Available online at http://www.ijims.com
ISSN: 2348 – 0343

Isolation and Optimization of Cellulolytic and Xylanolytic Microbes from Nepal and their Utilization for Lignocellulosic Biomass Degradation

Pranita Kaphle, Srishti Rana, Jay Bhandari, Asha Bhandari, Richa Aishwarya, Tika Bahadur Karki, Bhupal Govinda Shrestha*
Department of Biotechnology, Kathmandu University, Dhulikhel, Kavre, Nepal

*Corresponding Author: Bhupal Govinda Shrestha

Abstract

Microorganisms harbored by nature and guts of herbivorous animals can degrade different plant related biomass. One of the in-between steps for conversion of lignocellulosic biomass to ethanol entails isolation and identification of microorganisms that could convert pretreated biomass into a suitable form, which could then be fermented into bioethanol. We isolated 36 different microorganisms from hot spring, 6 from ruminant's (goat) gut, 2 sample from hay spray on the basis of their ability to secrete enzymes that hydrolyzed different plant constituents. Similarly 3 microorganisms were isolated from the rotten wood available around Kathmandu University on the basis of utilization of xylose and glucose. 3 varieties of thermophiles, gut microorganism and microorganism from hay spray that showed the highest cellulolytic and xylanolytic activities by saccharification of cellulose and xylan into their monosaccharide glucose and xylose units respectively were then applied on different biomass (rice straw, corn stover and sugarcane bagasse). Before Saccharification biomass was made accessible for the digestion by enzymes through 3 different pretreatment strategies (3.35% H_2SO_4, NaOH and H_2O_2 with 1:10 substrate: chemical ratio) following thermal strategy of steam explosion. Also, different conditions like incubation time, pH and temperature for saccharification were assessed with the highest liberation of reducing sugar at pH 5, temperature of 50^{0}C and incubation time of 4 days. Microorganism from rotten wood was able to utilize both xylose and glucose and yielded highest amount (5.567 mg/ml) of bioethanol.

Keywords: Lignocellulosic biomass, pretreatment, xylanolytic, cellulolytic, saccharification, fermentation.

I. Introduction

Owing to the increasing population of the world and subsequent industrialization, the call for fuels has grown worldwide in the last century to cope up with their demands. It is prognosticated that with the current rate of intake, the global oil reserves are expect to exhausted in 40 years[1]. Hence, all the nations round the world are committing in substitute sources of energy. One of such resources is ethanol derived from sugar-rich biomass (bioethanol).

After the Industrial Revolution, just before the break of the 20th century, fossil fuels, as a source of energy to run motor vehicles have been used extensively. Even though these resources are easy to use for energy generation requiring a simple direct combustion, they are quite expensive and also have a direct impact on the environment. Their combustion leads to muckle of air pollution and contributes to global warming, acid rain, etc. whereas; their mining from ground significantly alters the environment. In addition to this, being a non-renewable source of energy, it retains the threat of being eaten up. However, the use of renewable sources of energy overcomes certain shortcomings of the previous one. The use of these sources proffers significant opportunities like reduction in green house gas emission (run on zero carbon cycle) and assures

energy supply. The minuses of non-renewable sources of energy have been coercing scientists to look for the sustainable options[2].

Large amount of wastes from agriculture are unexploited and offers a workable option for development. Agricultural wastes or biomass are renewable sources of energy that refer to living and recently dead biological material which can be used as a fuel for industrial production. Of the many alternative sources of energy, lignocellulosic biomass is one of the promising sources that can be used to produce ethanol. This biomass represents approximately 90% of plant dry matter, is primarily composed of cellulose (35-50%) and hemicelluloses (20-35%) together with lignin (5-30%) [3].

Ethanol can blend with gasoline and run as neat alcohol in dedicated engines taking advantage of high octane number and heat of vaporization[4]. According to current technologies the ethanol produced from the biomass is relatively costly being low yield and high cost of the hydrolysis process[3].

At present times, United States and Brazil are leading in most of the ethanol being produced from renewable resources, mainly from sugarcane and starchy grains. Nonetheless, significant efforts are being made to produce ethanol from lignocellulosic biomass (almost 50% of all biomass in the biosphere) such as agriculture residues [5]. The main challenge to this attempt is efficient conversion of the complex carbohydrate into their simple sugars, which, can then be fermented to alcohol[6]. For the efficient conversion of carbