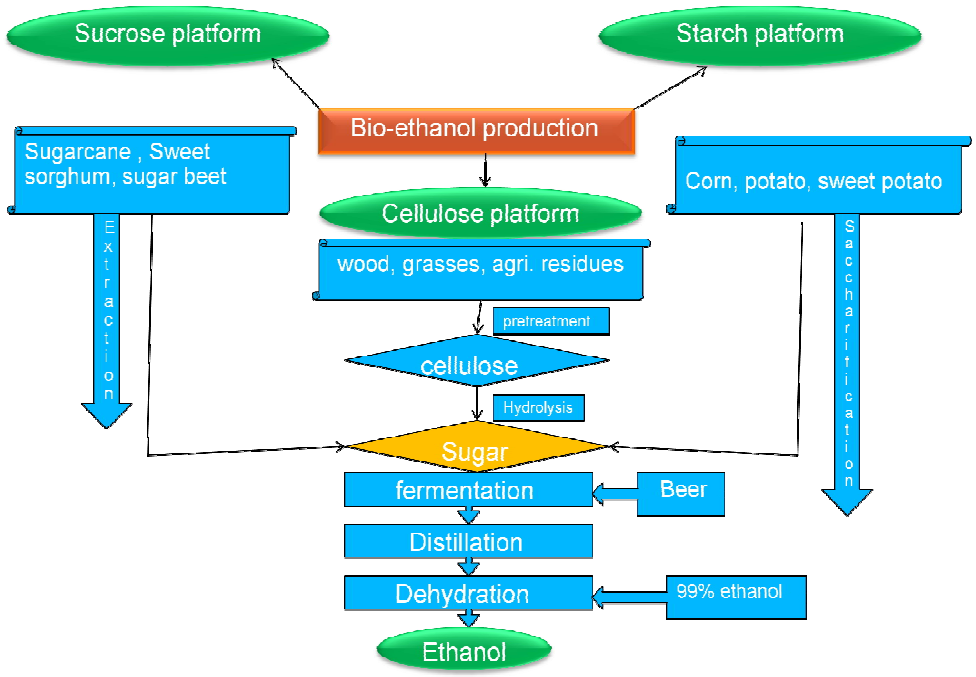


Fig. 5 Ethanol production source **(**Duhan et al., 2013**)**

The use of waste product like agriculture waste such as blackstrap molasses (Borzani *et al.*, 1998) , rice straw, sweet potato, (Wang *et al.*, 2013) manure, baggase (Yu *et al.*, 2007) and

household cooked waste food such as rice water (Pitch), potato, corns (core et al., 2004), etc has been the on the forefront **(**Duhan et al., 2013**).**

STARCH DEGRADING MICROORGANISM

Starch molecule is basically hydrolyzed by the action of amylolytic enzymes: α-amylase (for liquefaction) and glucoamylase (for saccharification).There is numerous bacteria and fungi which can produce α-amylases and glucoamylase. In bacterial culture, α-amylase is mostly produced by *Bacillus* spp like *Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus subtilis* and *Bacilus megaterium*. (Alvira *et al.,* 2010)Amylases are also produced by various fungi like *Aspergillus oryzae* and *Aspergillus niger*. It has been reported that *Aspergillus* amylase produces more sugar than *Bacillus* amylase. Fungal sources for the same are *Aspergillus niger, Aspergillus oryzae, Aspergillus saitai and Aspergillus awamori* (Rath et al., 2014), In the second stage of the process *Saccharomyces cerevisiae* can be effectively used as it is the most promising microorganism for ethanol production (Ahmad *et al.,* 2013).

TREATMENT PROCESS INVOLVED OF STARCH AND LIGNOCELLULOSIC BIOMASS IN BIOETHANOL PRODUCTION

1. **Biological pre-treatment**
    i. Bacterial treatment
    ii. Fungi treatment
2. **Physical pre-treatment**
    i. Mechanical comminuting
    ii. Extrusion
3. **Chemical pre-treatment**
    i. Alkali pre-treatment
    ii. Acid pre-treatment
4. **Physico-chemical pre-treatment**
    i. Steam explosion: SO_2-steam explosion
    ii. Liquid hot water
    iii. Microwave pre-treatment

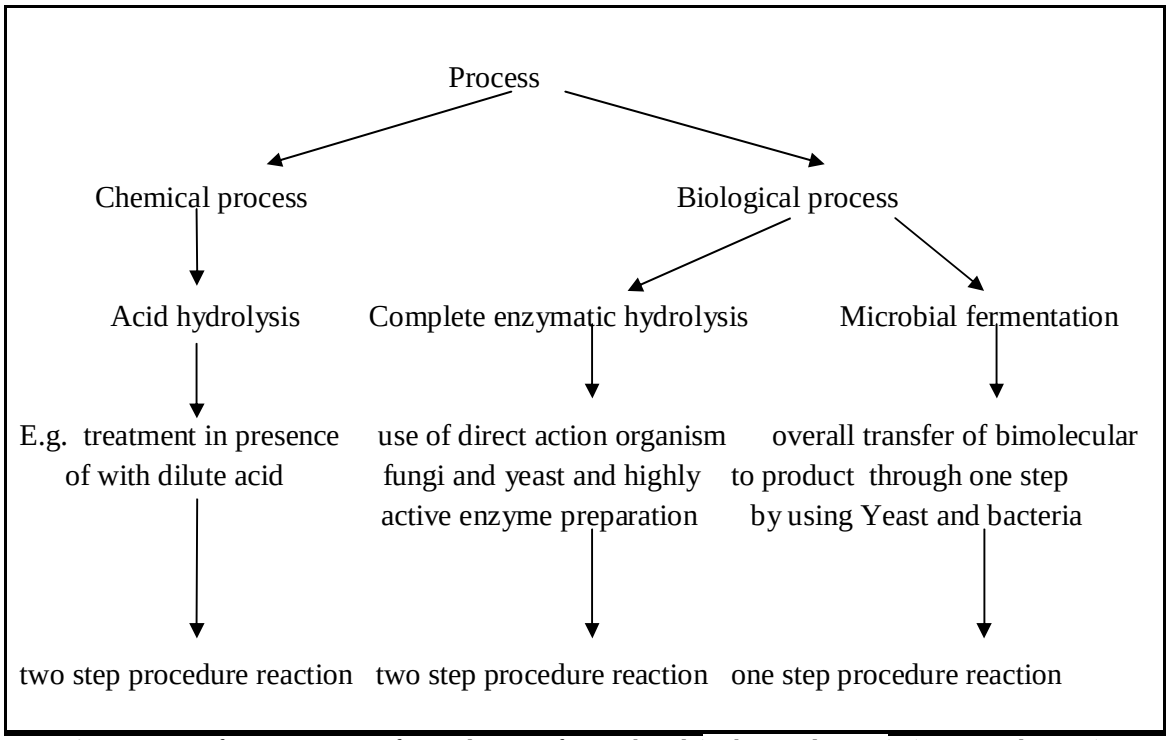


Fig. 6: Process for pre-treatment for production of Bio-ethanol **(**Duhan et al., 2013**),** (castro et al., 2014), (Bensah et al., 2013)

FERMENTATION

Fermentation techniques have gained immense importance due to their economic and environmental advantages (Talebnia *et al,* 2010). Two fermentation techniques have emerged as a result of this rapid development: Submerged Fermentation **(SMF)** and Solid State Fermentation **(SSF)** (Fig. 7; Fig. 8).

Submerged fermentation technique is used for liquid substrates such as sugarcane, whey, and molasses having high yield (Salam et al., 2014). While solid state fermentation technique is used for cellulose and starch based, bran, baggase, paper pulp etc. In these techniques, the substrates are utilized very slowly and steadily, so the same substrate can be used for long fermentation periods. Disadvantage of SSF is less yield than SMF (Neves *et al.*, 2007). Another technique developed in SHF i.e. separately hydrolysis and fermentation. In **SHF**, hydrolysis and fermentation are done separately for less interaction between them for better bio-ethanol production. In first step there is requirement for pre-treatment of hemicelluloses and lignin to convert in to glucose. After pre-treatment Liquefaction of cellulose, starch hydrolyzed to glucose by addition of celluloltic enzymes (that comprises of endo and exogluconases and beta gluconases activity), alpha-amylase, beta amylase and Saccharification by glucoamylase respectively. In second step, this glucose (hexose) is

fermented to bio-ethanol by of *Saccharomyces cerevisiae* (Ahmed et al., 2012). After complete hydrolysis the fermentation is conducted in a single step separately. Disadvantage of this technique is that is a long procedure with lesser yield of ethanol (Sanchez et al., 2008) (Fig. 7).

Schematic review of SHF:

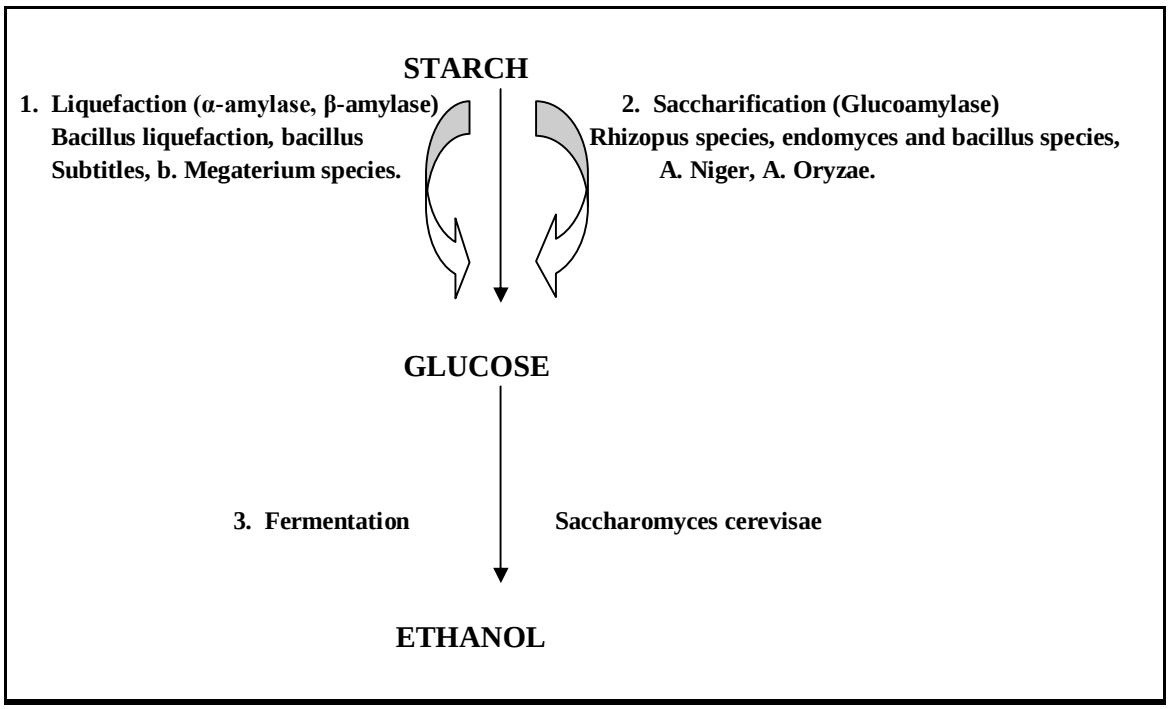


Fig. 7 Schematic review of SHF (Bothast et al., 2005), (Balat et., 2008)

In **SSF**, hydrolysis and fermentation are combined in one step to reduce the residence time. Hexose solution is fermented to ethanol using yeast. The solid must separate from the liquid but if we use enzymatic hydrolysis the two steps can reduce to one step. Enzyme can be added in flask without any separation of liquid from solid (Asnana et al., 2014). This process converts glucose (hexose) and hemicelluloses (pentose) to ethanol directly. It takes shorter time taken than SHF. (Duhan et al., 2013) (Fig. 8).

Advantage of this technique is that enzyme rate can be increased to maximum by reducing product (sugar) inhibition. In this single bioreactor is used with lower cost and maximum yield and presence of ethanol causes less invasion by undesirable microbe (Balat *et al*, 2008).

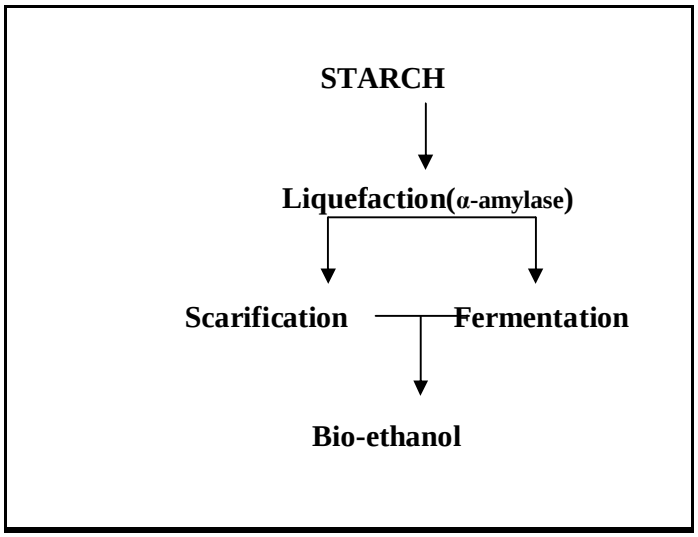


Fig. 8 Schematic review of SSF (Duhan et al., 2013)

CHAPTER 3
MATERIAL AND METHODS

Screening of bacterial isolates for amylase activity production using plate assay method

The *Bacillus licheniformis* NA8, NA9, NA11 were tested for amylase activity by employing zone clearing technique (Atlas *et al*, 1995) using starch agar medium 0.5%, 1%, 2%. Starch agar plates were spotted with different strains of *bacillus* and incubated at 37°C for 48 hrs. After incubation, the zone of hydrolysis of starch was detected by flooding the plates with iodine solution. The development of blue colour indicated the presence of starch, while the areas around the hydrolytic bacteria appeared clear. The best amylase activity was shown by strain of *Bacillus licheniformis* NA11 (Devi et al., 2013) on 1 % starch and was selected for further work.

Strains

Bacillus licheniformis NA11 and *Saccharomyces cerevisiae* were used. *Bacillus licheniformis* NA11 was maintained on (NA) Nutrient agar plate and *Saccharomyces cerevisiae* was maintained on (GYE) Glucose yeast extract agar respectively.

Microscopy of the strains

1. **Gram staining of B*acillus licheniformis* NA11**

Gram staining is a method of differentiating bacterial species into two large groups namely Gram-positively and gram negative. Gram Staining was done as per (Christian Gram *et al*, 1884).

Method

1. Bacterial smear from actively growing cells of culture *Bacillus licheniformis* NA11 were spread on a clean slide and was gently heat fixed.
2. Crystal violet was flooded for 1 minute on the smear.
3. Washed with tap water to remove excess of crystal violet.
4. Gram iodine was flooded for 1 minute and washed briefly with water
5. Decolorized with acetone and washed with the tap water.
6. Safranine was used to counter strain for 30 s.
7. Washed with tap water to remove excess strain.
8. Visualized under phase contrast microscope at different magnification. A Gram positive *Bacillus sp*. was taken as reference.

2. Methylene blue staining of *Saccharomyces cerevisiae*

This staining determines the proportion of viable yeast in a culture containing a mixture of live and dead yeast cells Viable cells are able to exclude the stain or reduce it to a colour less form.

Method

1. Place a small drop of alcohol (70%) on a clean microscope slide
2. Immersed the actively grown culture of Saccharomyces cerevisiae on slide.
3. Two drops of the 0.4 % methylene blue were added.
4. Flooding the cover slip between forefinger and thumb, touch one edge and avoiding air bubbles. The preparation was ready for examination.
5. Visualized under phase contrast microscope (Nikon eclipse 90i, Japan) at different magnification.

Production and purification of amylase from *Bacillus licheniformis* NA11

The bacterial isolates NA11 was initially grown on Nutrient Agar. From Petri plates one loop full was grown in nutrient broth of 50 ml and 2 ml soluble starch 1% and incubated at 37ºC for 24 hr at 120 rpm. This overnight grown culture *Bacillus licheniformis* NA11 was further 2 % inoculated in minimal media (M9 media) in 250 ml flask with 1% starch at 37ºC for 24-48 hr at 120 rpm. After 48 hrs of incubation, cell free supernatant collected after centrifugation (11000 rpm , 15 min.) was investigated for amylase activity.

Extraction of crude enzyme of *Bacillus licheniformis* NA11 (Miller 1959)

Amylase activity was determined by estimating the amount of reducing sugar using DNS (3,5-Dinitrosalicyclic acid) method as per miller, (1959).

a) Production of crude enzyme

The Amylase production was carried out in 500 ml conical flask using 100 ml medium with the following composition: The minimal media contained (g/100ml) (Soluble starch 1%= 2ml, Na_2HPO_4 = 6 g, KH_2PO_4 = 3g, NaCl = 0.5 g, NH_4CL = 1g, 1 M $CaCl_2$ = 0.01 µl, 1M $MgSO_4.7H_2O_4$ = 200 µl) incubated at 37°C under shaking condition (120 rpm) and inoculated with 24 hr old culture.

After incubation, production medium was centrifuged at 11000 rpm for 20 min. to separate the cells and supernatant was filtered by watt man filter paper. The filtrate contained crude enzyme and was stored at 4ºC till further use.

Enzyme Assay

Amylase activity in crude enzyme extract or purified enzyme was estimated by measuring amount of reducing sugar released during hydrolysis of 1% (w/v) starch in 0.1M phosphate at pH 7. The mixture was incubated at 60°C for 30 min.. 2 ml of DNS was added to the test tube and placed in water bath at 90ºC for 10 min.. The content of the test tubes were cooled and diluted up to 10 ml with distilled water (1 ml of reaction mixture sample + 9 ml of distilled water). The absorbance of reaction mixture was determined at 540 nm in spectrophotometer against dextrose/glucose as standard. One unit (U/ml) of activity as determined by the DNS (3, 5-Dinitrosalicyclic acid) method (Miller, 1959) assay is defined as an average of 1mg of glucose equivalents released per min in the assay reaction.

The amylase activity was determined in IU/ml/min by applying the standard formula:

$$\textbf{Amylase activity (IU/ml/min)} = \frac{\text{Amount of sugar released x 1000}}{\text{Molecular wt of glucose x Time of incubation x Volume of enzyme}}$$

Where,

Mol wt of glucose = 0.18, **Time of incubation** = 30 min., **volume of enzyme** = 1ml

b) Ammonium sulphate precipitation

A loop full of *Bacillus licheniformis* NA11 was inoculated in minimal media and was incubated for 24 hr. Cells were separated by centrifugation (11000 rpm for 20 min at 4ºC) and supernatant was taken. Now, 20%, 40%, 60%, 80% saturation with ammonium sulphate was done with 100 ml of supernatant. Ammonium sulphate was poured slowly in the supernatant over a period of 2-3 hrs allowing the salt to dissolve slowly. Supernatant was continuously stirred at 4ºC during dissolution of ammonium sulphate. The supernatant was kept for overnight at 4°C. Precipitates were recovered by centrifugation (11000 rpm, 10 min. at 4ºC), and dissolved in potassium phosphate buffer (7 pH). Further, precipitated fraction of protein was suspended in buffer and checked for amylase activity using starch as substrate and analyses by DNS and starch- iodine assay.

Effect of different parameters on amylase activity of NA11

Growth parameters of the isolate NA11 showing maximum hydrolysis were studied in term of effect of different starch concentration, temperature, pH, time of incubation by studying starch-iodine hydrolysis in test tube and micro-plate based starch iodine assay.

Optimal medium was designed using Central Composite Design response surface methodology software (Version 17.2.1.0, Minitab®) which was used to check the enzyme activity of *Bacillus licheniformis* NA11.

a) Effect of different substrate concentration of starch solution on enzyme activity

The hydrolytic ability of amylase produced *Bacillus licheniformis* NA11 was checked against different concentration of starch solution (w/v). The starch concentration was varied vary from 0.5%, 1%, 1.5%, 2%, 2.5%. The 1 ml of different starch concentration and 1 ml of crude enzyme was incubated at 37°C for 1 hr. The reaction was stopped by adding 2 ml of DNS (dinitrosalicylic acid). The reducing sugar released was determined by the method of Miller (1959). The absorbance was measured at 540 nm with spectrophotometer.

b) Determination of optimum pH

The optimum pH of the crude enzyme was determined by incubating the mixture of equal volume of crude enzyme and 1% (w/v) starch in the presence of appropriate buffers for different pH like 50mM sodium citrate (pH 4.0, 5.0), 50mM potassium phosphate (pH 6.0, 7.0) and 50mM NaOH buffer (8.0, 9.0). The reaction mixture of various pH buffers were incubated for 15 min. at 60°C. The enzyme assays were carried out by determining the amount of reducing sugars released as describe above.

c) Determination of optimum temperature

The optimum temperature of crude amylase enzyme activity was determined by incubating the appropriately enzyme with soluble starch medium at different temperature varying from (30, 40, 50, 60, 70, 80, 90ºC) and at pH 7.0 with incubation time 15 min. in water bath. Enzyme activity was measured by determining the amount of reducing sugar released by DNS method.

d) Effect of incubation time on enzyme activity at optimum temperature

The effect of incubation time on the activity of crude amylase was studied by performing the assay at different time intervals from (15, 30, 60, 90, 120, 150 min.) at different temperature ranges varies from (30, 40, 50, 60, 70°C). The eppendorfs containing 1 ml of 1% starch solution and 1 ml of enzyme and incubated in water bath at different temperature at different time intervals. These incubated enzyme activity was measured by DNS method.

e) Estimation of starch hydrolysis by iodine assay

For the estimation of starch –iodine hydrolysis assay, took the 1% starch medium and 1 ml of enzyme and 1 drop of iodine solution in test tubes in water bath and incubated at different Temperature (30, 40, 50, 60, 70 °C) for different time interval (15, 30, 60, 90 min.). Blank

was set by 1ml of 1% starch in 1 ml distilled water at OD 580 nm. Similarly treated samples were also measured at this absorbance.

f) Micro-plate based starch iodine assay

Micro-plate-based starch–iodine assay was carried out as follows. Assay reactions were initiated by adding 100 micro litre of starch (1%) solution (1.0 g/100mL) and 100 micro litre crude enzyme and partially purified enzyme separately in 0.1M phosphate buffer at pH 7.0 to micro-plate wells. To minimize evaporative loss during incubation, a plastic mat was used to cover the micro-plate in combination with using a temperature block equipped with a hot lid. After 30min of incubation at 60 °C, when the enzyme is most active, 20 micro litre of 1M HCl was added to stop the reaction, followed by the addition of 20 micro litre of iodine reagent (5mM I_2 and 5mM KI). Following colour development, 200 micro litre of the iodine-treated sample was transferred to a transparent flat-bottomed 96-well micro-plate and the absorbance at 580 nm (*A*580) was measured using micro-plate reader. Micro-plate format assay accurately measures the residual of starch in 100 micro litre samples containing 10–100 micro gram of starch. All experiments were performed in triplicates

One unit (U) for the micro plate-based starch–iodine assay is defined as the disappearance of an average of 1mg of iodine binding starch material per min in the assay reaction.

U/ml was calculated using the formula:

$$\text{Amylase activity (IU/ml/min)} = \frac{\text{(A580 control - A580 sample)}}{\text{Molecular wt of starch x time of incubation x volume of enzyme}}$$

Where,

Mol wt of starch (0.342), time of incubation= 30 min, volume of enzyme=0.01ml

A580 control is the absorbance obtained from the starch without the addition of enzyme,

A580 sample is the absorbance for the starch digested with enzyme.

g) Designing of Optimal Medium using Central Composite Design

To derive the optimum level of screened variables, a central composite design with three coded levels and three variables was used to study the combined influence of Starch (A), yeast extract (B), $MnCl_2.4H_2O$ (C). Twenty experiments were performed in the present study. Using least squares, the regression model was fit to evaluate the linear interaction and the quadratic effects of screened variables.

Response surface methodology is a stepwise approach, used to evaluate the interaction effects of the screened parameters for optimization of amylase production by *Bacillus licheniformis*

NA11. Analysis of the screened variables from a three-level response surface design was complex but led to identification of the key variables affecting amylase production. The results obtained were fed into the Minitab software (Version 17.2.1.0, Minitab®) and analyzed using ANOVA as appropriate to the experimental design used.

Bioconversion of starchy and other starchy waste by *Bacillus licheniformis*

a) Collection

Substrate like Potato peel waste, banana peel waste and orange peel waste was obtained from Hostel and Juice corner of Thapar University, Patiala. It was washed and air dried for 2 days at 60ºC for 24 hr, ground and sieved to obtain particle size. It was stored at room temperature in tight container until further use.

b) Proximate analysis

Determination of moisture content

The moisture content of the biomass were determined using hiven in ASTM 3173-87.Thoroghly mixed, 5.0 g of Biomass were dried at 105 C in hot air oven till a consistency in weight was observed. Following drying, the samples were moved to desiccators for cooling, without absorbing moisture. After one hour of cooling, a dry weight measurement was recorded. The percentage difference of weight is expressed as the moisture content of the sample.

The percentage moisture was calculated as follows:

$$\textbf{Moisture content} = \frac{\text{Weight of sample loss on drying X 100}}{\text{Weight of sample}}$$

Determination of ash content

"Ash" is defined as the amount of inorganic material present in the biomass. The ash content in biomass were determined by NREL protocol (LAP NREL.TP-510-42622; 2008). Oven dried samples (2.0 g) were taken in crucible and mass of crucible along with specimen was determined. Crucible and mass of crucible along with the specimen was determined. Crucible was placed in muffle furnace and heated up to 575 C for 4 hr. Crucible was removed from the furnace and placed in the desiccators. The above process of heating and cooling was repeated until the specimen is completely ash (no change of mass occurs after a further period of

heating) and after cooling dry weight was recorded. This process allows removal of the volatiles and un-burnt carbon.

Ash content was calculated as follows:

$$\textbf{Ash content (\%)} = \frac{\text{Weight of crucible sample before processing} - \text{Weight of crucible sample after processing}}{\text{Weight of sample}} \times 100$$

Determination of volatile matter

The volatile matter in the biomass was determinant by the procedure given in ASTM D 3175-07. Approximately 1.0 gram of the biomass was taken in crucible with cover and initial weight was determined. The covered crucible was placed in muffle furnace regulated at 950 C for 7 min to obtain heating. Then the covered crucible was cooled to room temperature in a desiccators and weight was recorded. The percentage weight loss was regulated as the percentage of volatile matter.

Volatile matter was calculated as follows:

$$\textbf{Volatile matter (\%)} = 100 - \left(\frac{\text{Weight of crucible \& sample} - \text{Weight of Crucible \& sample after drying X 100}}{\text{Weight of sample}} \right)$$

Determination of biomass extractives

Extractives in agriculture and kitchen waste were determined using NREL protocol (Sluiter et al., 2008).

Hexane extraction of biomass

Biomass sample (10 g) was added to a tared extraction thimble and weight was recorded to the nearest 0.1 mg. Soxhlet apparatus was assembled and thimble was inserted in to the soxhlet tube. 190 ml of hexane was added to hexane tared receiving flask and receiving flask was placed on the soxhlet apparatus and heating mantles were adjusted to provide a minimum of 4-5 siphon cycles per hour. Samples were refluxed for 12 hr. After refluxing, heating mantles were turned off and glassware was allowed to cool to room temperature. Solid was washed with approximately 100 ml of hexane and allowed to dry.

Methanol extraction of biomass

Biomass sample (10 g) was added to a tared extraction thimble and weight was recorded to the nearest 0.1 mg. Soxhlet apparatus was assembled and thimble was inserted in to the soxhlet tube. 190 ml of Methanol was added to methanol tared receiving flask and receiving flask was placed on the soxhlet apparatus. Heating mantles were adjusted to provide a minimum of 6-10 siphon cycles per hour. Samples were refluxed for 12 hr. After refluxing, heating mantles were turned off and glassware was allowed to cool to room temperature, Thimble was removed and extracted Solid was transferred, on to Watts-man paper in a buncher funnel. Solid was washed with approximately 100 ml of methanol and allowed to dry.

Water extraction of biomass

Biomass sample (5 g) added to 100 ml Distilled water and placed on shaker for overnight at room temperature. Filter the sample and measured the 25 ml extracted sample. Weight the Petri-plate. Now, pour the extracted sample (25 ml) in Petri-plate (triply). Placed each Petri-plate on water bath incubation for 1- 2 hrs. Placed the petri-plate in hot air incubator for 1 hr at 105°C temperature. Measured the weight of petri-plate with dried sample without moisture.

Estimation of cellulose

Estimation of cellulose in agriculture and kitchen waste biomass was done by Anthrone Assay (Updegraff, 1969).

About 3 ml acetic acid/nitric acid reagent(15 ml of 80% acetic acid mixed with 1.5 ml of concentrated nitric acid) was added to 0.1 g of waste biomass and the mixture was kept in a water-bath at 100 C for 30 min.. After cooling the sample was centrifuged at 8000 rpm for 10 min and supernatant was discarded. The pallet was washed twice with distilled water and 10 ml sulphuric acid (67%, v/v) was added and resulting suspension was allowed to stand for 1 hr. the suspension was diluted to 10 times, and 2 ml of anthrone reagent (200 mg of Anthrone in 100 ml of concentrated sulphuric acid) was added to 1 ml of diluted mixture. The mixture was inoculated in a boiling water bath for 15 min. for colour development. After cooling the tubes, the optical density was ready 620 nm against the reagent blank (1ml distilled water and 2 ml anthrone reagent). The concentration of cellulose in the sample was calculated using cellulose standard (Updegraff *et al.,* 1969) (Fig. 9).

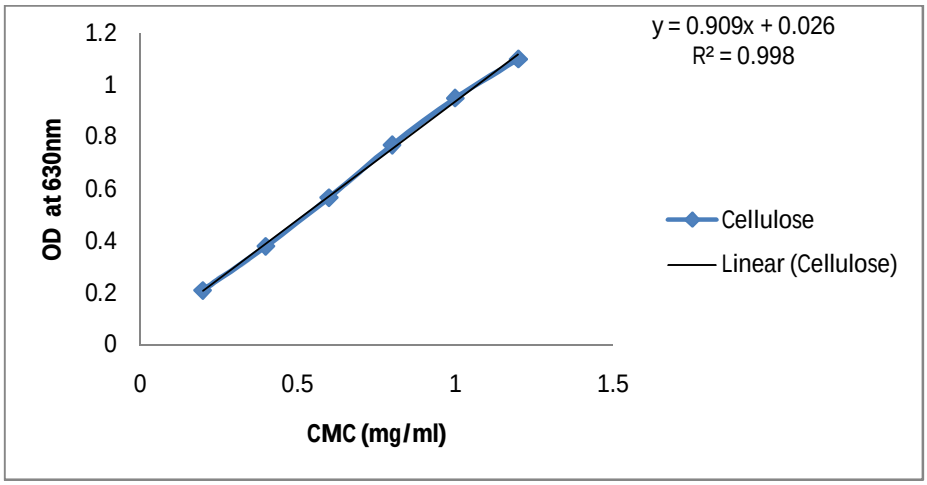


Fig. 9 Standard graph of cellulose concentration

Estimation of Starch concentration by phenol solution

Reagent: Phenol solution 5 % (w/v), concentrated H_2SO_4 (95%)

Procedure

Starch (Hi media labs, Mumbai, India) standard solutions were prepared in the range of concentration (0.2, 0.4, 0.6, 0.8 and 1 mg/ml). 200µl of Starch was pipette out for standard curve. 0.5 ml of phenol solution was added and mixed well and add 2.5 ml of H_2SO_4, and incubated at 25°C for 15 min. Then O.D was taken at 490 nm (Dubois *et al.*, 1956) (Fig. 10).

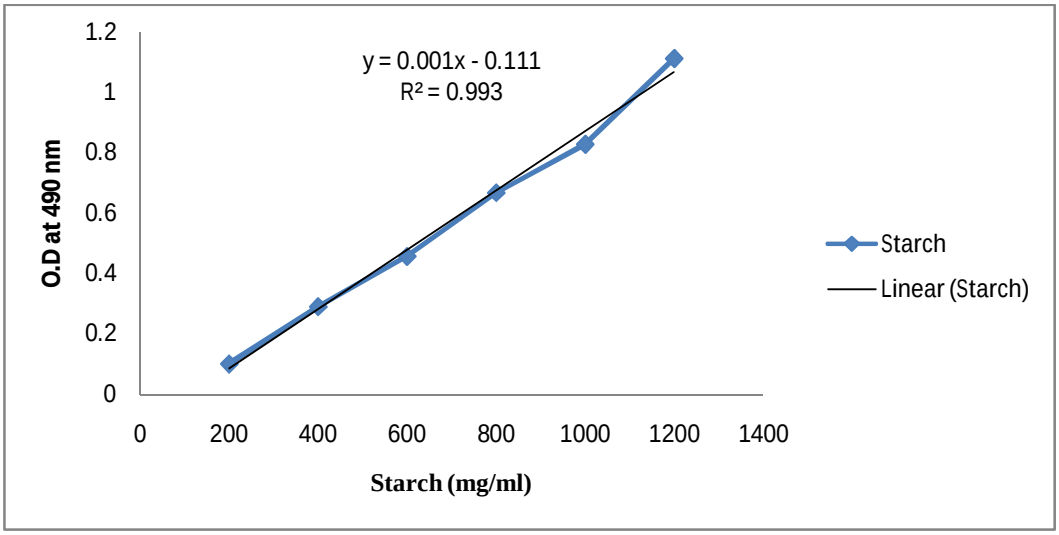


Fig. 10 Standard graph of starch concentration

Estimation of Protein concentration

To study the protein concentrations of bacterial isolates, 1% (v/v) overnight grown bacterial inoculums was added to 100 ml media in 250 ml flask for incubated at 37°C under shaking condition (120 rpm) for 36 h. The aliquots were an interval of 4h, centrifuged (8000 rpm, 20 min) and culture supernatants were used for protein estimation by Folin- Lowery method (Lowery *et al*., 1951).

Reagent

Reagent A: 20 g Na_2CO_3, 4 g NaOH, add distilled water to make 1000 ml

Reagent B: 1 g $CuSO_4.5H_2O$, 2 g Na-K tartarate and add distilled water to make 100 ml.

Reagent C: Mixed these in order 100 ml of Reagent A and 2 ml of Reagent B.

Procedure

Bovine serum albumin (Hi media labs, Mumbai, India) standard solutions were prepared in the range of concentration 0.2, 0.4, 0.6, 0.8 and 1 mg/ml). 200µl of BSA was pipette out for standard curve. 5 ml of Reagent C was added and mixed well with taken BSA sample and incubated for 10 min. 1 N phenol reagent (0.5) was added and mixed at once and incubated for 30 min and then O.D was taken at 660 nm (Fig.11).

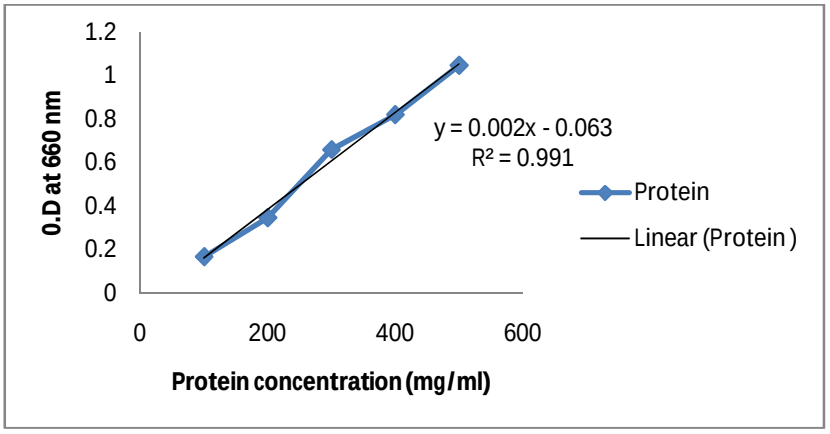


Fig. 11 Standard graph of protein concentration

Estimation of reducing sugars

Reducing sugars were estimated by using DNS (3,5-dinitrosalicyclic acid) method given by Miller, 1959.

Material

1) Stock : 1mg/ml
2) DNS reagent
    a. Distilled water= 100 ml
    b. Potassium sodium tartatate = 18.2 g
    c. NaOH pallet= 1g
    d. DNS= 1g
    e. Sodium meta bi-sulphite= 0.5g
    f. Phenol= 0.2 ml

Method of preparing DNS assay reagent

1. Boil the 50 ml distilled water in 150 ml beaker.
2. Add NaOH pallet in boiled Distilled water and dissolve it
3. Add DNS in above mixture and again dissolve it
4. Add potassium tartarate and sodium meta bi-sulphite and phenol.
5. Make up the distilled water up to 100 ml.
6. Now pour this reagent in to dark colored reagent bottle covered with aluminum foil.

Method

1) Different concentrations of glucose were prepared ranging from 0.2 to 1 mg/ml and volume was made to 1 ml
2) Then the 3 ml of DNS was added and placed in boiling water bath for 10 minute.
3) OD was taken at 540 nm
4) A standard graph was prepared using stock concentration.
5) In the blank: 2 ml distilled water and 3 ml DNS assay reagent.
6) In control: 1 ml control sample, 1 ml distilled water and 3 ml DNS assay reagent.
7) For sample analysis: cell free supernatant (8000 rpm) 1ml was taken and the same procedure was followed. Make the dilution if sample colour is dark (1 ml sample and 9 ml distilled water). Then the dilution factor comes out to be 10. (Table 2; Table 3; Fig. 12).

Formula:

$$\textbf{Activity (IU/ml/min)} = \frac{\text{Amount of sugar released X 1000}}{\text{Molecular wt of glucose X Time of incubation X Volume of enzyme}}$$

Where ,

Mol wt of glucose = 0.18

Time of incubation = 10 min.

Volume of enzyme = 1 ml

Table 2 Standard curve of glucose using DNS method

Glucose (mg/ml)	**Distilled water (ml)**	**DNS reagent (ml)**	**Incubation**	**OD**
0.2 or 200µl	0.8 or 800µl	3	Incubation at 90° C for 10 min. in waterbath	O.D taken at 540 nm
0.4	0.6	3		
0.6	0.4	3		
0.8	0.2	3		
1.0	0	3		

Table 3 Optical density of reducing sugar at 540 nm

Reducing sugar (mg/ml)	**O.D. (540 nm)**
0.2	0.233
0.4	0.435
0.6	0.659
0.8	0.910
1.0	1.2

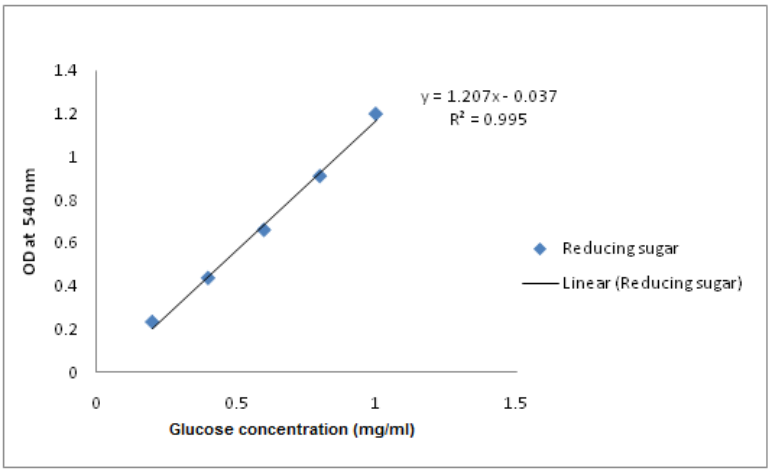


Fig. 12 Standard Graph of Reducing sugar.

c) Pre-treatment methods

Pre-treatment methods of processed substrate

Two methods of pre-treatment of starchy waste by dilute acid-alkali hydrolysis (Taherzadeh *et al.*, 2007) and enzymatic hydrolysis were done

Acid-alkali hydrolysis

1. Processed potato, orange and banana peel starchy waste (5 gm) were taken in different each (250) Erlenmeyer flask.
2. One percent of H_2SO_4 (10 ml) was added to each flask.
3. The each agriculture flask waste were then autoclaved at 121 C for 15 min.
4. Residues were again boiled with 1N NaOH (5ml) in water bath at 60° C for 5 min.
5. Residues were washed with distilled water to neutralize pH.
6. It was filtered by what man filter paper no 42 and then dried in oven at 70°C for 12-24 hrs.
7. Take the 1 gm of dried residues and add 1 ml distilled water.
8. Add 2 ml of DNS reagent, boiled at 90°C for 10 min. and check the reducing sugar at OD 540 nm.

Enzymatic hydrolysis

1. Processed potato, orange and banana peel starchy waste (1 gm) and soluble (1%) starch were taken in test tube and volume was made to 1 ml with distilled water
2. Add 1ml crude Supernatant enzyme in Residues and taken in to water bath at 60° C for 1 hour.
3. The reaction mixture should centrifuge at 11000 rpm and pallets were discarded and took supernatants 1 ml each were taken.
4. Add 2 ml of DNS reagent, boiled at 90°C for 10 min. and check the reducing sugar at OD 540 nm.

Biological treatment

Inoculum preparation

The bacterial isolate NA11 was grown in 100 ml enriched media in 250 ml flask was inoculated with 5 % overnight grown culture (O.D. 0.6 at 600 nm) and incubated at 37 C under shaking condition (120 rpm) for 24 h.

Biological treatment

The biological treatment was carried out in 250 ml flask with (5 g) of biomass such as potato peel waste, orange peel waste and banana peel waste and add in to (95) ml of distilled water. The samples were sterilized in autoclave for 15 min. (121 C, 15lbs) and inoculated with 5 ml inoculums .The samples were incubated at 37 C, for 1 month with agitation at 150 rpm. At the end of incubation, substrate was thoroughly washed with Distilled water and dried at 60 C in an oven for 12 h and further these samples were used for the analysis of SEM, XRD and FTIR techniques.

SEM, XRD, FTIR analysis

Scanning Electron Microscopy (SEM) analysis of starchy waste biomass

SEM of dried untreated and pretreated starchy biomasses were carried such as potato peel, banana peel and orange peel waste out using Scanning Electron Microscope (Model: EV- 40, Carl Zeiss, India) at 20 kV. Prior to viewing, the samples were sputter coated with thin layer gold. Micrographs were taken at different magnifications from 1,000 x to 10,000 x depending upon the feature to be traced.

X-ray Diffraction (XRD) analysis of starchy waste biomass

Treated and untreated biomass was vacuum dried to remove the water content from the cellulosic substrate. The effect of treatment by co-inoculation of *Bacillus licheniformis* NA11 on the crystalline structure of starchy waste biomass were determined by X-ray diffraction at room temperature in the scanning angle of 5-50° at the scan speed of 0.5 min-1 using a PANalytical X'Pert PRO system with Ni-filter and diffract meter operating at 45 kV and 130 mA with Q (CuKR) = 1.5406 A°.

Fourier transform infrared (FTIR) spectroscopy analysis

Fourier transform infrared (FTIR) spectroscopy analysis was carried out to detect changes in functional groups of native and treated starchy biomass, were obtained using Nicolet instrument (Model: MAGNA 550, USA). The biomass (10 mg) was well mixed with 200 mg of KBr and the mixture was compressed for preparation of pellets. Each spectrum was the average of 64 scans with a total scan time of 15 s in the IR range of 400 - 4000 cm -1 at 1 cm -114.

Ethanol production from starchy wastes by Simultaneous saccharification and fermentation (SSF)

Media composition for SSF

The fermentation media were prepared in 100 ml Erlenmeyer flask for SSF using amylase from *Bacillus licheniformis* NA11 and *Saccharomyces cerevisiae*.

The fermentative microorganism, *Saccharomyces cerevisiae* (NCIM 3215) was independently grow at 4°C on 5 ml of GYP slants. After 24 h incubation at 30 C the agar slants were stored at 4°C. Starter culture was prepared by inoculation of one loop-full from agar slant cultures into 50 ml of glucose yeast extract medium in two separate 100 ml Erlenmeyer flask. Flasks were incubated at 30° C and 120 rpm for 48 h prior to inoculation into fermentation media.

Table 4 SSF media ingredient for ethanol production

Ingredient	**Amount(g/l)**
Distilled water	90ml
Substrate	5g
Na_2HPO_4	6g
KH_2PO_4	3g
NaCL	0.5g
NH_4CL	1g
Yeast extract	0.5g
peptone	0.5g

Experimental procedure

1. Minimal media 100 ml supplemented with processed waste biomass such as potato, orange and banana peel agriculture waste (5 gm) and soluble (1%) starch were taken in different each (250) Erlenmeyer flask and pH of media were adjusted to 7 (Table 4).
2. The each flask containing starchy wastes were then autoclaved at 121°C for 15 min. and cooled at room temperature
3. **Stage 1**: Two percent of overnight grown cellulose and starch degrading bacterial strain (NA11) was inoculated in flasks contain fermentation media and incubated at 37° C for 2 days under shaking condition at 120 rpm. (In case of enzymatic

hydrolysis SSF the crude supernatant enzyme (10 ml) taken and flasks were boiled at 60°C in water bath for 1 hour and placed the flask at 37°C for 24 hour).

4. **Stage 2**: Twenty four hour grown (10%) *Saccharomyces cerevisiae* was added to each flask after 24 hr of incubation and placed in incubator at 30°C under shaking condition at 120 rpm for 7 days.
5. Samples were withdrawn at a regular interval of 48 h and analyzed for reducing sugar and ethanol as per methods explained earlier.

1. Ethanol estimation by Chemical method (potassium dichromate ($K_2Cr_2O_7$))

Ethanol was estimated by potassium dichromate method, it involves extraction of ethanol from a culture broth followed by dichromate oxidation. When ethanol is present in aqueous solution, chromium ions oxidize ethanol, and these ions are reduced from +6 oxidation state to +3, changing the colour from orange to green.

Material

a. Ethanol
b. Potassium dichromate: it was prepared by dissolving 10 g potassium dichromate in 100 ml of 5 M sulhuric acid solution.
c. Di-n-butyl phthalate (DBP)

Method

Different concentration of ethanol was prepared ranging from 2 to 10 % and volume was raised to 2 ml.

1. 2 ml of sample was taken in test tube with different concentration
2. 2 ml of DBP was added to each above tubes of different concentration.
3. The mixture was shake vigorously for 1 hour on shaker at 250 rpm.
4. The lower phase (1.5ml) was transferred to new micro-tube.
5. Then 1.5 ml of potassium dichromate was added to mico-tube.
6. The mixture was shake vigorously for 30 min. on shaker at 250 rpm.
7. Then O.D. of the lower phase was taken at 595 nm.
8. Standard curve was prepared and ethanol concentration was estimated from unknown samples

Sample of standard ethanol concentration sample should be diluted to 10 times (1 ml sample + 9ml DW). In blank only 1.5 ml Distilled water and potassium dichromate 1.5 ml should be taken and set on auto zero before O.D taken of samples (Table 5; Table 6; Fig. 13).

Table 5 Standard curve of ethanol using potassium dichromate method

Ethanol (µl)	Distilled Water (ml)	Ethanol (%)	DBP (ml)	Kept on shaker for 1 hour and there after 1.5 ml of lower layer was taken	Potassium dichromate (ml)	Kept on Shaker for 30 min. and there after 1.5 ml of lower layer was taken for absorbance	OD ` ` Optical density taken at 595 nm
0	2	0	2		1.5		
40	1.96	2	2		1.5		
80	1.92	4	2		1.5		
120	1.88	6	2		1.5		
160	1.84	8	2		1.5		
200	1.80	10	2		1.5		

Table 6 Standard curve for ethanol production using potassium dichromate method

Ethanol (%)	O.D AT 595 (nm)
0	0
2	0.228
4	0.428667
6	0.623333
8	0.856667
10	1.259333

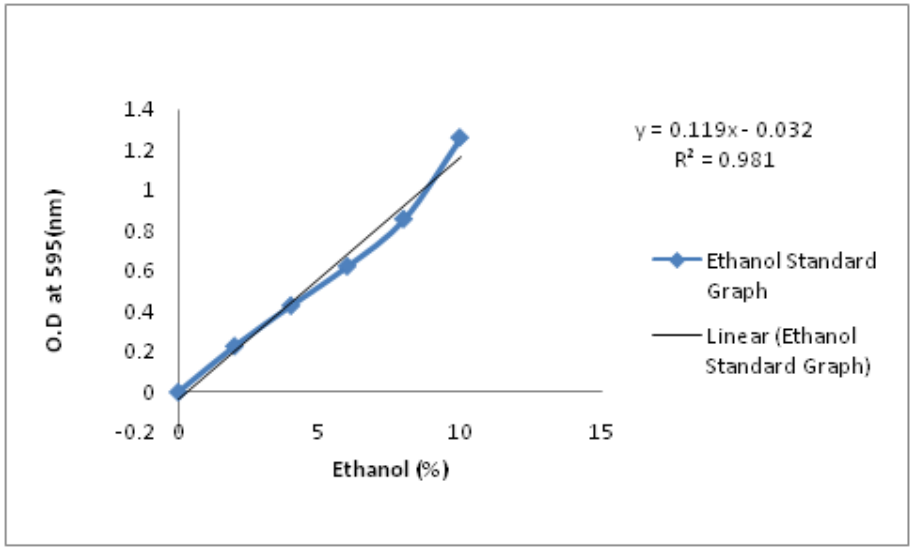


Fig. 13 Standard graph of Ethanol using potassium dichromate method

2. Ethanol estimation by Gas chromatography (GC)

Gas chromatography is used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. In gas chromatography, the mobile phase carrier gas, usally an inert gas such as helium or nitrogen.

The stationary phase is microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column. The instrument used to perform gas chromatograph was Agilent head space.

Method

1. Different concentration of ethanol (2 to 10%) were prepared, samples (1µl) were injected and chromatogram was obtain using flame ionization detector with GC under following condition:

   Oven temperature: 155° C

   Inlet temperature: 175° C

   Detector temperature: 250° C

   Carrier gas flow: 30 ml/min

   Hydrogen and air flow rate: 30 ml/min and 300 ml/min, respectively.
2. Samples were prepared by centrifugation at 8000 rpm for 15 min. and then filtered through membrane using a membrane filter of 0.45 µm. the samples were kept at 4 C prior to analysis. The samples should not be preserved for more than 30 days.
3. Ethanol concentration of unknown samples was calculated from standard curve.

$$\textbf{Calculation} = \frac{\text{Sample area x Weight of standard x 100}}{\text{Standard area x Weight of sample}}$$

Here, given standard area average = 12222122.67 and weight of standard = 30 mg (provided by Herbal Health Research Consortium Pvt. Ltd. in Amritsar HHRC, Amritsar lab)

CHAPTER 4
RESULTS AND DISCUSSION

Bacillus licheniformis NA 8, 9, 11 were grown and checked for starch hydrolysis activity. *Bacillus licheniformis* NA11 was grown and cell free supernatant was checked for amylase activity in minimal media containing 1% starch. Precipitation of amylase from cell free supernatant was carried by ammonium sulphate precipitation. Effect of temperature, pH and substrate concentration on starch hydrolysis was checked on micro-plate based starch iodine assay.

Screening of bacterial isolates for amylase activity production using plate assay method

Bacillus licheniformis NA8, NA9, NA11 were tested for amylase activity by employing zone clearing technique (Atlas et al 1995) using starch (0.5%, 1%, 2%) agar medium. The development of blue colour indicated the presence of starch, while the areas around the hydrolytic bacteria appeared clear. The best amylase activity was shown by strain NA11 on 1 % starch and was selected for further work (Fig. 14).

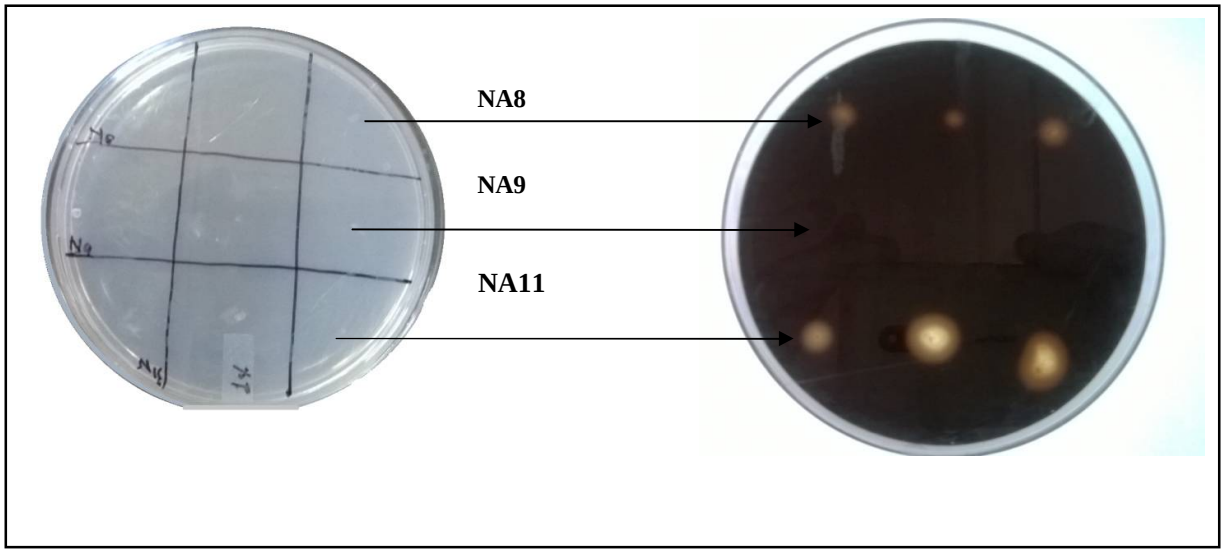


Fig. 14 Zone of inhibition shown by *Bacillus licheniformis* NA8, NA9, NA11 using iodine plate method

Strains

To check the purity of the cultures morphology study of strain *Saccharomyces cerevisiae* and *Bacillus licheniformis* NA11 was done using light microscope.

Microscopy of the strains

1. **Gram staining of *Bacillus licheniformis* NA11(Christian Gram, 1884)**

   The strain *Bacillus licheniformis* was visualized under light microscope at 40 x magnification and was Gram positive rod shaped bacteria (Fig. 15).

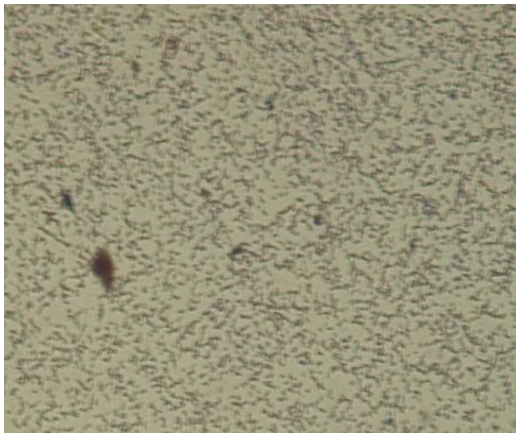


Fig. 15 Gram staining of *Bacillus licheniformis* NA11 (40x magnification)

2. **Methylene blue staining of *Saccharomyces cerevisiae***

   Saccharomyces cerevisiae was stained with methylene blue dye. This strain was visualized under microscope at 40 x magnification (Fig. 16)

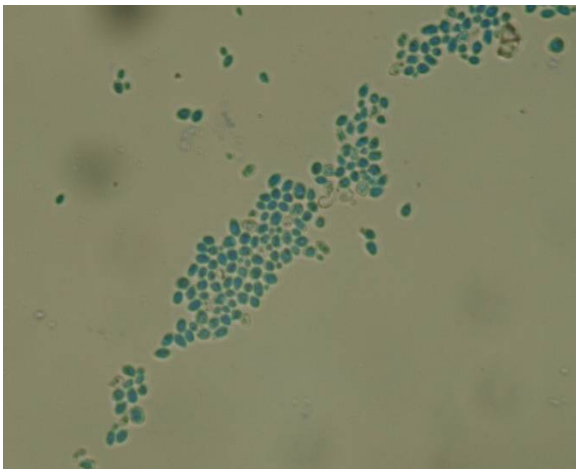


Fig. 16 Methylene blue staining of *Saccharomyces cerevisiae (*40 x magnification)

Amylase activity of crude enzyme from *Bacillus licheniformis* NA11 (Miller, 1959)

Amylase activity was determined by estimating the amount of reducing sugar using DNS method as per Miller, 1959 using glucose (dextrose) as the standard. Amylase activity of *Bacillus licheniformis* was 1.15 U/mg/min at 60°C in 30 min..

Ammonium sulphate precipitation

Ammonium sulphate precipitation of cell free supernatant of 24 hr old grown culture was done up to 80% saturation. And amylase activity of partially purified enzyme was studied by iodine method as described earlier in the presence of 1 %(w/v) starch at pH 7 at 60ºC for 30 min. and was found to be 20% =1.41 U/mg, 40% =1.59 U/mg, 60% =2.07 U/mg, 80% =1.57 U/ml/min at 60°C for 30 min (Table 7).

Table 7 Purification of amylase from *Bacillus licheniformis* NA11 by ammonium sulphate precipitation

Procedure	Reducing sugar (mg/ml)	Total activity (U)	Volume (ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification (fold)
Crude enzyme	0.467	116.7	250	0.415	1.15	1
Ammonium sulphate 20%	0.494	49.4	100	0.348	1.41	1.22
Ammonium sulphate 40%	0.538	53.8	100	0.338	1.59	1.38
Ammonium sulphate 60%	0.677	67.7	100	0.326	2.07	1.8
Ammonium sulphate 80%	0.475	47.5	100	0.301	1.57	1.36

Effect of different parameters on amylase activity of NA11

Effect of starch concentration, temperature, pH and time of incubation on starch hydrolysis by *Bacillus licheniformis* was studied by starch iodine assay in test tube and Micro-plate based starch iodine assay. The enzyme activity was also checked using response surface methodology (RSM).

a) Effect of substrate concentration

Crude enzyme from cell supernatant of *Bacillus licheniformis* NA11 was checked for amylase activity using different substrate concentration. Starch was taken as substrate at varying concentration. The crude enzyme showed the highest hydrolytic activity of 0.53 U/ml/min was against 1% (w/v) starch with production of 0.22 mg/ml of reducing sugar. At 0.5 % (w/v) starch concentration it was less than 1% (0.462 U/ml) and at 1.5%(w/v) starch amylase activity was 0.414, at 2% (w/v) it was 0.385 U/ml and at the 2.5%(w/v) the amylase activity was 0.371 U/ml (Table 8 ; Fig. 17). Data showed that enzyme hydrolysis 1% starch with maximum amylase activity. Below and above 1% starch amylase activity was less because the amount of substrate was less for reactivity or amount of enzyme fully occupied with substrate respectively. In crude enzyme some inhibitors may be present which may decrease enzyme activity above 1% starch (Khawla *et al.*, 2014)

Table 8 Effect of starch concentration on amylase activity from NA11

Starch (%)	Reducing sugar (mg/ml)	Enzyme activity (U/ml/min)
0.5	0.192	0.462
1.0	0.220	0.530
1.5	0.172	0.414
2.0	0.160	0.385
2.5	0.154	0.371

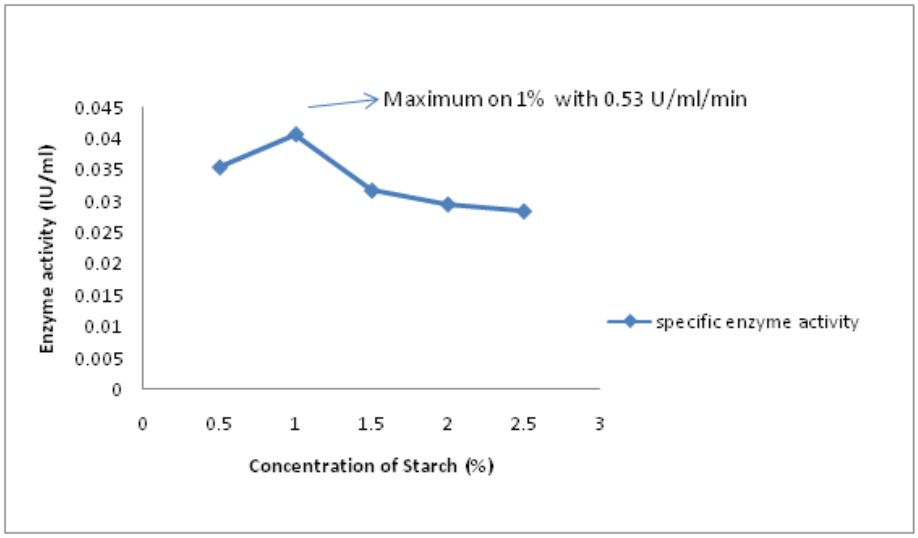


Fig. 17 Effect of starch concentration on amylase activity from NA11

b) Effect of pH on amylase activity

Because most of the *Bacillus* strains used for the production of amylase have an optimum pH between 6.0–9.0 pH for growth in minimal media. The optimum pH of the crude enzyme from *Bacillus licheniformis* NA11 was found to be at pH 7 (1.11 U/ml/min) on 1% (w/v) when incubated for 15 min at 60°C .which was in accordance with the previous study by (Shukla *et al.*, 2006), where pH 7.0 - 9.0 was more suitable for cellulase produced by *Bacillus subtillus* and *Bacillus licheniformis*. (N Shinde *et al.*, 2014) also reported amylase from *Bacillus licheniformis*, which were active at an optimum pH of 6.0 – 9.0 for wild type and mutants (Table 9; Fig. 18).

Table 9 Effect of pH on amylase activity from NA11 using 1% starch

pH	Reducing sugar (mg/ml)	Enzyme activity (U/ml/min)
5	0.312	0.751
6	0.405	0.975
7	0.461	1.11
8	0.258	0.621
9	0.198	0.477

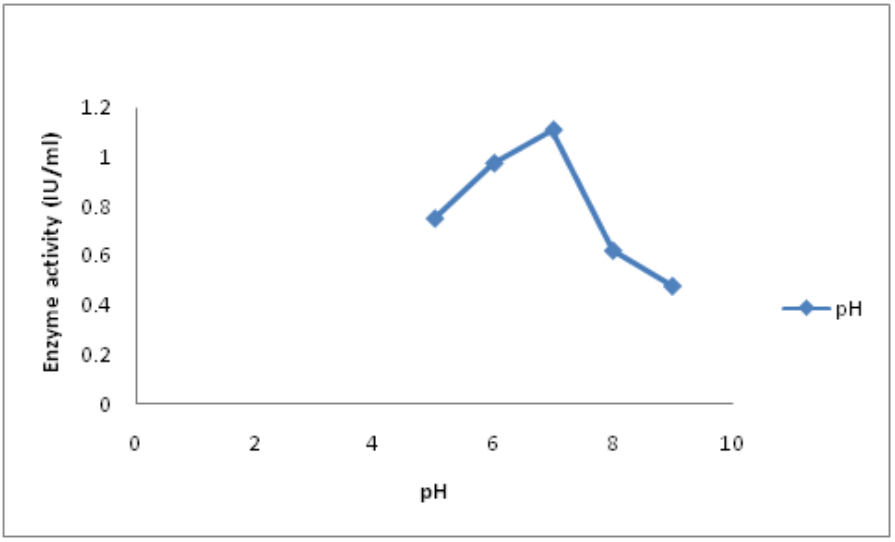


Fig. 18 Effect of pH on amylase activity from NA11 using 1% starch

c) Effect of temperature on amylase activity

The result indicates that up to 60°C enzyme is thermostable (Table 10 ; Fig. 19). The optimum temperature for amylase activity from *Bacillus licheniformis* NA11 was 1.12 U/ml/min at 60°C with production of 0.467 mg/ml of reducing sugar. The enzyme was stable between 50°-60°C. Vengadaramana *et al*., 2013 reported the activity of crude and partially purified amylase at temperature ranging from 40°C – 90°C, and showed optimal activity at 85°C on pH 7. Deb et al., 2013 also reported the optimal activity of *Bacillus amyloliquefaciens* P001 on 50°C.

Table 10 Effect of temperature on amylase activity from NA11 using 1% starch

Temperature (°C)	Reducing sugar (mg/ml)	Amylase specific activity (U/ml/min)
30	0.296	0.713
40	0.38	0.915
50	0.422	1.01
60	0.467	1.12
70	0.299	0.720
80	0.215	0.518
90	0.175	0.421

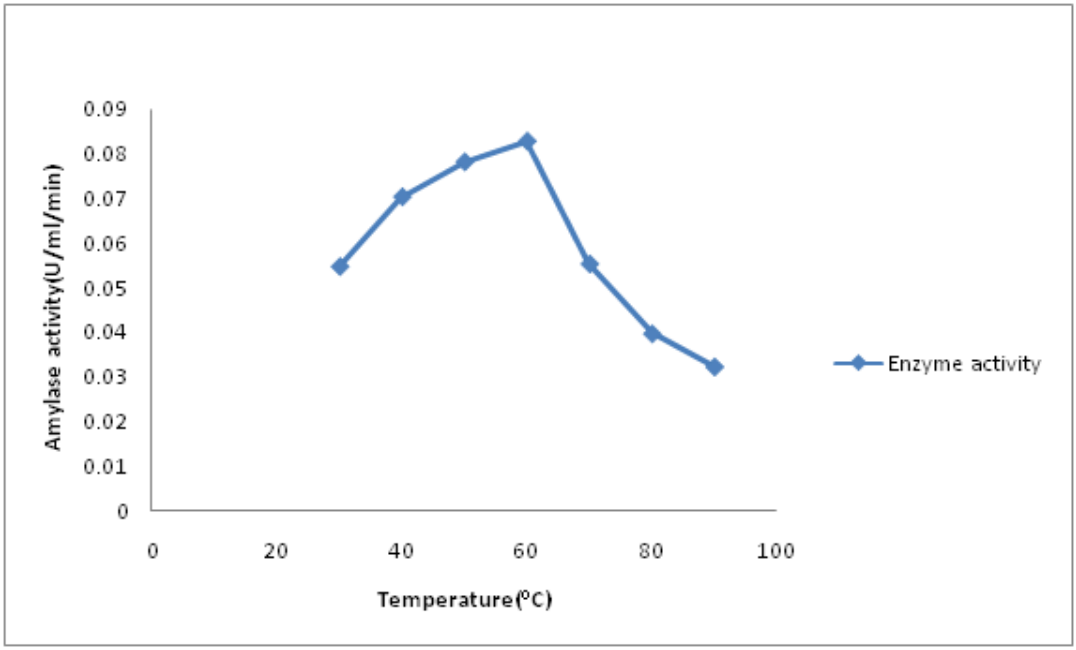


Fig. 19 Effect of temperature on amylase activity from NA11 using 1% starch

d) Effect of temperature and incubation time on amylase activity

Thermo-stable enzymes have more applicability in industry. Maximum enzyme activity of 1.12 U/ml/min was observed at 60°C in 30 min of incubation where 0.467 mg/ml of reducing sugar was produced from starch and stability up to between 50°-60°C (Table 11; Fig. 20).

Table 11 Estimation of reducing sugar at different temperature (mg/ml)

Time (min)	30° C	40° C	50° C	60° C	70° C
0	0	0	0	0	0
10	0.105	0.158	0.192	0.209	0.123
15	0.216	0.324	0.332	0.339	0.28
30	0.296	0.38	0.422	0.467	0.299
60	0.29	0.272	0.277	0.287	0.268

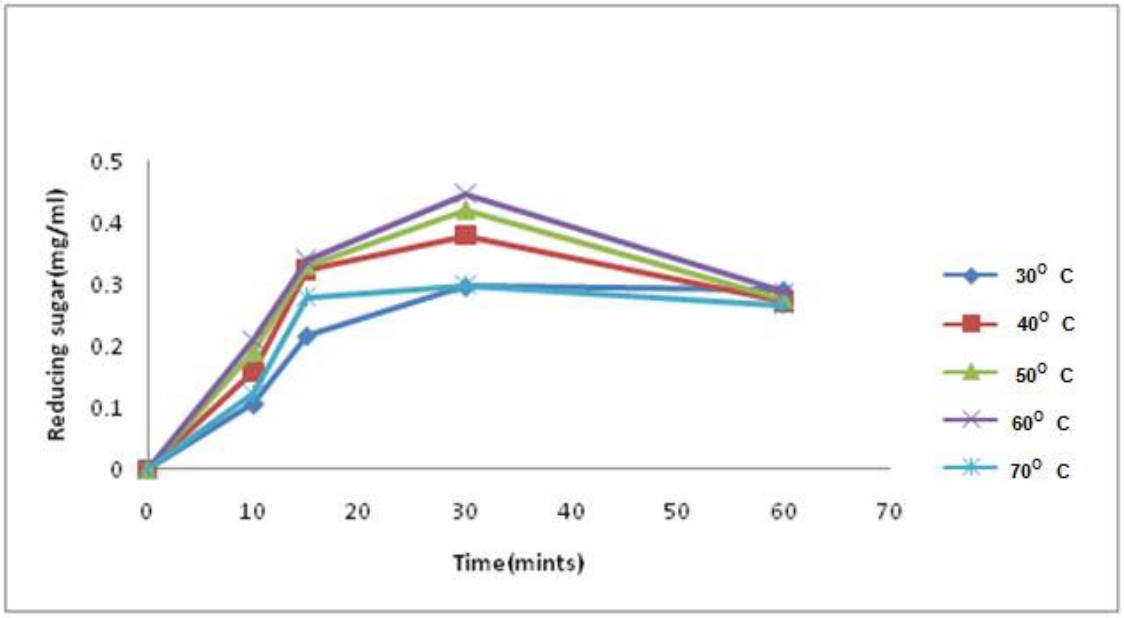


Fig. 20 Estimation of reducing sugar at different temperature (mg/ml)

Bioconversion of starchy wastes

Amylase activity of partially purified enzyme from *Bacillus licheniformis* showed maximum activity of 1.12 U/ml/min, with production of 0.467 mg/ml reducing sugar when 1% starch was used as substrate. When starchy wastes were used as substrate the enzyme activity in 160 min at 60°C was 1.048 U/ml/min for orange peel waste (OPW) with production of 0.435 mg/ml of reducing sugar, 0.99 U/ml/min for banana peel waste (BPW) with production of 0.411 mg/ml of reducing sugar and 0.959 U/ml/min for potato peel waste (PPW) with

production of 0.398 mg/ml of reducing sugar. These were used as substrate for hydrolysis by crude enzyme from NA11 at parameters optimized, (Table 12; Fig. 21).

Table 12 Bioconversion of starchy waste in to reducing sugar by crude enzyme from NA11 at different temperature and incubation time

Time	PPW T40	PPW T50	PPW T60	BPW T40	BPW T50	BPW T60	OPW T40	OPW T50	OPW T60
0	0	0	0	0	0	0	0	0	0
10	0.022	0.031	0.045	0.03	0.056	0.067	0.077	0.087	0.089
15	0.146	0.167	0.213	0.176	0.197	0.246	0.198	0.211	0.266
30	0.266	0.279	0.323	0.275	0.298	0.365	0.286	0.31	0.395
60	0.302	0.383	0.398	0.325	0.391	0.411	0.324	0.395	0.435

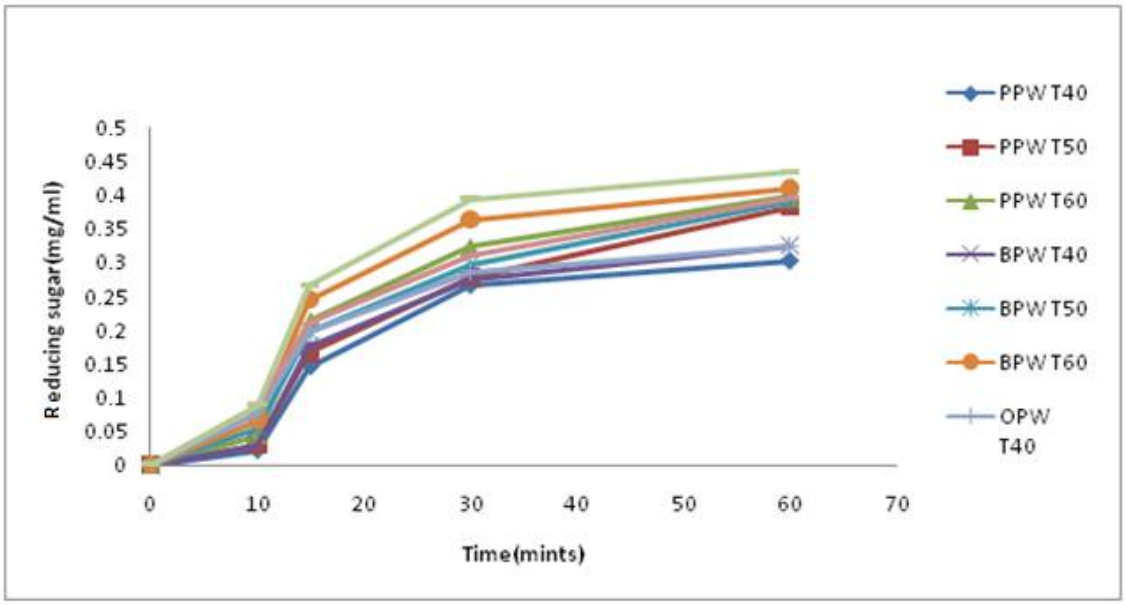


Fig. 21 Bioconversion of starchy waste in to reducing sugar by crude enzyme from NA11 at different temperature and incubation time

e) Estimation of starch hydrolysis by iodine assay

Maximum degradation of 1% starch was at 60°C and 30 min of incubation with crude enzyme from *Bacillus licheniformis* NA11. As judged by iodine assay this was performed in test tube which showed blue colour in the presence of starch- iodine reaction. When enzyme is added and kept at different temperature in water bath for 15- 90 min the blue colour disappeared due to starch hydrolysis. It was observed seen that at 60°C and 70°C all blue color disappeared after 60 min. (Table 13; Fig. 22; Fig. 23).

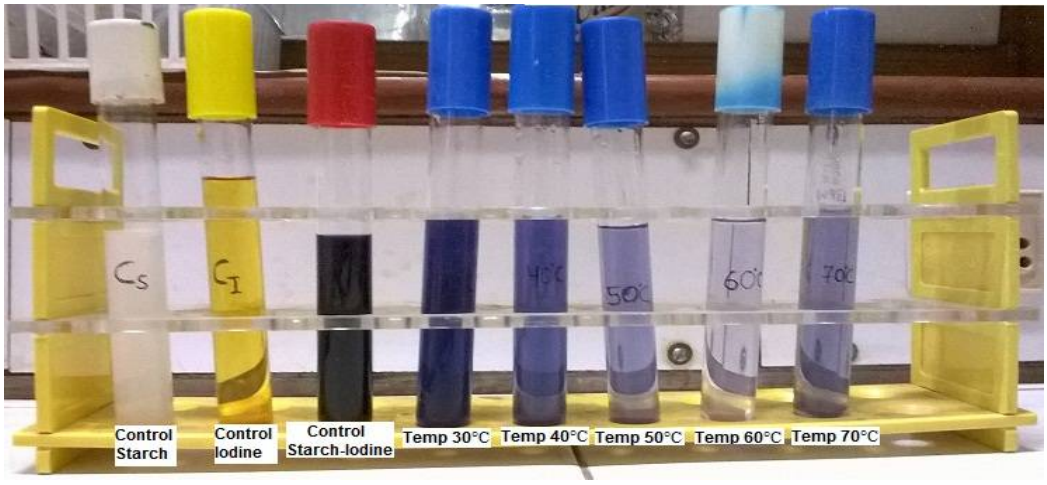


Fig. 22 Starch hydrolysis from NA11 at different temperature by Starch-Iodine method (Time: 60 min.)

Table 13 Starch hydrolysis from NA11 at different temperature and time of incubation

Time	30° C	40° C	50° C	60° C	70° C
0	3.684	3.681	3.168	3.673	3.692
15	2.818	2.784	2.48	2.262	2.465
30	2.518	2.462	1.816	0.536	1.5656
60	1.095	0.964	0.935	0.177	0.787
90	0.232	0.154	0.1316	0	0.1

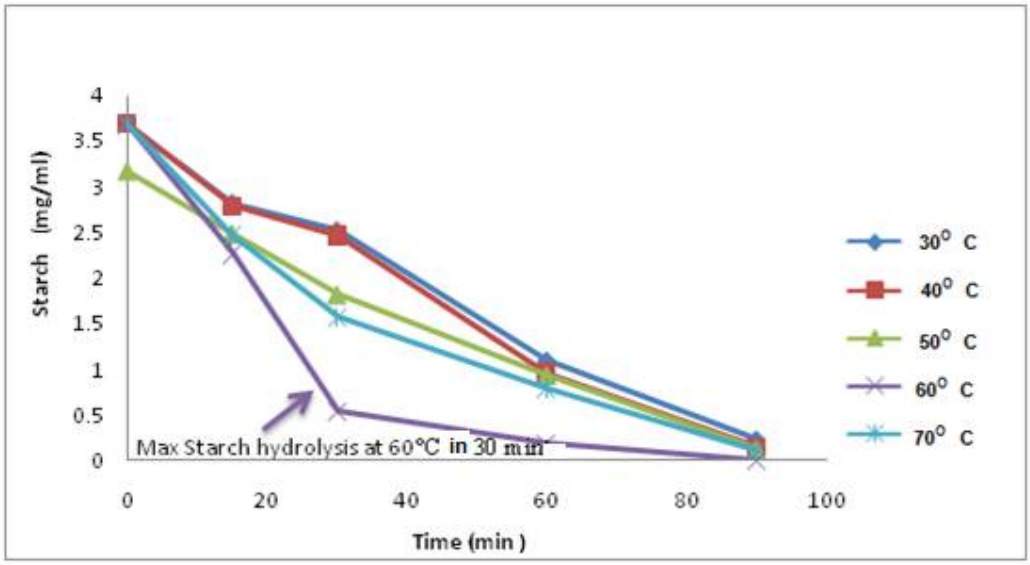


Fig. 23 Starch hydrolysis from NA11 at different temperature and time of incubation

f) Micro-plate based starch iodine assay

During the development of micro-plate-based starch–iodine assay, following observations were made. Maximum absorbance for the starch–iodine complex was at 580 nm using 2.5mM iodine for blue colour (Table 14 and Fig. 24).

Table 14 Starch hydrolysis by crude and partially purified enzyme by NA11 using iodine method

Concentration of distilled water	Concentration of starch µl	Crude enzyme	Ammonium sulphate precipitate enzyme			
			20%	40%	60%	80%
0	**100**	3.4501	3.7171	2.927	2.918	3.098
10	**90**	3.3533	3.652	2.768	2.81	2.999
20	**80**	3.1896	3.4081	2.698	2.441	2.812
30	**70**	3.03906	3.1882	2.567	2.224	2.752
40	**60**	2.8351	2.456	2.431	1.799	2.685
50	**50**	2.6646	2.281	1.865	1.86	2.329
60	**40**	2.3257	2.103	1.64	1.66	1.983
70	**30**	1.8669	1.7302	1.255	1.189	1.712
80	**20**	1.5861	0.989	0.957	0.897	1.432
90	**10**	0.9561	0.767	0.636	0.511	0.735

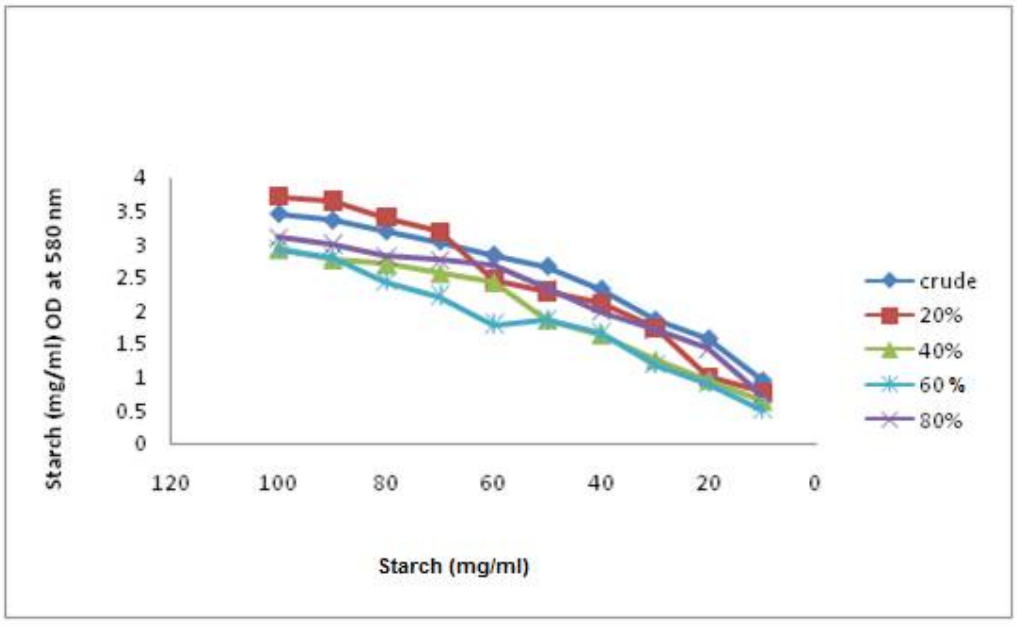


Fig. 24 Starch hydrolysis by crude and partially purified enzyme by NA11 using iodine method

(g) Enzyme kinetics of crude and purified amylase

Michelis-menton constant (Km) and maximum velocity (Vmax) of crude and purified extracellular amylase was determined by line weaver Burk double plot at varying substrate (Starch) concentration and were found to be for crude amylase enzyme Km= 3.198 mg/ml, Vmax=0.0446 µmol/min/ml and for purified amylase enzyme Km=0.621(mg/ml), Vmax=0.0354µmole/min/ml of *Bacillus licheniformis* strain (Table 15; Table 16; Fig. 25; Fig. 26).

Table 15 Enzyme kinetics of crude enzyme from NA11 at different substrate concentration and V□

CRUDE ENZYME	
1/S (mg/ml)	**1/V□**
0.1	22.22
0.2	35.71
0.3	41.46
0.4	55.67
0.5	66.66
0.6	70
0.7	75.3
0.8	80.3
0.9	82.5
1	88.8

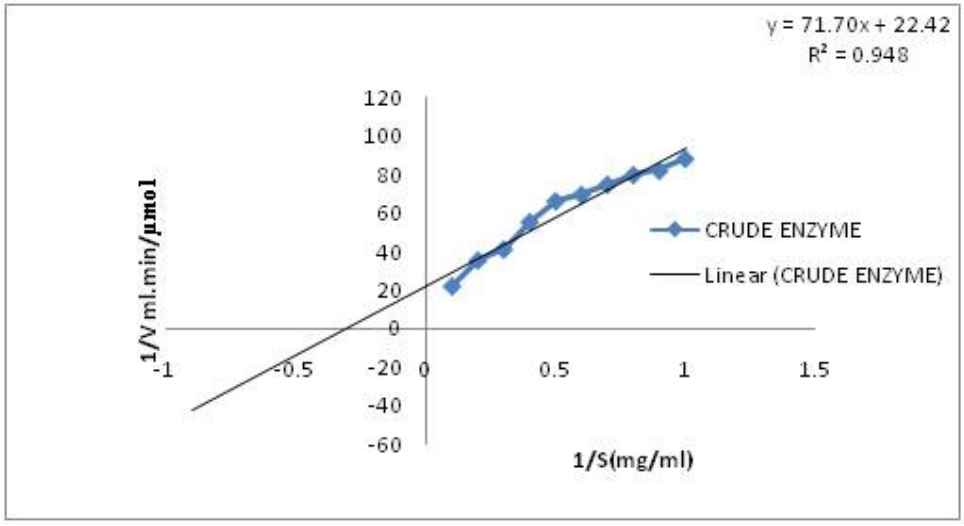


Fig. 25: Enzyme Kinetics of crude enzyme from NA11 at different Substrate and V□

Table 16 Enzyme kinetics of purified enzyme from NA11 at different substrate concentration and V□

PURIFIED ENZYME	
1/S (mg/ml)	**1/V□**
0.1	29
0.2	32.1
0.3	34.48
0.4	35.971
0.5	37.03
0.6	38.46
0.7	39.21
0.8	41.8
0.9	43.47
1	47

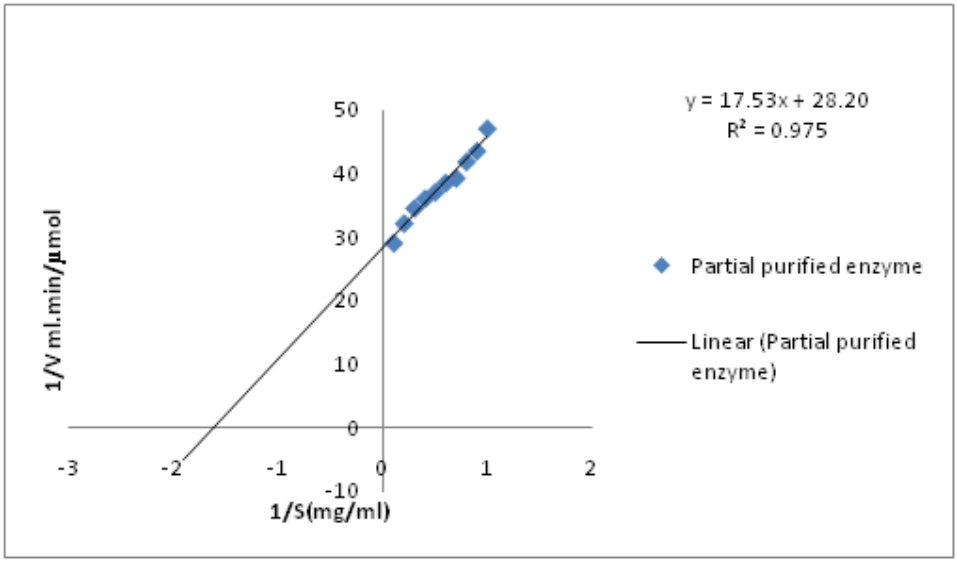


Fig. 26 Enzyme kinetics of Purified enzyme from NA11 at different substrate concentration and V□

h) Designing of Optimal Medium using Central Composite Design

To derive the optimum level of screened variables, a central composite design with three coded levels (Table 17) and three variables was used to study the combined influence of Starch (A), yeast extract (B), $MnCl_2.4H_2O$ (C). Twenty experiments were performed in the present study (Sivamani et al., 2015), Using least squares, the regression model was fit to evaluate the linear interaction and the actual values ranges of the screened variables were presented in (Table 18). To test the fit of the model equation, the regression-based determination coefficient R2 was evaluated. The predicted R2 (0.97) was in close proximity with adjusted R2 (0.95), advocated for greater significance of model (Table 20). The

application of RSM yielded the following regression equation (Eq. 1), which established an empirical relationship between Amylase Activity (Y) and the test variables in coded units:

Regression Equation in Coded Units

Amylase activity (Y) = - 0.0435 + 0.05050 * A + 0.0074 * B + 0.1326 * C - 0.001361 * A^2 + 0.00078 * B^2 - 0.0184 *C^2 - 0.000215 * A*B - 0.01023 * A*C + 0.00227 * B*C

Where,

Y= Amylase activity

A= Starch

B= Yeast extract

C= $Mncl_2.4H_2O$

The contour plots were generated to investigate the interaction among the four significant parameters and to evaluate their combined effects on the response of Amylase activity. Based on the interactions between the variables, an increase in Amylase activity on increasing the concentration of each variable reached to optimum level. From (Fig. 27), it was observed that at a concentration of 10 g/L Starch (A) and 6 g/L of yeast extract (C), the Amylase activity was maximum (0.47 U/mL). Similarly in an interactive study of yeast extract (B) and $Mncl_2.4H_2O$ (C) maximum Amylase activity was observed at their respective concentrations of 6.0 and 0.1 g/L, respectively (Fig. 28). A number of numerical solutions were suggested by the Minitab software within the experimental range of parameters for maximum Amylase activity. In order to validate the predicted model experiments suggested was conducted in duplicates. The suggested solutions revealed that the variables were optimized at points located in the rightmost region of the plot (Fig. 29). The maximum experimental valAmylase activity was 0.46 U/mL, while the predicted response based on RSM was estimated to be 0.47 U/mL at Starch (10 g/L), yeast extract (6 g/L), and $MnCl_2.4H_2O$ (1.0 g/L).Using RSM, optimized Amylase activity (0.47 U/mL) of *B.subtilis* NA11 obtained in this study was higher than that of Geo *bacillus sp*., *Brevibacillus sp*., and *Bacillus sp.* which was 0.12, 0.02, 0.01, 0.43 U/mL, respectively.

Table 17 Range of values for response surface method

Independent Variable (g/l)		-1 Low (g/l)	0 Medium (g/l)	+1 High (g/l)
A	Starch	2	8	14
B	Yeast extract	2	6	8
C	$MnC_2.4H_2O$	0.01	0.05	1

Table 18 Response surface method result

Runs				Coded value (g/l)			Experimental Predicted
StdOrder	RunOrder	PtType	Blocks	Starch	Yeast extract	$Mncl_2.4H_20$	Amylase activity (U/ml)
16	1	0	1	8	5	0.505	0.351
9	2	-1	1	2	5	0.505	0.145
13	3	-1	1	8	5	0.010	0.301
14	4	-1	1	8	5	1.000	0.364
4	5	1	1	14	8	0.010	0.471
7	6	1	1	2	8	1.000	0.258
2	7	1	1	14	2	0.010	0.426
8	8	1	1	14	8	1.000	0.477
1	9	1	1	2	2	0.010	0.070
17	10	0	1	8	5	0.505	0.325
5	11	1	1	2	2	1.000	0.184
6	12	1	1	14	2	1.000	0.375
10	13	-1	1	14	5	0.505	0.431
19	14	0	1	8	5	0.505	0.398
12	15	-1	1	8	8	0.505	0.401
20	16	0	1	8	5	0.505	0.351
15	17	0	1	8	5	0.505	0.350
3	18	1	1	2	8	0.010	0.174
11	19	-1	1	8	2	0.505	0.287
18	20	0	1	8	5	0.505	0.358

Table 19 Analysis of Variance

Source	Sum of square	Df	Mean square	F - value	P- value	
Model	0.224937	9	0.024993	43.64	<0.0001	Significant
Linear	0.205918	3	0.068639	119.85	<0.0001	
Starch	0.181980	1	0.181980	317.75	<0.0001	
Yeast extract	0.019272	1	0.019272	33.65	<0.0001	
$Mncl_2.4H_20$	0.000056	1	0.004666	8.15	0.017	
A^2	0.006603	1	0.006603	11.53	0.007	
B^2	0.000135	1	0.000135	0.24	0.063	
C^2	0.000056	1	0.000056	0.10	0.076	
AB	0.000120	1	0.000120	0.21	0.045	
BC	0.007381	1	0.007381	12.89	0.005	
AC	0.000091	1	0.000091	0.16	0.069	
Residual	0.002914	5	0.000583			
Lack of result	0.002814	19	0.000563	1.04	0.485	Not- significant

Table 20 Analysis of model summary

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0239314	97.52%	95.28%	83.98%

As a result, the amylase activity in cell free supernatant of *Bacillus licheniformis* NA11 showed variation from 0.07 to 0.477 U/ml. this variation reflected the significance of factors on the response. The analysis of regression coefficients and t-value of three ingredients were shown in (Table 20). In general, a large t- value associated with low P- value of a variable indicated a high significance of the corresponding model terms. The model F value of 43.64 as well as values of P>F (<0.0001) indicated that the model terms (A, B, C, A^2, B^2, C^2, AB, BC, AC) were significant (Table 19).

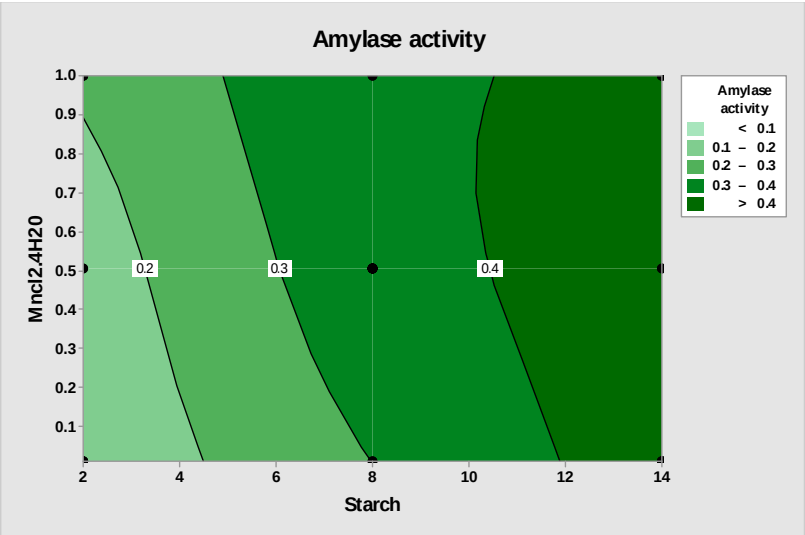


Fig. 27 Contour Plot of Amylase activity Vs $MnCl_2.4H_2O$, Starch

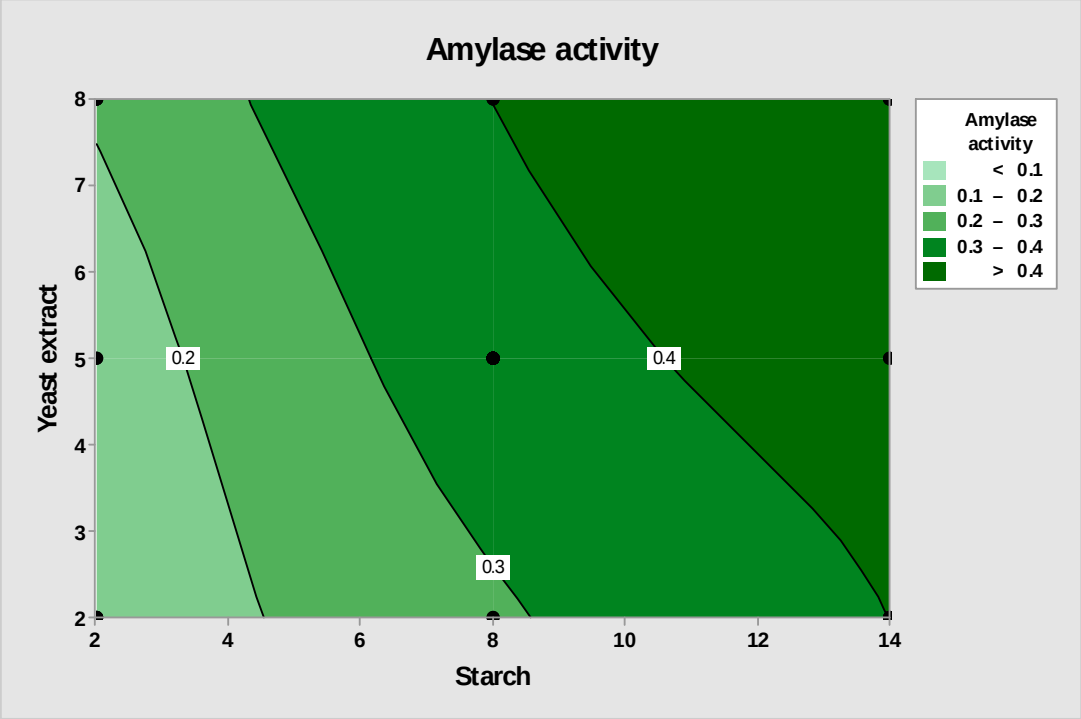


Fig. 28 Contour Plot of Amylase activity Vs Yeast extract, Starch

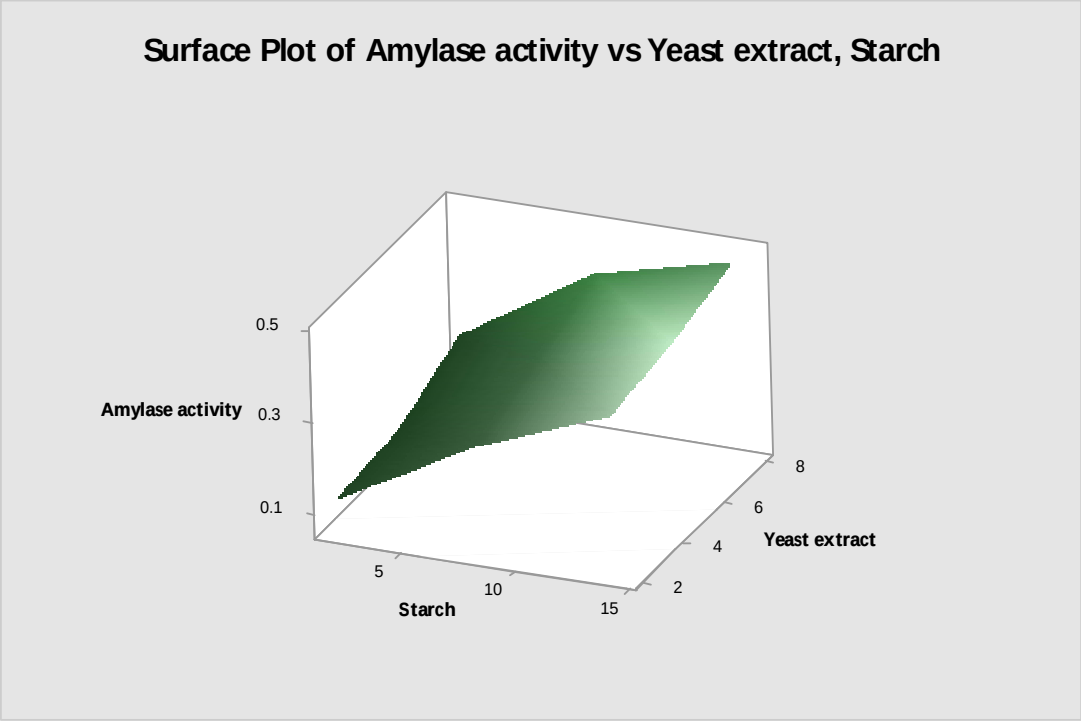


Fig. 29 Surface Plot of Amylase activity Vs Yeast extract, Starch

Table 21 One-way ANOVA: Amylase activity versus Starch

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0469062	83.78%	81.88%	76.39%

Means

Yeast extract	N	Mean	StDev	95% CI
2	5	0.1662	0.0680	(0.1219, 0.2105)
8	10	0.3486	0.0367	(0.3173, 0.3799)
14	5	0.4360	0.0411	(0.3917, 0.4803)

Pooled StDev = 0.0469062

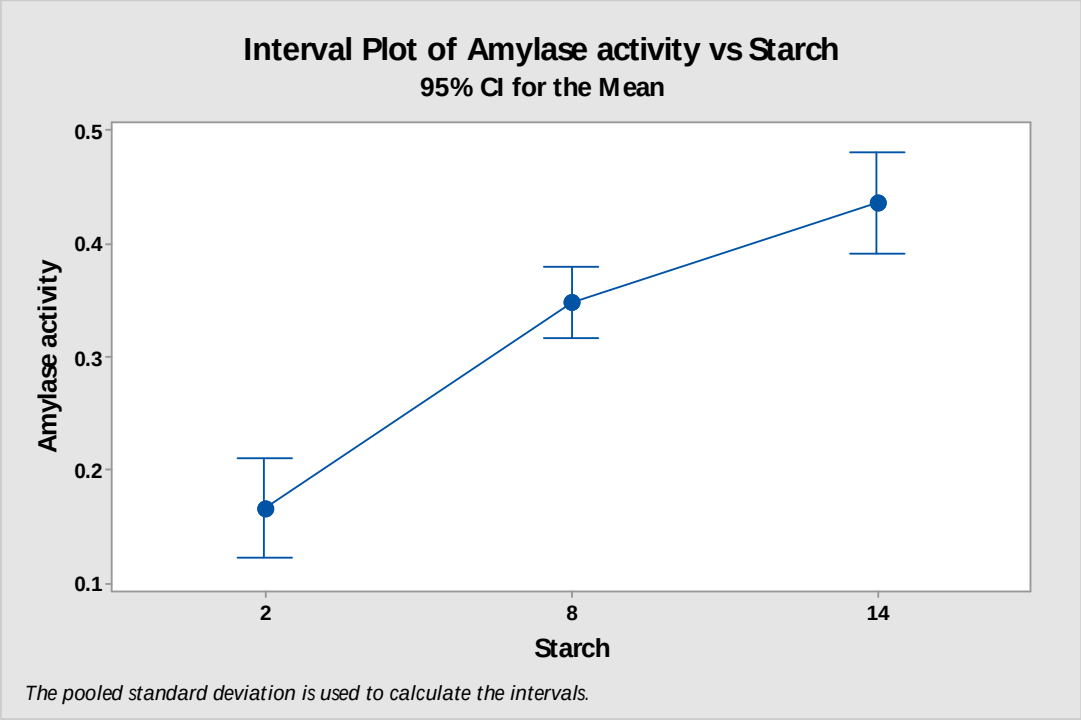


Fig. 30 Interval Plot of Amylase activity vs Starch

Table 22 One-way ANOVA: Amylase activity versus Yeast extract

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.110678	9.72%	0.00%	0.00%

Means

Yeast extract	N	Mean	StDev	95% CI
2	5	0.2684	0.1441	(0.1640, 0.3728)
5	10	0.3374	0.0764	(0.2636, 0.4112)
8	5	0.3562	0.1347	(0.2518, 0.4606)

Pooled StDev = 0.110678

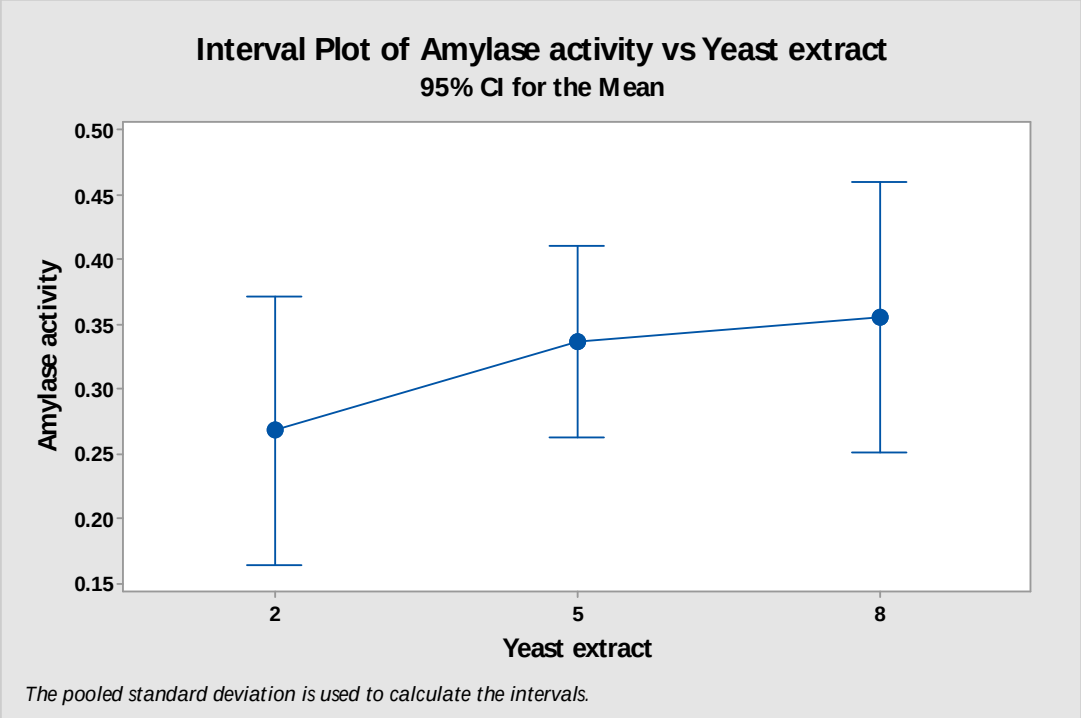


Fig. 31 Interval Plot of Amylase activity Vs Yeast extract

Table 23 One-way ANOVA: Amylase activity versus $MnCl_2.4H_2O$

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.114169	3.93%	0.00%	0.00%

Means

$Mncl_2.4H_20$	N	Mean	StDev	95% CI
0.010	5	0.2884	0.1682	(0.1807, 0.3961)
0.505	10	0.3397	0.0796	(0.2635, 0.4159)
1.000	5	0.3316	0.1132	(0.2239, 0.4393)

Pooled StDev = 0.114169

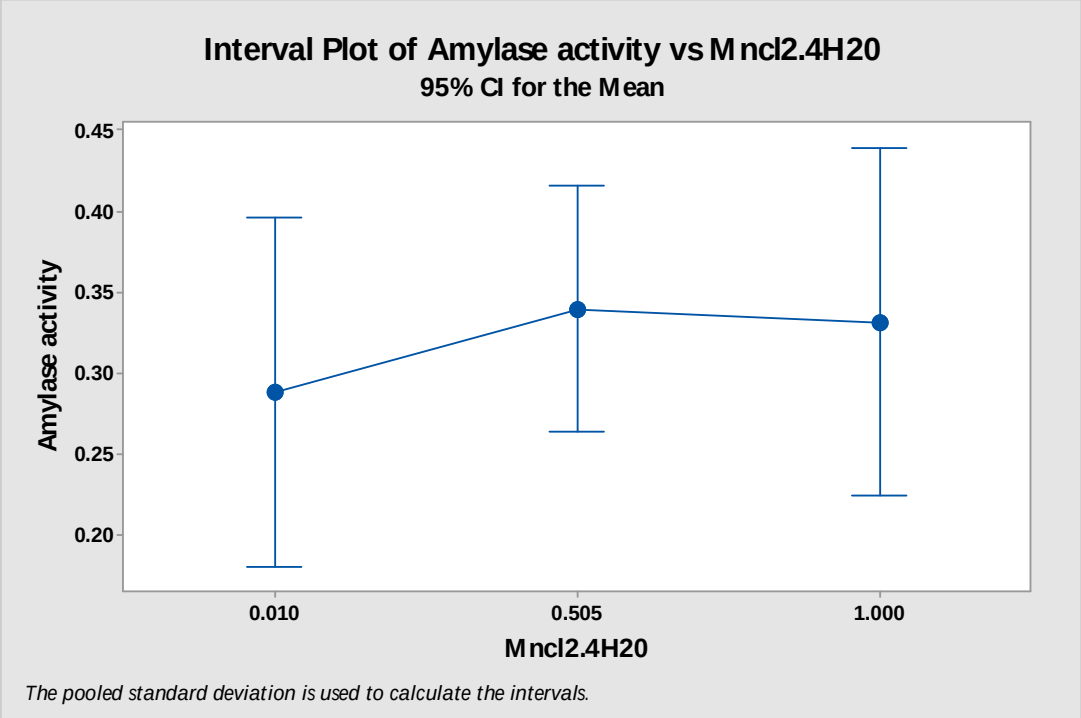


Fig. 32 Interval Plot of amylase activity Vs $Mncl_2.4H_20$

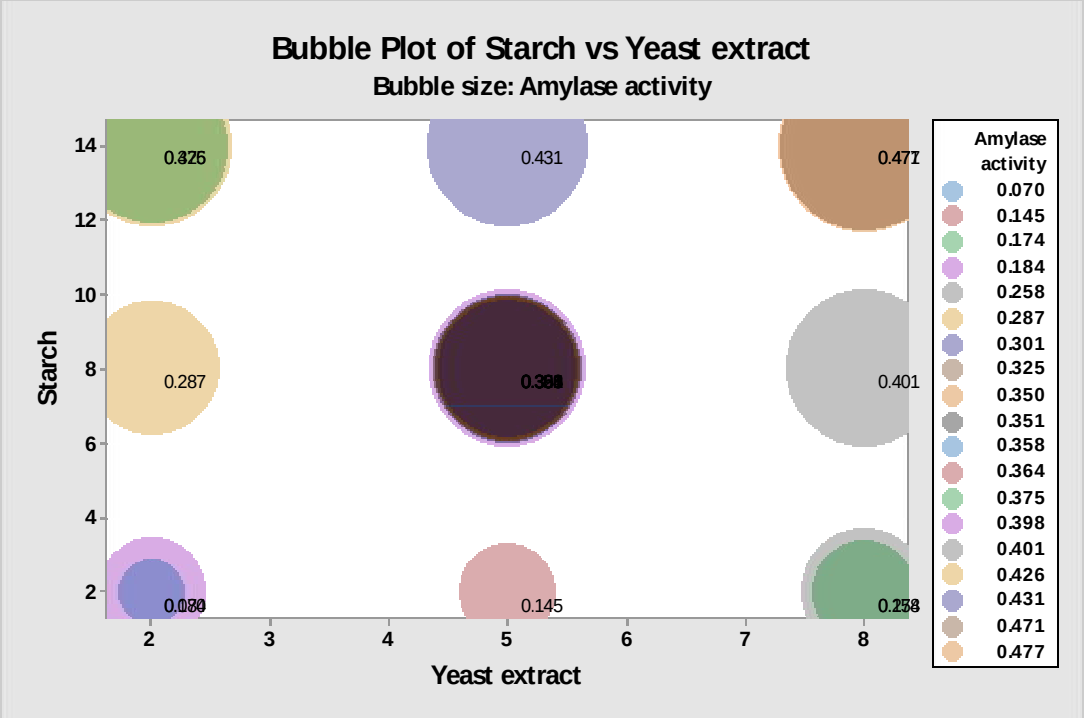


Fig. 33 Bubble Plot of Starch Vs Yeast extract

Bubble plot of starch and yeast extract shows the maximum 0.477 U/ml enzyme activity (Fig. 33).

Bioconversion of starch and other starchy wastes by *Bacillus licheniformis* NA11

a) **Microorganism and Substrate**

Substrate like potato peel, banana peel, and orange peel collected from Hostel G and Juice counter from Thapar University, Patiala. Biomass after drying was grinded using a mechanical blender and sieved to a mesh size ill a of 0.2-0.5 mm The biomass sample was stored at room temperature in air tight container for further study (Fig. 34).

1. Banana peels waste 2. Orange peel waste 3. Potato peel waste

Fig. 34 Washed and air dried a) Banana peel, b) orange peel and c) potato peel d) powder form of biomass

b) Proximate analysis

Orange peel, banana peel and potato peel were subjected to physicochemical characterization to determine the feasibility for use as feedstock to obtain ethanol. Further, pre-treatments of OPW, BPW and PPW biomasses using various approaches were employed to enhance

reducing sugar yield. Compositional and structural characteristics were revealed using SEM, XRD, and FTIR analysis. Pre-treated and untreated biomasses were subjected to SSF for ethanol production for one week and ethanol estimated by both gas chromatography (GC) and potassium dichromate method maximum production of ethanol was reported by enzyme hydrolysis pretreatment than dilute acid-alkali hydrolysis pre-treatment. Proximate analysis is the most often employed practice for bio-fuel characterization. Proximate analysis was done to determine the weight percentage of moisture, volatile matter (VM), ash and ash acid soluble content of biomasses. The percentage of moisture, ash, volatile matter of OPW, BPW, PPW biomasses are given in (Table 24). Potato peel waste (PPW) was found to contain 6.64% moisture, 5.48% ash and 75.79 % volatile matter, similarly orange peel waste (OPW) was found to contain 5.451 % moisture, 9.717 % ash, 84.52 % volatile matter and banana peel waste (BPW) was found to contain 5.01 % moisture, 7.35 % ash, 81.40 % volatile matter. This proximate analysis shows that with increase in moisture there is reduction in the volatile matter and ash content. By increase in the reducing sugar decrease in the ash content but increase in the volatile matter.

Table 24 Proximate analysis

Proximate analysis (wt %)			
Waste Biomass	**Orange peel waste**	**Banana peel waste**	**Potato peel waste**
Moisture	5.451 ± 0.147	5.012 ± 0.382	6.641 ± 0.256
Volatile matter	84.52 ± 1.25	81.40 ± 1.92	75.79 ± 2.31
Ash	9.717 ± 0.441	7.353 ± 0.254	5.484 ± 0.241
Acid soluble Ash	0.742 ± 3.24	0.551 ± 3.24	0.286 ± 3.24
Cellulose	9.06 ± 4.53	15.43 ± 8.52	22.31 ± 2.54
Starch	29.56 ± 2.56	31.6 ± 3.26	48.24 ± 2.67

Orange peel waste (OPW) contains cellulose 9.06 % and starch 29.56 %. Banana peel waste (BPW) contains cellulose 15.43 % and starch 31.6 %. Potato peel waste (PPW) contains cellulose 22.31 % and starch 48.24 % (Table 24). PPW had high cellulose and starch as compared OPW and BPW and therefore represents most suitable biomass for its bioconversion to ethanol. Banana peel waste contains 3% starch, and 6.9-9.6% cellulose (Mohapatra et al., 2010). Orange peel is in fact constituted by 16.9 % soluble sugars, 3.75 % starch, fiber (cellulose, 9.21 % wt; hemicelluloses, 10.5 % wt; lignin 0.84 % wt; and pectins, 42.5 % wt), 3.50 % ashes, 1.95 % fats, and 6.50 % proteins (Rivas et al.,2014). Potato peel

waste 3.4% pectin, 2.2% cellulose, 14.7% protein, 66.8% starch, and 7.7% ash (Torrado et al., 2011).

Extractive determination of starchy waste

The yield of hexane, methanol and water extracts for waste biomasses samples are presented on (Table 25).

Table 25 Extractive composition of starchy waste biomass (g/ 100 g biomass)

	*Weight (%)		
Extractive	**Orange peel waste**	**Banana peel waste**	**Potato peel waste**
Hexane	7.88 ± 0.511	8.59 ± 0.345	6.59 ± 0.651
Methanol	18.96 ± 0.745	19.85 ± 0.675	28.873 ± 0.54
Water extract	15.66 ± 0.867	20.42 ± 0.778	33.066 ± 412

**Values are mean ± SD (n=3)*

Polar and non polar compounds need to be extracted before processing of starchy biomass for ethanol production due to their use as other value added chemicals. Extraction process was performed to separate the class of compounds such as non polar lipids, hydrocarbon compounds and terpenoids, etc using hexane extraction and methanol extraction process to separate polar compounds such as chlorophyll, polar waxes, sterol and other minor constitutes. Water extraction was carried out to separate inorganic material and non-structural sugars, etc. Total amount of extractive content were detected to be around for Orange peel waste, Banana peel waste, Potato peel waste of hexane extract 7.88%, 8.59%, 6.59 % respectively, of methanol 18.96%, 19.85%, 28.87 % respectively and of water extract 15.66 %, 20.42 %, 33 % respectively (Table 25).

c) Pretreatment of starchy waste biomass

Pretreatment of starchy waste was done to hydrolyse starch component to increase yield reducing sugar. Two methods for pre-treatment for starchy waste dilute acid-alkali hydrolysis and enzymatic hydrolysis were used.

Table 26 Pretreatment of starch, OPW, BPW, PPW and estimation of reducing sugars

Reducing sugar (mg/ml)			
Substrate	**Untreated**	**Dilute Acid-alkali pretreatment**	**Enzymatic pretreatment**
Starch	0.195	0.358	0.467
OPW	0.173	0.248	0.435
BPW	0.128	0.214	0.411
PPW	0.099	0.117	0.398

The starchy substrates were pre-treated with diluted acid-alkali and enzymatic pretreatment which showed the increase of reducing sugar due to hydrolysis of starch and cellulose present in the biomass. In the native biomass reducing sugar was less. Enzymatic pre-treatment resulted in enhanced reducing sugar as compared to dilute acid-alkali pre-treatment. Amylase activity of partially purified enzyme from *Bacillus licheniformis* showed maximum production of 0.467 mg/ml reducing sugar when 1% starch was used as substrate. When starchy wastes were used as substrate the enzyme activity in 160 min at 60°C was production of 0.435 mg/ml of reducing sugar for orange peel waste (OPW), production of 0.411 mg/ml of reducing sugar for banana peel waste (BPW) and for potato peel waste (PPW) production of 0.398 mg/ml of reducing sugar (Table 26).

SEM, XRD, FTIR of native and enzymatic pretreated starchy wastes

SEM

Scanning electron microscope (SEM) observation of native (untreated) and treated biomass of potato peel waste, banana peel waste, orange peel waste showed pre-treatment induced structural changes in the biomass. SEM revealed ordered and compact structure which was destroyed in pre-treated biomass. Three starchy waste biomasses were pre-treated such as potato peel waste, banana peel waste, orange peel waste. Each starchy waste biomass showed hydrolysis of starch and lignocellulosic components and modification of structural changes.

1. Native potato peel waste

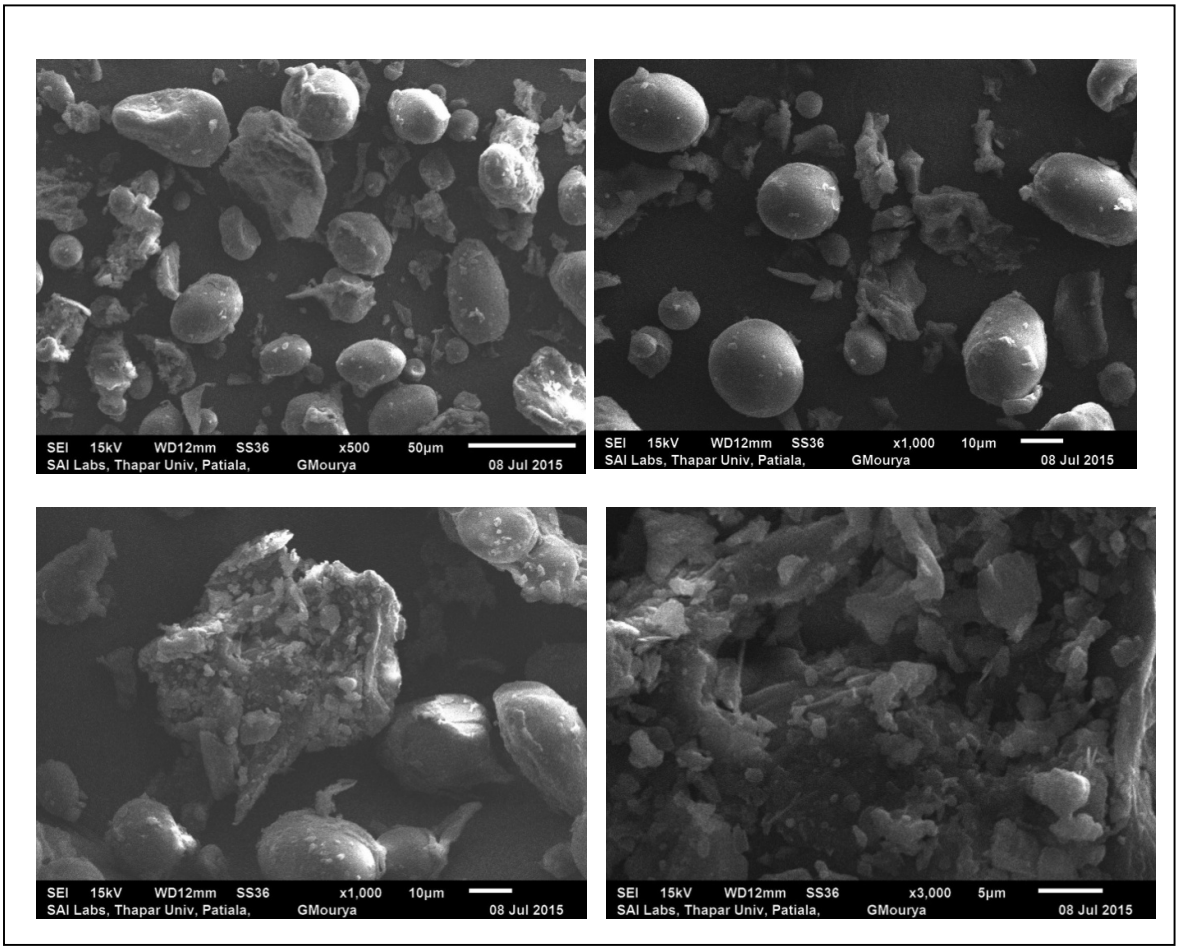


Fig. 35 SEM of native potato peel waste at different magnification x500, x1000, and x3000

Fig. 35 demonstrates smooth, compact and spherical structures of starch granules with average size ranges from 30-45 µm. The presence of some degree of roughness is an indicative of protein matrices, such as lignin. The results obtained through electron microscopy corroborate with previous results where a less proportion of lignin was also found in native, i.e., untreated waste biomass of potato peel along with starch.

2. Treated potato peel waste

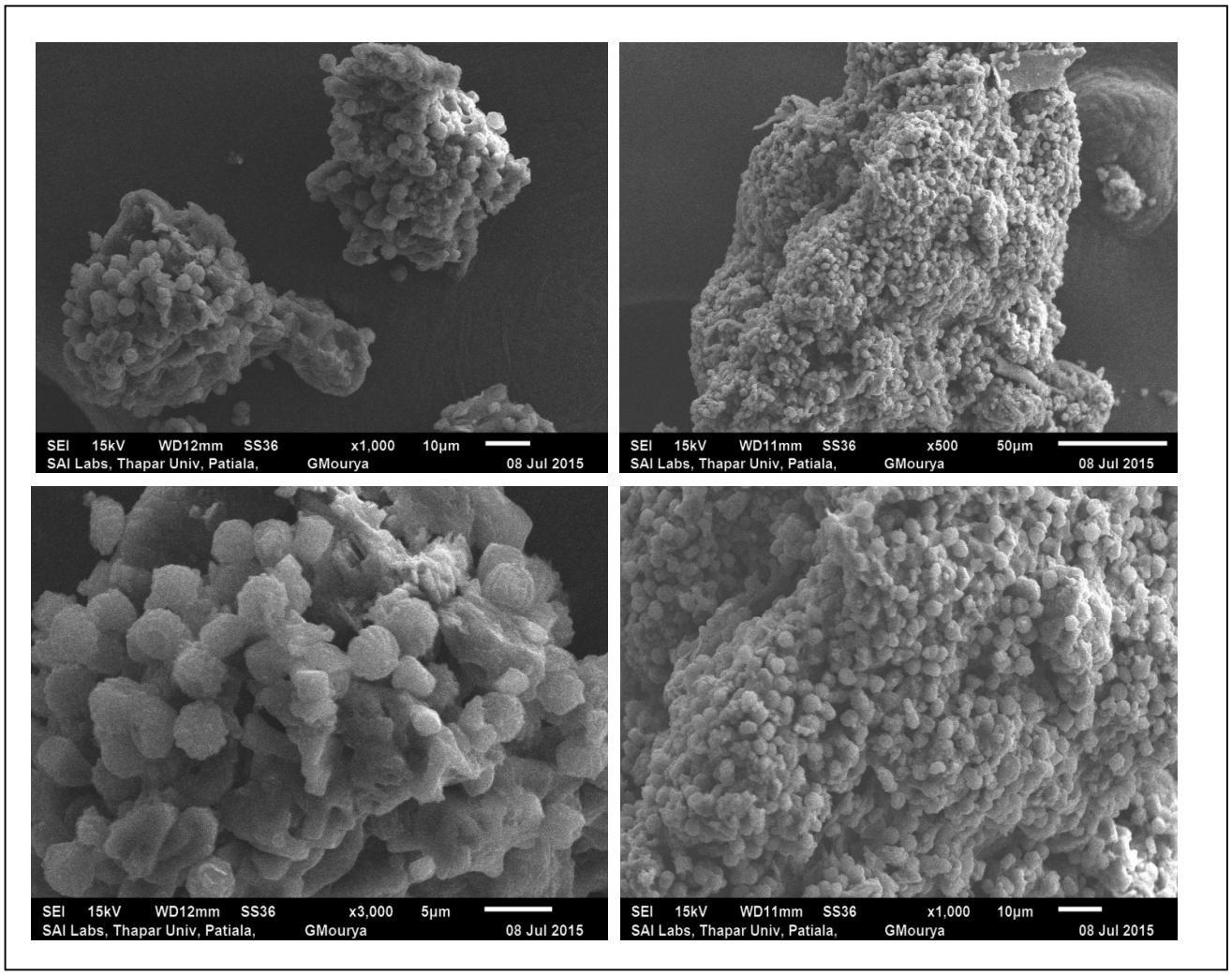


Fig. 36 SEM of treated potato peel waste at different magnification x500, x1000, and x3000 showed hydrolysis of biomass.

As shown in Fig. 36, there was change in surface morphology of starch granules with size ranges from 3- 10 µm, which shows the roughness structure surface of granules and complete hydrolysis of lignin i.e. treated waste biomass of potato peel along with starch.

3. Native banana peel waste

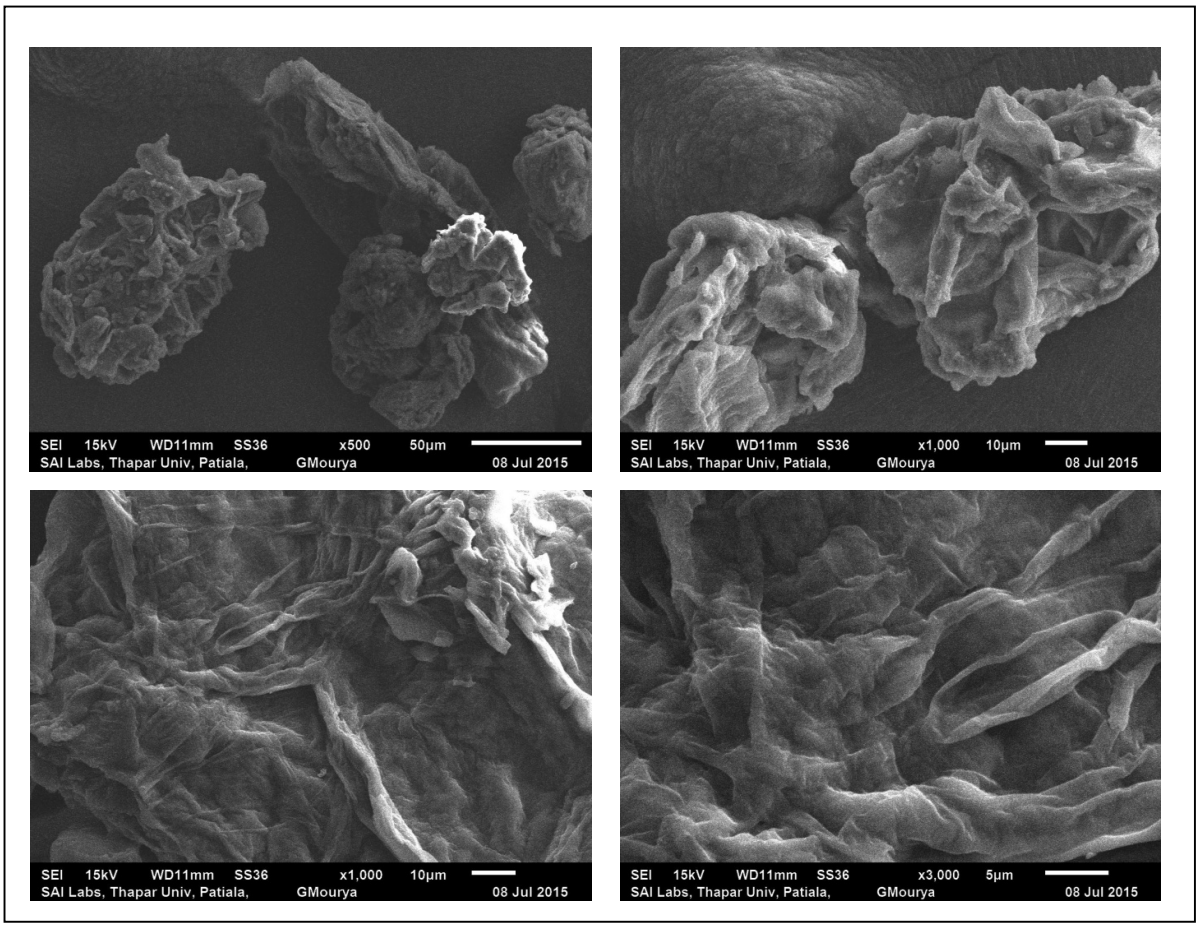


Fig. 37 SEM of native banana peel waste at different magnification x500, x1000, and x3000 showed hydrolysis of biomass.

Fig. 37 demonstrates crude fibrous structures and carbohydrate .The results obtained through electron microscopy corroborate with composition of banana peel where a less proportion of cellulose and lignin were also found in native, i.e., untreated waste biomass of banana peel along with starch.

4. Treated banana peel waste

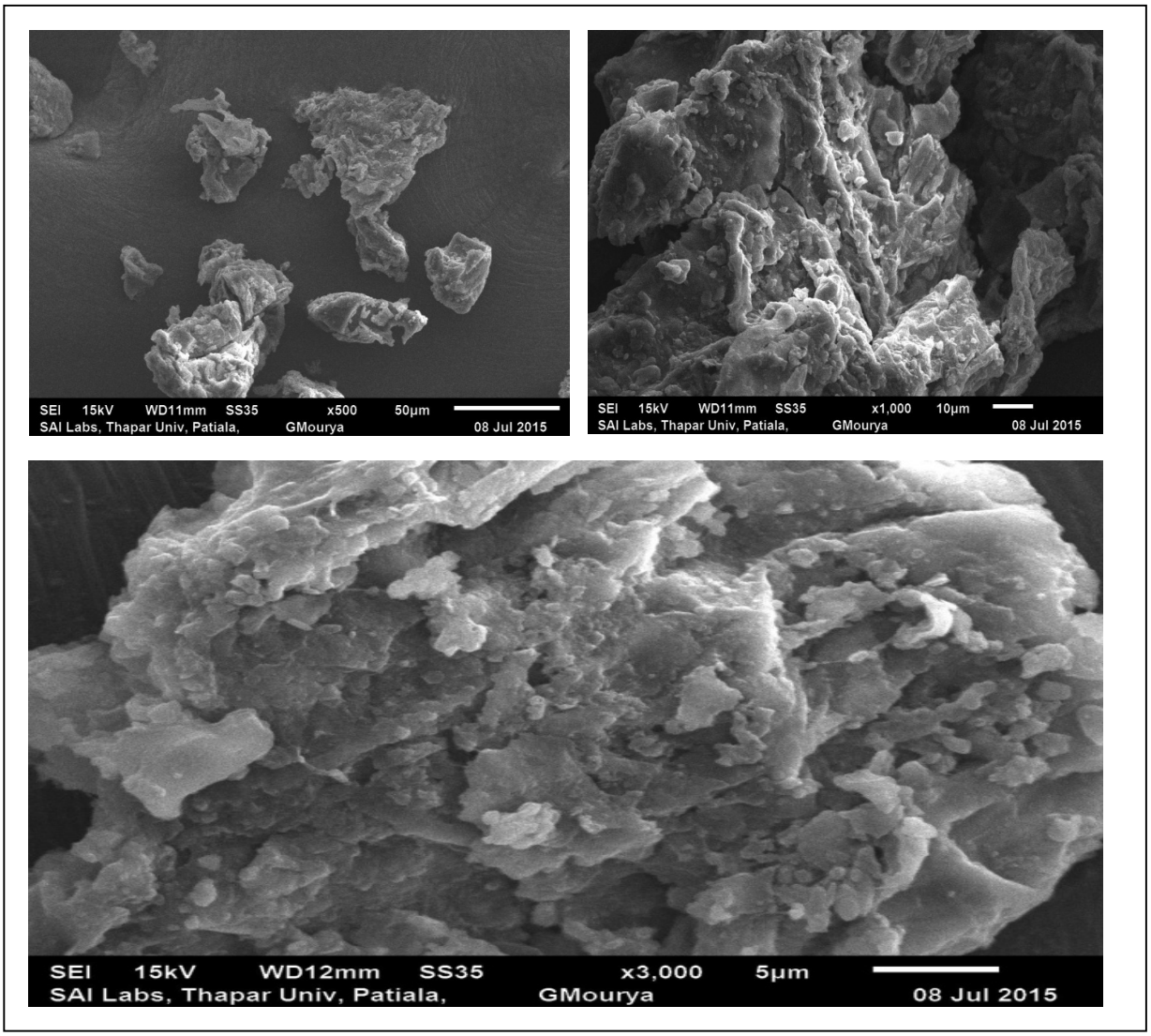


Fig. 38 SEM of treated banana peel waste at different magnification x500, x1000, and x3000 showed hydrolysis of biomass.

As shown in Fig. 38, there was change in surface morphology attributed to heavy distortion of fibrous structure due to complete hydrolysis of carbohydrate , lignin and hemicelluloses i.e. treated waste biomass of banana peel along.

5. Native orange peel waste

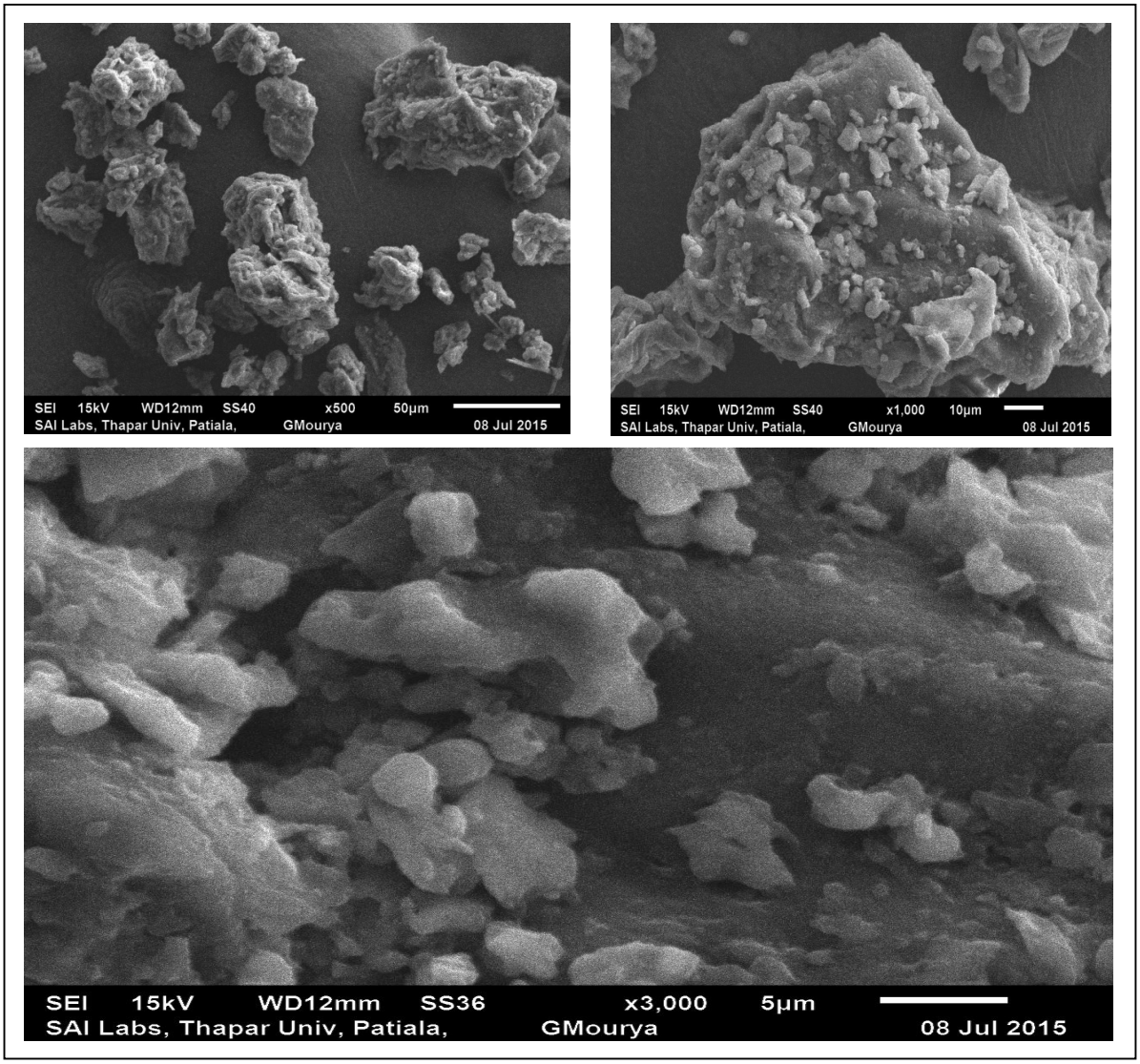


Fig. 39 SEM of native orange peel waste at different magnification x500, x1000, and x3000 showed hydrolysis of biomass.

Fig. 39 demonstrates smooth, compact structures of starch granules .The presence of some degree of roughness is an indicative of protein matrices, such as lignin. The results obtained through electron microscopy matches with previous results where a less proportion of lignin was also found in native, i.e., untreated waste biomass of orange peel.

6. Treated orange peel waste

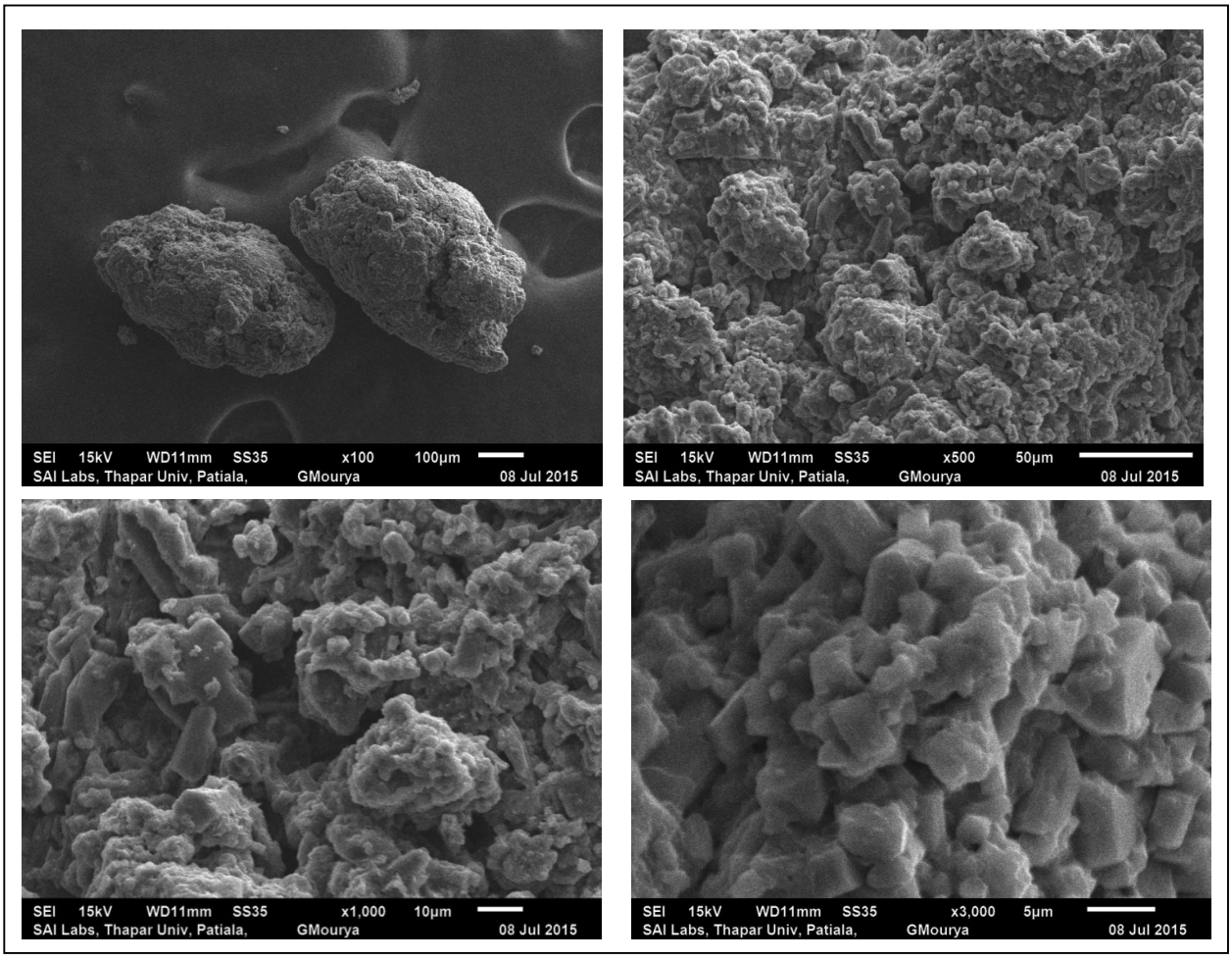


Fig. 40 SEM of treated orange peel waste at different magnification x100, x500, x1000, and x3000 showed hydrolysis of biomass.

As shown in Fig. 40, there was a significant change in surface morphology of starch granules. Starch granules were shown to be of smaller size with spherical morphology and high degree of aggregation indicating efficient hydrolysis of lignin i.e. treated waste biomass of orange peel.

XRD

X-ray diffraction analysis showed intense crystalline peak at 2θ angle of 21.5°. After 30 days of biodegradation, a significant increase in CrI from the peaks was recorded in biomasses treated with *Bacillus licheniformis* NA11under natural environmental conditions.

1. **Native potato peel waste**

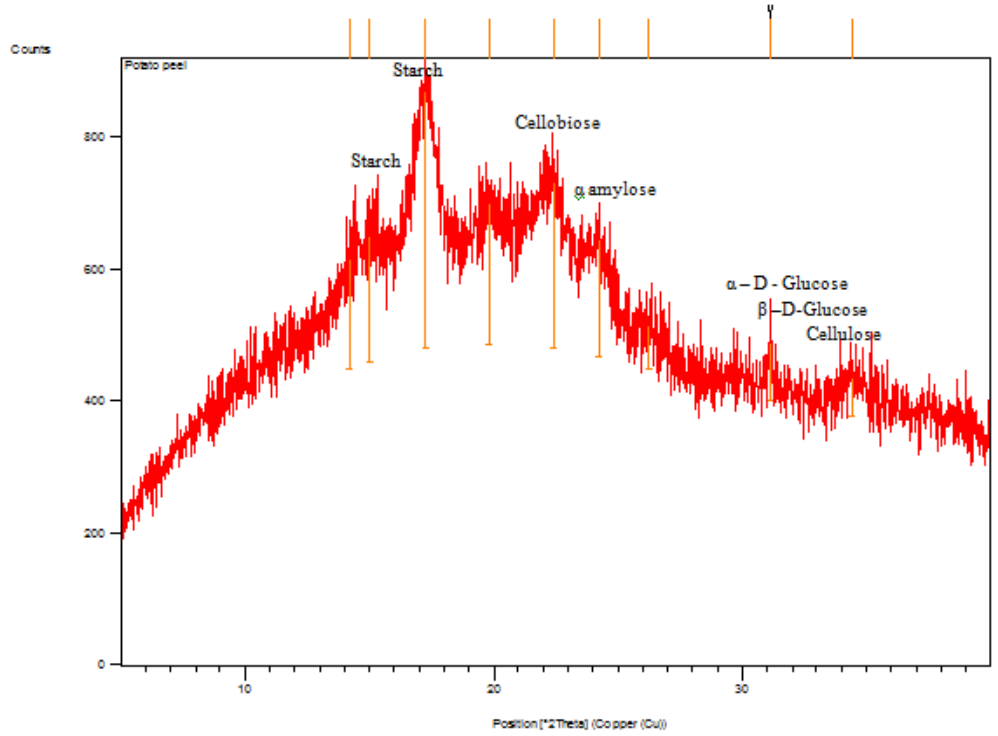


Fig. 41 Native potato peel waste

2. Treated Potato peel waste

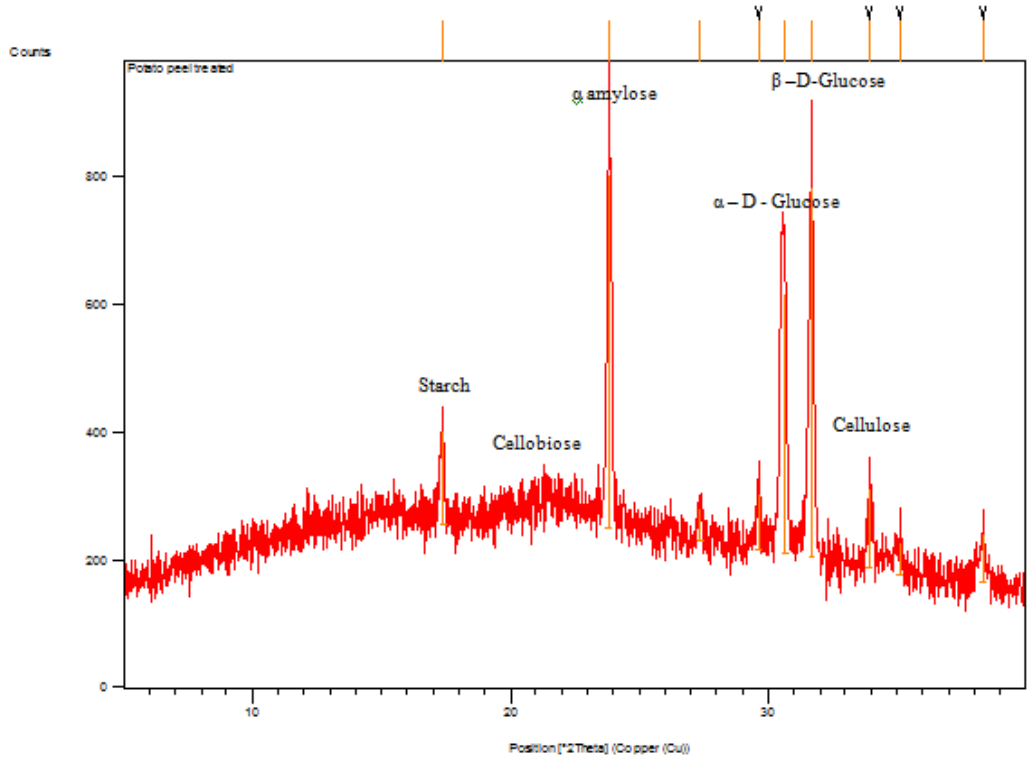


Fig. 41 X-ray crystallography of enzyme **t**reated Potato Peel waste

X-ray crystallograph of untreated (Fig. 41) and treated potato peel (Fig. 42) shows reduction in peaks of cellobiose and starch in treated biomass. Also there was appearance of peaks of amylose and glucose after treatment (Fig. 42). X-ray crystallography confirms enzymatic bioconversions of starch in potato peel waste to monomeric units.

3. Native Banana peel waste

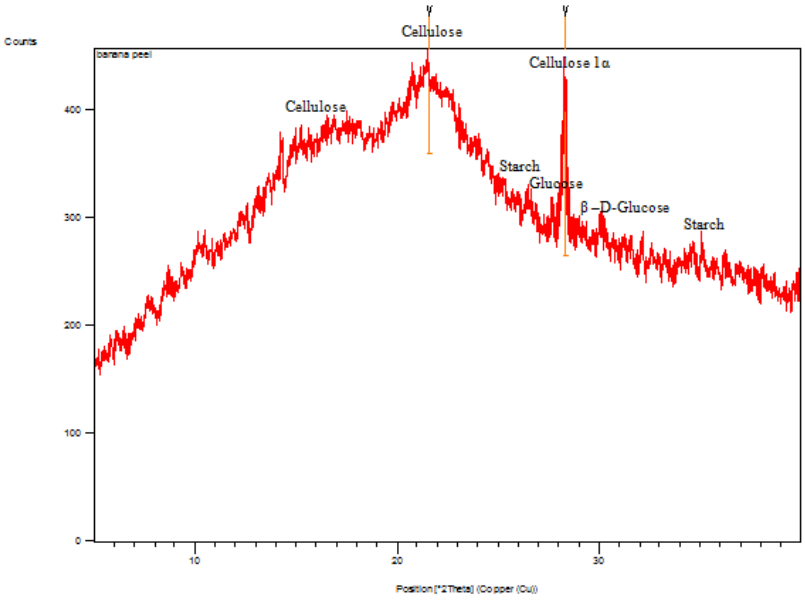


Fig. 43 Native Banana Peel waste

4. Treated Banana peel waste

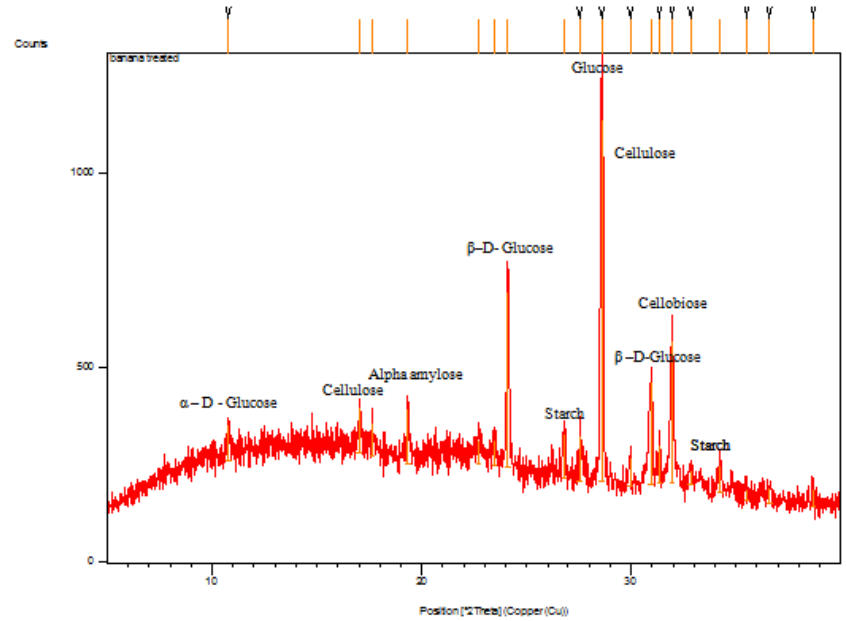


Fig. 44 Treated Banana Peel Waste

X-ray crystallograph of untreated (Fig. 43) and treated potato peel (Fig. 44) shows reduction in peaks of cellobiose and cellulose in treated biomass. Also there was appearance of peaks of Beta-D-Glucose and glucose after treatment (Fig. 44). X-ray crystallography confirms enzymatic bioconversions of starch in banana peel waste to monomeric units.

5. Native Orange peel waste

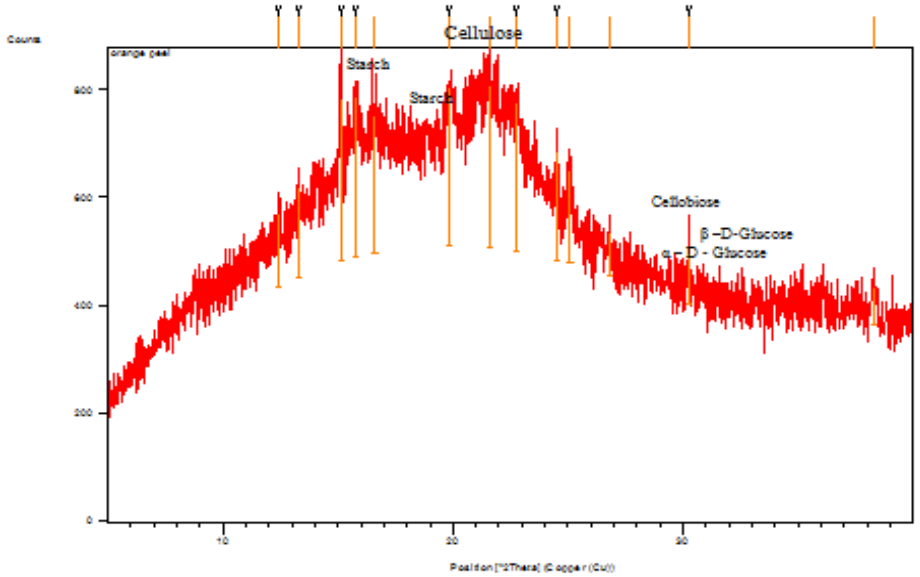


Fig. 45 Native Orange Peel Waste

6. Treated Orange peel waste

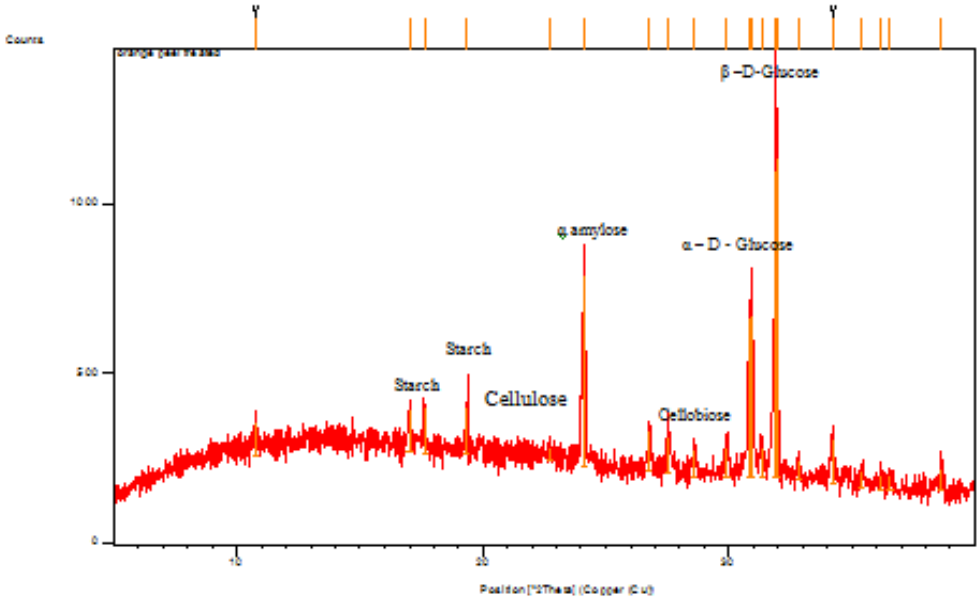


Fig. 46 Treated Orange Peel Waste

X-ray crystallograph of untreated (Fig. 45) and treated potato peel (Fig. 46) shows reduction in peaks of cellobiose and starch in treated biomass. Also there was appearance of peaks of alpha amylose and beta-D-Glucose after treatment (Fig. 46). X-ray crystallography confirms enzymatic bioconversions of starch in potato peel waste to monomeric units.

FTIR Results

1. Native banana peel waste

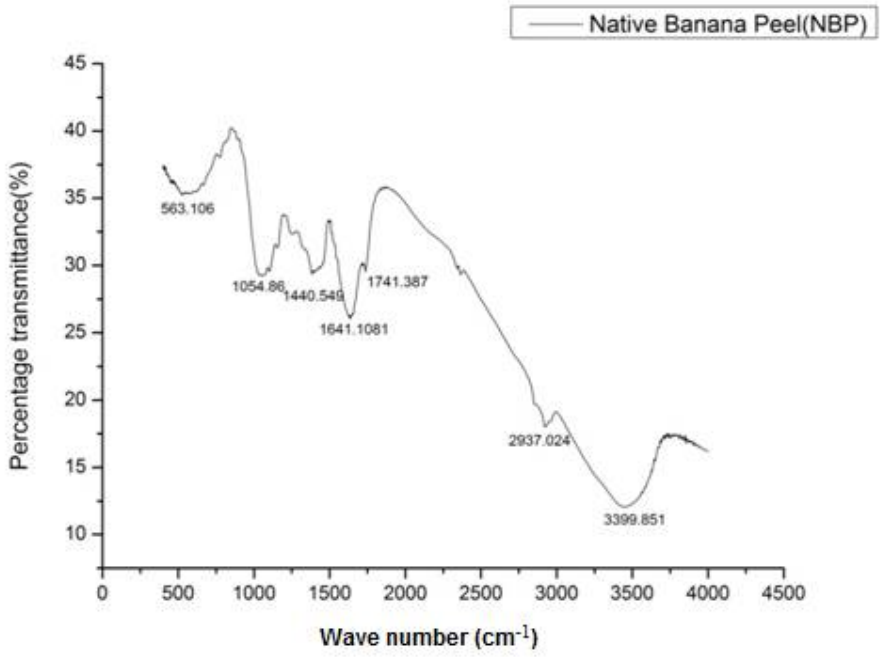


Fig. 47 Native Banana Peel waste

Table 27 FTIR of native banana peel waste

Frequency(cm^{-1})	Bond	Functional Group
690–515 (m)/ 590	C–Br stretch	alkyl halides
1300–1150 (m)/ 1054.86	C–H wag (–CH2X)	alkyl halides
1470–1450 (m)/1440.549	C–H bend	alkanes
1650–1580 (m)/ 1641.1061	N–H bend	1˚ amines
1750–1735 (s) / 1741.387	C=O stretch	esters, saturated aliphatic
3000–2850 (m) / 2937.024	C–H stretch	alkanes
3400–3250 (m)/ 3399.651	N–H stretch	1˚, 2˚ amines, amides

FTIR spectroscopy analysis of untreated banana peel waste was carried out to detect changes in functional groups of biomass and FTIR spectrum is shown in (Figure 47). The main characteristics were attributed to different chemical groups of cellulose, starch, hemicelluloses and lignin. Most prominent functional groups and bands in biomass sample were alkyl halides, alkanes, esters, saturated aliphatic, 1˚, 2˚ amines, and amides and 590, 1054.86, 1440.549, 1641.1061, 1741.387, 2937.024, 3399.651 (Figure 47). The peaks in fingerprint based on literature values are summarized in Table 27 (Adel at al., 2011; Meng et al ., 2012; Xiao et al., 2011).

2. Native potato peel waste

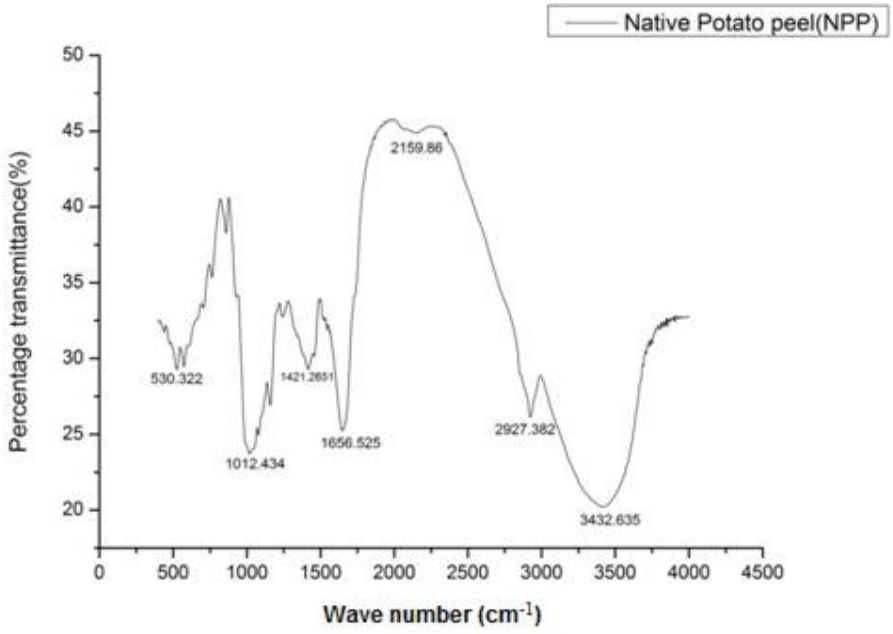


Fig. 48 Native Potato Peel waste

Table 28 FTIR of native potato peel waste

Frequency, cm–1	Bond	Functional Group
690–515 (m)/ 530.322	C–Br stretch	alkyl halides
910–665 (s, b)/765.59	N–H wag	1˚, 2˚ amines
900–675 (s)/765.59	C–H "oop"	aromatics
1250–1020 (m)/1064.502	C–N stretch	aliphatic amines
1500–1400 (m)/1423.1936	C–C stretch (in–ring)	aromatics
1650–1580 (m)/1650	N–H bend	1˚ amines
3000–2850 (m)/2933.167	C–H stretch	alkanes
3500–3200 (s,b)/3422.992	O–H stretch, H–bonded	alcohols, phenols

FTIR spectroscopy analysis of untreated potato peel waste was carried out to detect changes in functional groups of biomass and FTIR spectrum is shown in (Figure 48). The main characteristics were attributed to different chemical groups of cellulose, starch, hemicelluloses and lignin. Most prominent functional groups and bands in biomass sample were alkyl halides, aromatics , alkanes, aliphatic amines, 1˚, 2˚ amines, and alcohols, phenols and 530.322,765.59, 765.59, 1064.502, 1423.1936, 1650, 2933.167, 3422.992 (Figure 48). The peaks in fingerprint based on literature values are summarized in Table 28 (Adel *et al*., 2011; Meng *et al* ., 2012; Xiao *et al.,* 2011).

3. Native orange peel waste

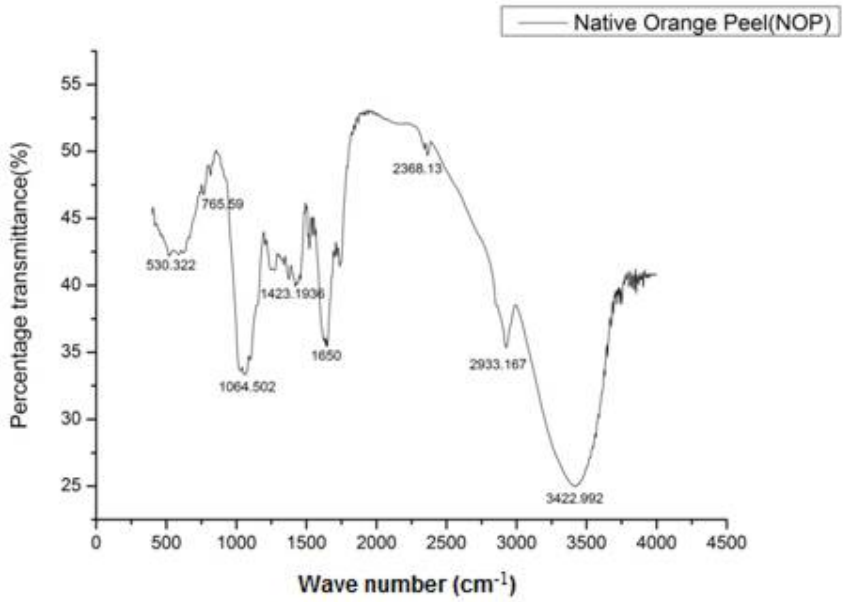


Fig. 49 Native Orange Peel Waste

Table 29: FTIR of native orange peel waste

Frequency, cm–1	Bond	Functional Group
690–515 (m)/ 530.322	C–Br stretch	alkyl halides
1320–1000 (s)/1012.434	C–O stretch	alcohols, carboxylic acids, esters, ethers
1500–1400 (m)/1421.2651	C–C stretch (in–ring)	aromatics
1650–1580 (m)/1656.525	N–H bend	1˚ amines
2260–2100 (w)/2159.86	–C≡C– stretch	alkynes
3000–2850 (m)/2927.382	C–H stretch	alkanes
3300–2500 (m)/ 2927.382	O–H stretch	carboxylic acids
3500–3200 (s,b)/3432.635	O–H stretch, H–bonded	alcohols, phenols

FTIR spectroscopy analysis of untreated orange peel waste was carried out to detect changes in functional groups of biomass and FTIR spectrum is shown in (Figure 49). The main characteristics were attributed to different chemical groups of cellulose, starch, hemicelluloses and lignin. Most prominent functional groups in biomass sample were alkyl halides, alcohols, carboxylic acids, esters, ethers , aromatics , alkanes, alkynes, 1˚amines and bands were 530.322, 1012.434, 1421.2651, 1656.525, 2159.86, 2927.382, 2927.382, 3432.635 (Figure 49). The peaks in fingerprint based on literature values are summarized in Table 29 (Adel *et al.*, 2011; Meng *et al*., 2012; Xiao *et al.*, 2011).

4. Treated banana peel waste

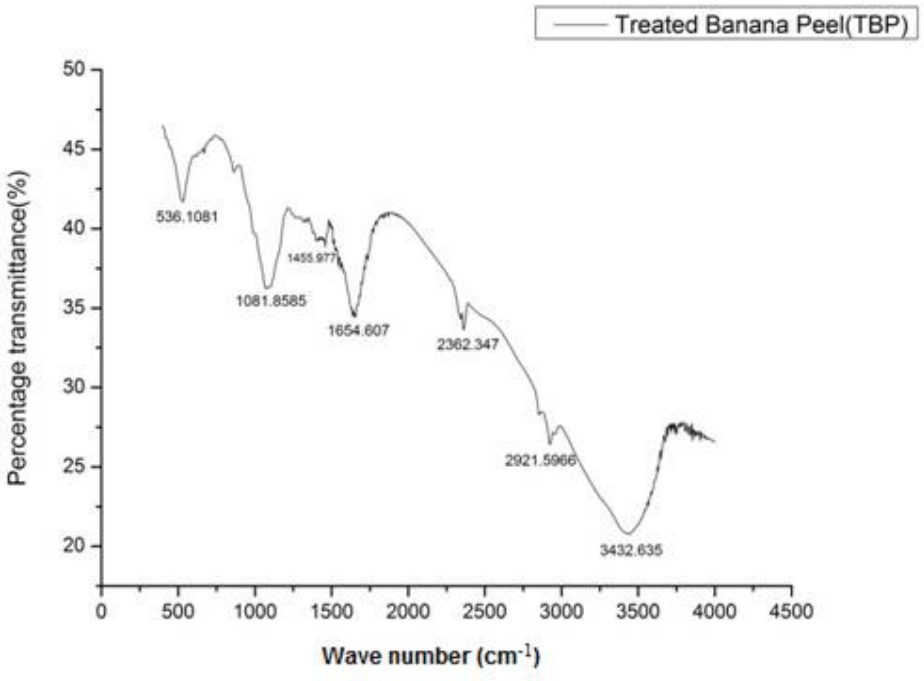


Fig. 50 FTIR of treated Banana Peel Waste

Table 30 FTIR of treated banana peel waste

Frequency, cm–1	Bond	Functional Group
690–515 (m)/ 536.1081	C–Br stretch	alkyl halides
1250–1020 (m)/1081.8585	C–N stretch	aliphatic amines
1470–1450 (m)/1455.977	C–H bend	Alkanes
1680–1640 (m/1654.607	–C=C– stretch	Alkenes
3000–2850 (m/ 2921.5966	C–H stretch	Alkanes
3300–2500 (m)/ 2921.5966	O–H stretch	carboxylic acids
3500–3200 (s,b)/ 3432.635	O–H stretch, H–bonded	alcohols, phenols

FTIR spectroscopy analysis of treated banana peel waste was carried out to detect changes in functional groups of biomass and FTIR spectrum is shown in (Figure 50). The main characteristics were attributed to different chemical groups of cellulose, starch, hemicelluloses and lignin. Most prominent functional groups in biomass sample were alkyl halides, aliphatic amines, alcohols, phenol, carboxylic acids, alcohols and phenols and bands were 536.1081, 1081.8585, 1455.977, 1654.607, 2921.5966, 2921.5966, 3432.635 (Table 50). The peaks in fingerprint based on literature values are summarized in Table 30 (Adel *et al.*, 2011; Meng *et al*., 2012; Xiao *et al*., 2011). It is found that the C=O stretching of un-conjugated ketones, carbonyls, esters groups, hemicellulose group was removed from treated banana peel waste after the treatement from *Bacillus licheniformis* NA11.

5. Treated potato peel waste

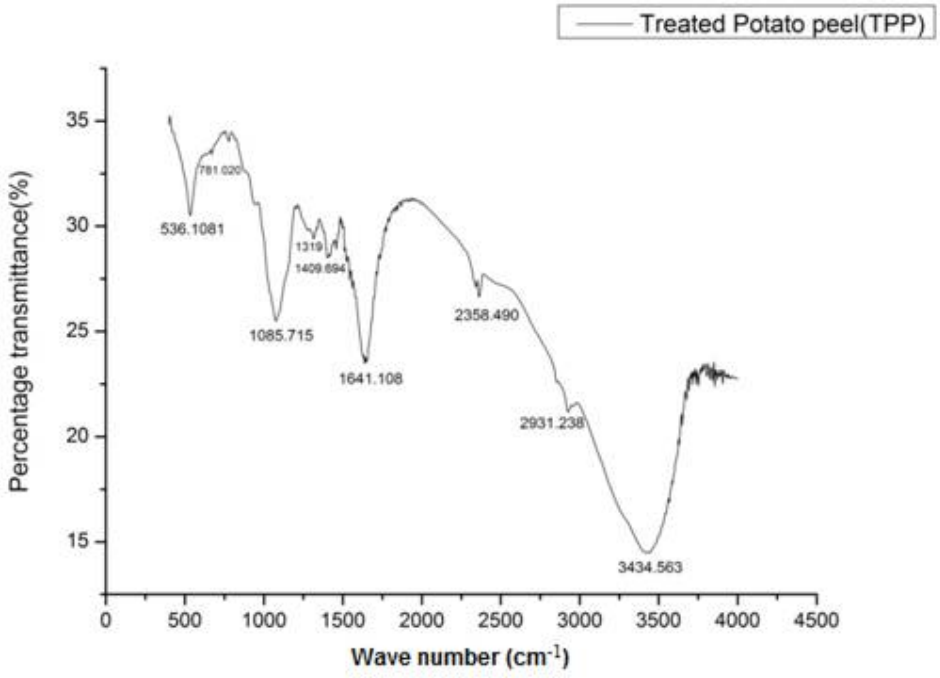


Fig.51 Treated Potato Peel Waste

Table 31 FTIR of treated potato peel waste

Frequency, cm–1	Bond	Functional Group
690–515 (m)/ 536.1081	C–Br stretch	alkyl halides
900–675 (s)/781.020	C–H "oop"	aromatics
910–665 (s, b)/781.020	N–H wag	1˚, 2˚ amines
1250–1020 (m)/ 1085.715	C–N stretch	aliphatic amines
1320–1000 (s)/ 1085.715	C–O stretch	alcohols, carboxylic acids, esters, ethers
1320–1000 (s)/ 1319	C–O stretch	alcohols, carboxylic acids, esters, ethers
1335–1250 (s)/1319	C–N stretch	aromatic amines
1500–1400 (m)/ 1409.694	C–C stretch (in–ring)	Aromatics
1680–1640 (m)/1641.108	–C=C– stretch	alkenes
3000–2850 (m)/2931.238	C–H stretch	Alkanes
3300–2500 (m)/2931.238	O–H stretch	carboxylic acids
3500–3200 (s,b)/3434.563	O–H stretch, H–bonded	alcohols, phenols

FTIR spectroscopy analysis of treated banana peel waste was carried out to detect changes in functional groups of biomass and FTIR spectrum is shown in (Figure 51). The main characteristics were attributed to different chemical groups of cellulose, starch, hemicelluloses and lignin. Most prominent functional groups in biomass sample were alkyl halides, aromatics, aromatic amines, aliphatic amines, alcohols, phenol, carboxylic acids, esters, ethers and bands were 536.1081, 781.020, 1085.715, 1319, 1409.694, 1641.108, 2931.238, 3434.563 (Figure 51). The peaks in fingerprint based on literature values are summarized in Table 31 (Adel *et al.*, 2011; Meng *et al.*, 2012; Xiao *et al.*, 2011). It is found

that C-O stretching vibration in starch, cellulose and hemicelluloses group were present more in peaks which shows reduction of lignin.

6. Treated orange peel waste

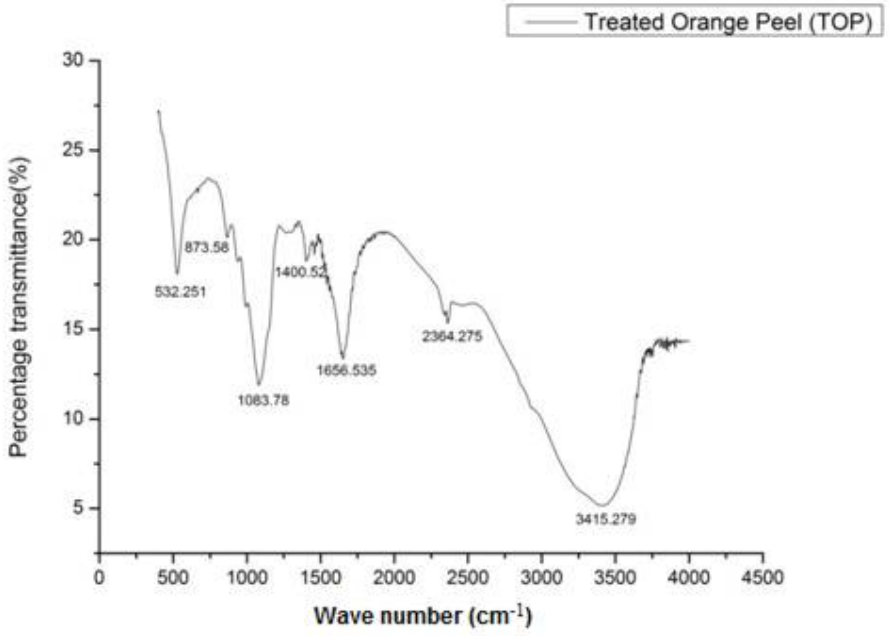


Fig. 52 Treated orange peel waste

Table 32 FTIR of treated orange peel waste

Frequency, cm–1	Bond	Functional Group
690–515 (m)/ 532.251	C–Br stretch	alkyl halides
900–675 (s)/873.58	C–H "oop"	aromatics
910–665 (s, b)/ 873.58	N–H wag	1˚, 2˚ amines
1250–1020 (m/1083.78	C–N stretch	aliphatic amines
1320–1000 (s)/1083.78	C–O stretch	alcohols, carboxylic acids, esters, ethers
1500–1400 (m)/1400	C–C stretch (in–ring)	aromatics
1680–1640 (m)/1656.535	–C=C– stretch	Alkenes
3500–3200 (s,b)/3415.279	O–H stretch, H–bonded	alcohols, phenols

FTIR spectroscopy analysis of treated banana peel waste was carried out to detect changes in functional groups of biomass and FTIR spectrum is shown in (Figure 52). The main characteristics were attributed to different chemical groups of cellulose, starch, hemicelluloses and lignin. Most prominent functional groups in biomass sample were alkyl halides, aromatics, 1˚, 2˚ amines , aliphatic amines, alkenes, alcohols, phenol, carboxylic acids, esters, ethers and bands were 532.251, 873.58, 1083.78, 1400, 1656.535, 3415.279, (Figure 52). The peaks in fingerprint based on literature values are summarized in Table 32 (Adel *et al.*, 2011; Meng *et al*., 2012; Xiao *et al*., 2011). It is found C-O stretching vibration in cellulose and hemi-cellulose were showed.

Ethanol production using simultaneous saccharification and fermentation

Ethanol production from different starchy substrate by using enzyme from NA11 and *Saccharomyces cerevisiae* using potassium dichromate method for 5 days time of incubation at 30° C **,** 120 rpm. This result showed the maximum ethanol production on 5th day of starch, OPW, BPW, PPW as 2.68%, 1.39%, 0.949 %, and 0.873% respectively. In comparison of starchy waste biomass orange peel showed maximum ethanol production than the banana peel and potato peel waste (Table 33; Fig. 54; Fig. 55). Recent research has demonstrated that orange peel waste represents a potentially valuable resource that can be developed into high value products (Angel et al., 2010).

Table 33 Ethanol production from different starchy substrate by using enzyme from NA11 and Saccharomyces cerevisiae using potassium dichromate method

Absorbance at O.D. 595								
Days	**STARCH**	**Ethanol (%)**	**OPW**	**Ethanol (%)**	**BPW**	**Ethanol (%)**	**PPW**	**Ethanol (%)**
0	0.082	0.957	0.042	0.621	0.038	0.588	0.025	0.42
1	0.098	1.09	0.051	0.697	0.043	0.63	0.031	0.436
2	0.158	1.59	0.074	0.89	0.056	0.739	0.038	0.495
3	0.178	1.76	0.088	1.008	0.064	0.806	0.043	0.56
4	0.211	2.04	0.091	1.033	0.075	0.899	0.051	0.677
5	0.287	2.68	0.133	1.39	0.081	0.949	0.071	0.873

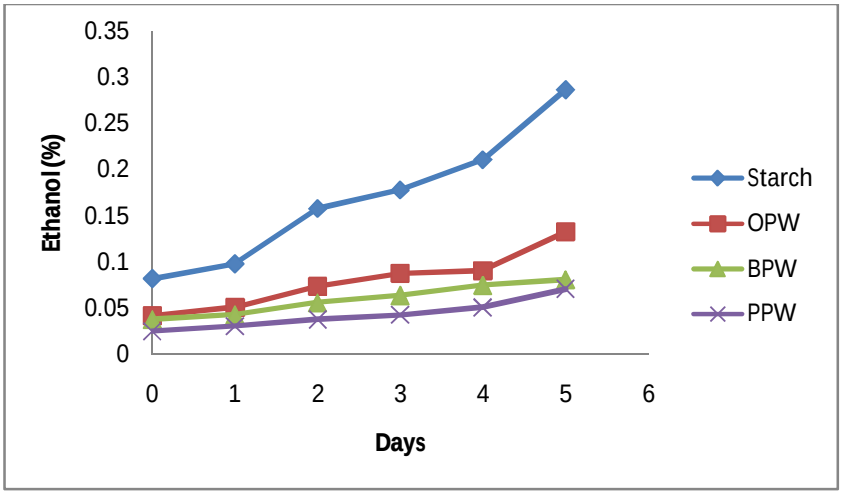


Fig.: 53 Ethanol production from different starchy substrate by using enzyme from NA11 and Saccharomyces cerevisiae using potassium dichromate method

Table 34 Ethanol production from different starchy substrate after 6 days of SSF using enzyme from NA11 and Saccharomyces cerevisiae

S. No.	Biomass	Ethanol (%) by GC (g / 100 ml)		Ethanol (%) by Potassium Dichromate Method for Treated
		Untreated	Treated	
1	Starch	0.002	2.51	2.68
2	OPW	0.433	1.33	1.39
3	BPW	0.317	1.03	0.94
4	PPW	0.033	0.89	0.87

The result shows maximum ethanol production using pre-treated biomass than the untreated one. The maximum ethanol was produced using starch which was 2.51% (Table 34). Comparison of starchy biomasses waste orange peel waste showed maximum ethanol production than the banana peel waste and potato peel waste such as 1.33%, 1.03%, and 0.89% under estimation by GC (Table 34; Fig. 54 ; Fig 55).

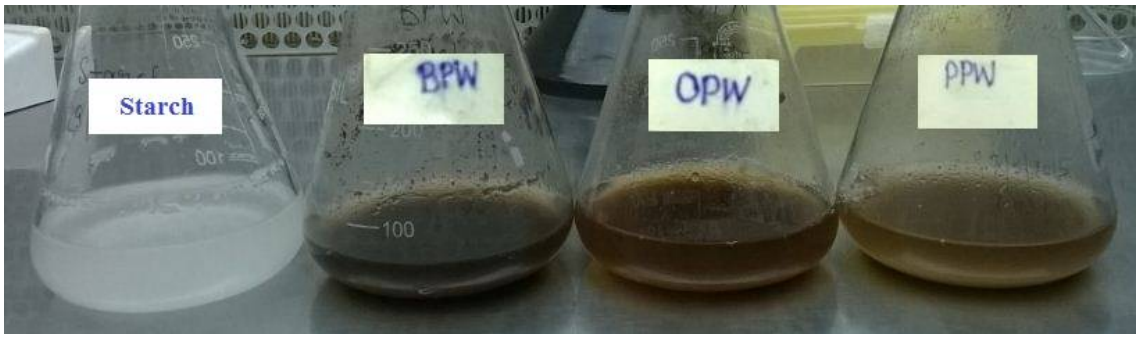


Fig. 54 Starch, banana peel waste, orange peel waste and potato peel waste

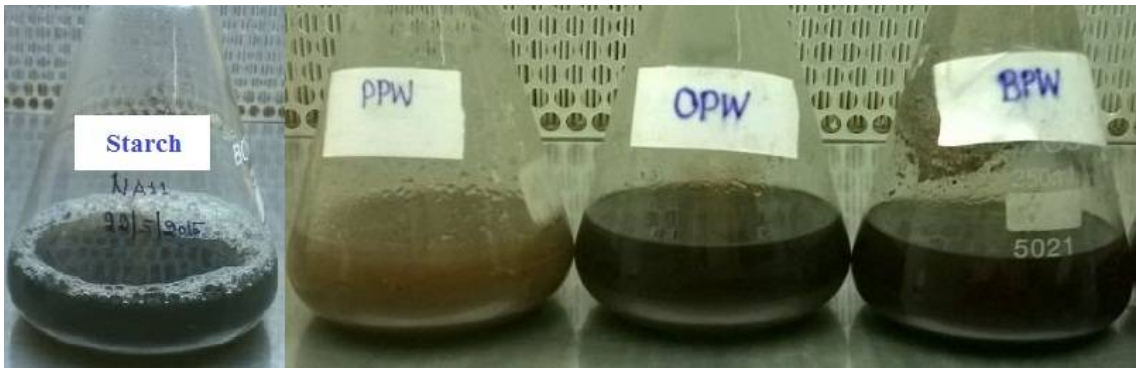
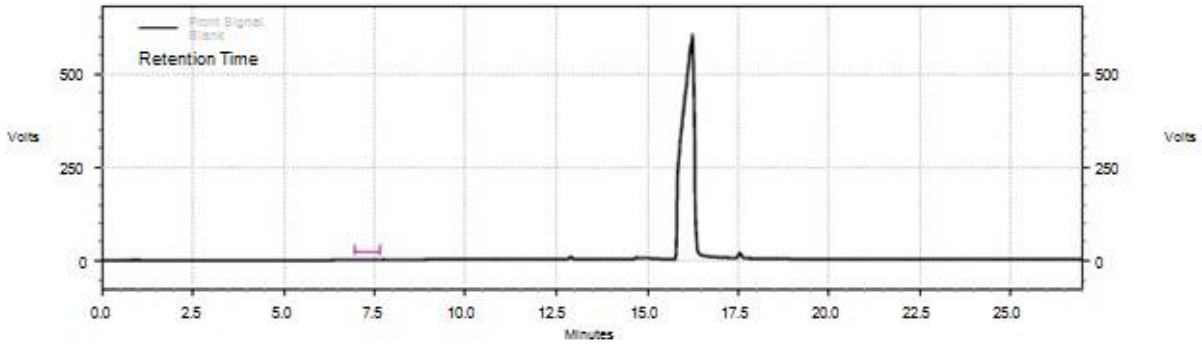


Fig. 55 Starch, potato peel waste, orange peel waste, banana peel waste

GC RESULT

1) Blank

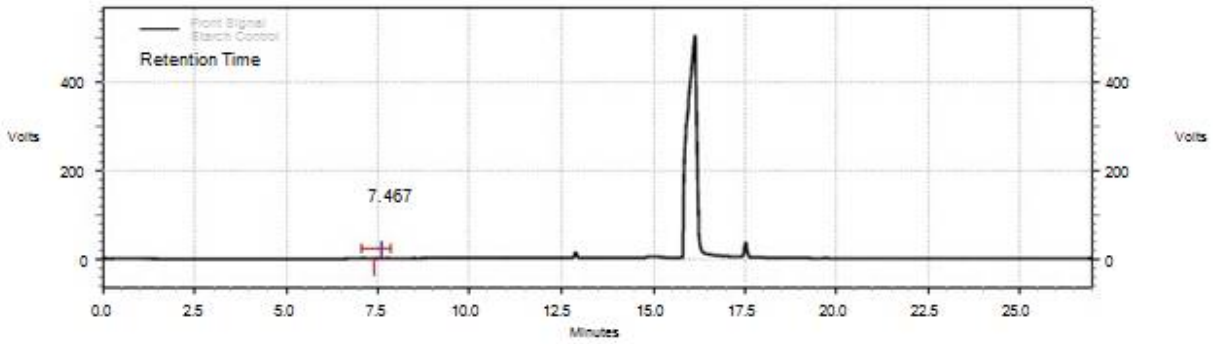


Front Signal
Results

Name	Retention Time	Area	Area %	Height	Height %

Fig. 56 No ethanol production in blank

2) Starch control

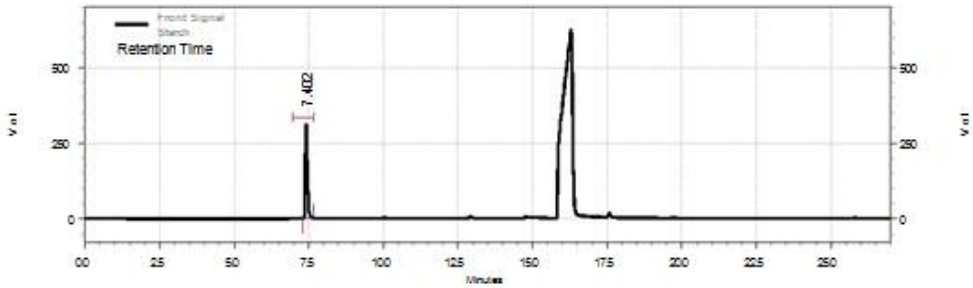


Front Signal
Results

Name	Retention Time	Area	Area %	Height	Height %
Ethanol	7.467	1125	100.00	5199	100.00
Totals		1125	100.00	5199	100.00

Fig. 57 No ethanol production in control starch biomass

3) Starch

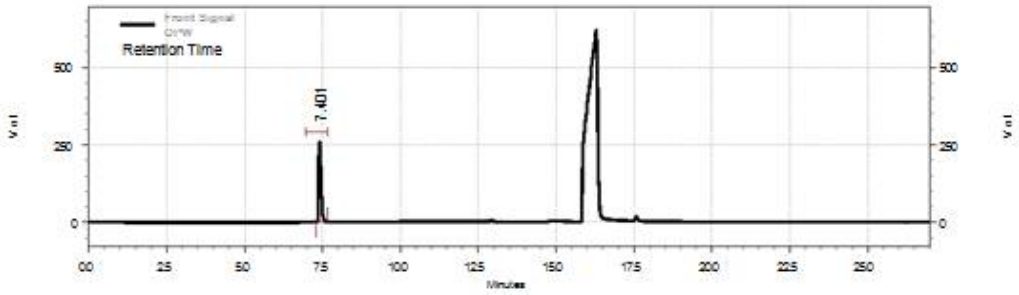


Front Signal
Results

Name	Retention Time	Area	Area %	Height	Height %
Ethanol	7.402	12225204	100.00	**212571**	100.00
Totals		12225204	100.00	**212571**	100.00

Fig. 58 Estimation of ethanol (2.5%) produced starch

4) OPW

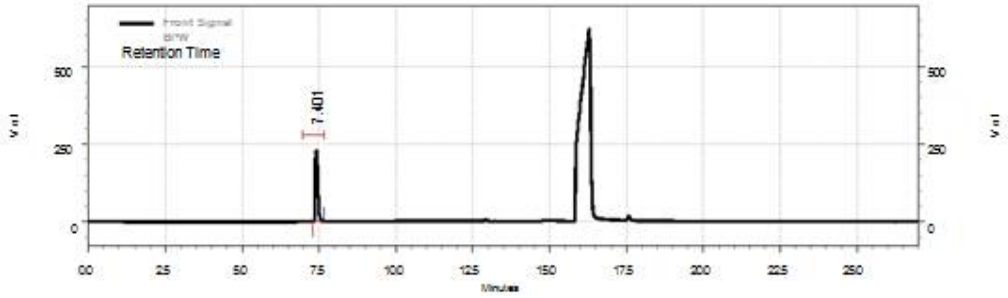


Front Signal
Results

Name	Retention Time	Area	Area %	Height	Height %
Ethanol	7.401	1085581	100.00	**222520**	100.00
Totals		1085581	100.00	**222520**	100.00

Fig. 59 Estimation of ethanol (1.33%) produced orange peel waste biomass (OPW)

5) BPW

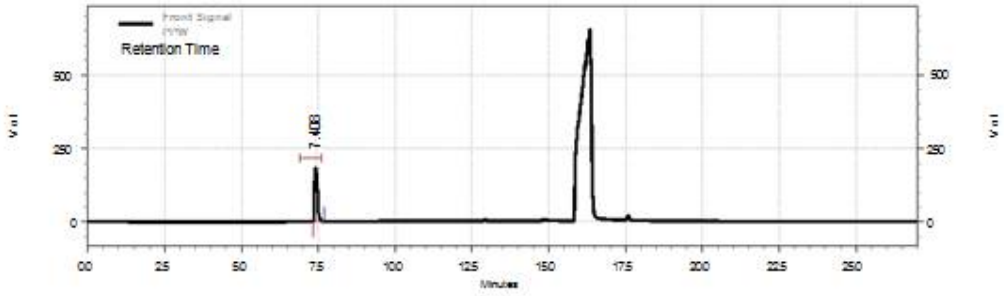


Front Signal
Results

Name	Retention Time	Area	Area %	Height	Height %
Ethanol	7.401	1055323	100.00	212571	100.00
Totals		1055323	100.00	212571	100.00

Fig. 60 Estimation of ethanol (1.03 %) produced from banana peel waste biomass (BPW)

6) PPW

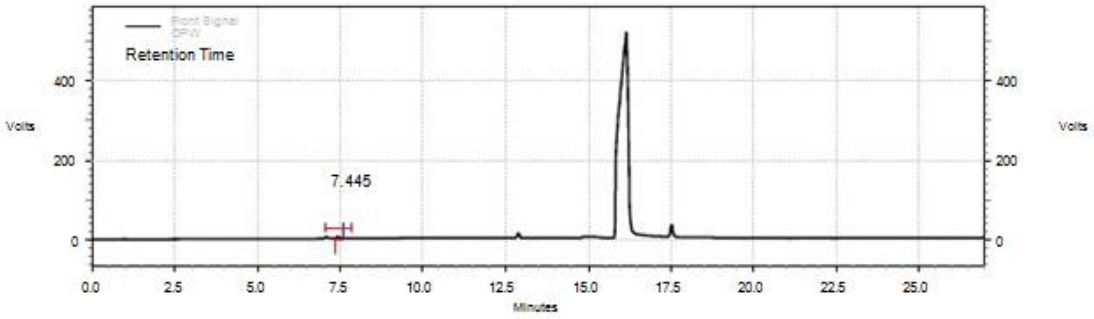


Front Signal
Results

Name	Retention Time	Area	Area %	Height	Height %
Ethanol	7.408	1043025	100.00	111421	100.00
Totals		913025	100.00	111421	100.00

Fig. 61 Estimation of ethanol (0.89 %) produced from potato peel waste biomass (PPW)

7) Untreated OPW

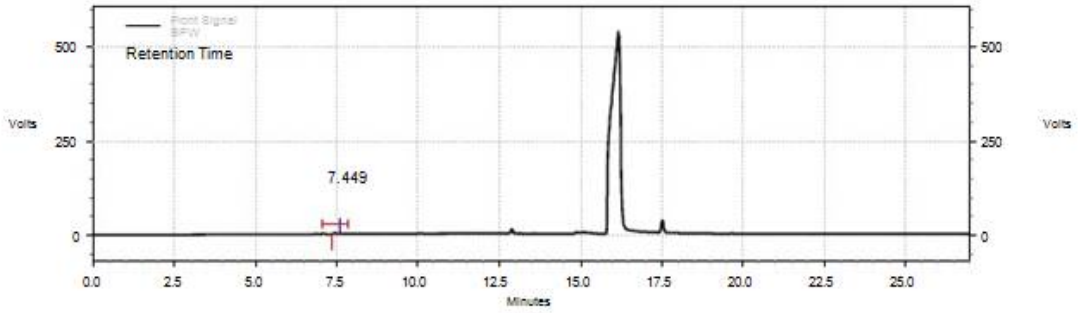


Front Signal
Results

Name	Retention Time	Area	Area %	Height	Height %
Ethanol	7.445	211734	100.00	37572	100.00
Totals		211734	100.00	37572	100.00

Fig. 62 Estimation of ethanol (0.433%) produced from orange peel waste biomass (OPW)

8) Untreated BPW

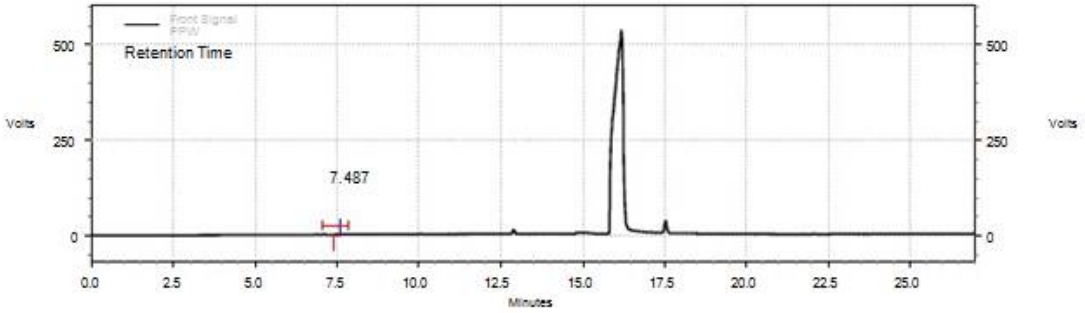


Front Signal
Results

Name	Retention Time	Area	Area %	Height	Height %
Ethanol	7.449	155316	100.00	27245	100.00
Totals		155316	100.00	27245	100.00

Fig. 63 Estimation of ethanol (0.317 %) produced from banana peel waste biomass (BPW)

9) Untreated PPW



Front Signal
Results

Name	Retention Time	Area	Area %	Height	Height %
Ethanol	7.487	16440	100.00	2967	100.00
Totals		16440	100.00	2967	100.00

Fig. 64 Estimation of ethanol (0.033 %) produced from potato peel waste biomass (PPW)

CHAPTER
CONCLUSION

1. A potential Amylase activity in the cell free supernatant of starch degrading bacteria *Bacillus licheniformis* NA11 was studied using 1% starch. with optimal activity at pH 7.0 and temperature 60°C. The Km value of the partially purified enzyme for starch substrate was 0.621 mg/ml and Vmax=0.0354 µmole/min/ml.
2. Amylase activity of partially purified enzyme from *Bacillus licheniformis* showed maximum activity of 1.12 U/ml/min, with production of 0.467 mg/ml reducing sugar when 1% starch was used as substrate. When starchy wastes were used as substrate the enzyme activity in 160 min at 60°C was 1.048 U/ml/min for orange peel waste (OPW) with production of 0.435 mg/ml of reducing sugar, 0.99 U/ml/min for banana peel waste (BPW) with production of 0.411 mg/ml of reducing sugar and 0.959 U/ml/min for potato peel waste (PPW) with production of 0.398 mg/ml of reducing sugar.
3. Iodine assay in test tube and micro-plates, showed blue colour in the presence of starch- iodine and at 60-70°C, all blue color disappeared after 60 min.
4. Statistical optimization using response surface methodology predicted amylase activity of 0.47 U/mL, which coincided with the experimental amylase activity of 0.46 U/mL using starch (10 g/L), yeast extract (6 g/L), and $MnCl_2.4H_2O$ (1.0 g/L).
5. Starchy waste such as Potato peel waste (PPW), orange peel waste (OPW), banana peel waste were (BPW) biomass were washed to remove adhering debris, dried and powdered (0.3- 0.5 mm) to investigate its physico-chemical properties. Potato peel waste (PPW) was found to contain 6.64% moisture, 5.48% ash and 75.79 % volatile matter, similarly orange peel waste (OPW) was found to contain 5.451 % moisture, 9.717 % ash, 84.52 % volatile matter and banana peel waste (BPW) was found to contain 5.01 % moisture, 7.35 % ash, 81.40 % volatile matter.
6. Scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier transform infrared analysis (FTIR) analysis of native and enzyme pre-treated biomass was done to study physical and chemical changes.
7. Enzymatically pre-treated starchy wastes (PPW, OPW and BPW) were used as substrate for ethanol production using cell free supernatant from *Bacillus licheniformis* NA11 followed by ethanol fermentation using *Saccharomyces*

cerevisiae (NCIM 3215) via SSF. Ethanol production using starch as substrate was 2.51%, with orange peel waste it was 1.33%, with banana peel waste it was 1.03% and with potato peel waste it was 0.89%. The high catalytic activity and its stability to temperature, pH, indicated that the amylase enzyme from *Bacillus licheniformis* NA11 is a good candidate for hydrolysis of starchy waste into bio-refinery processes.

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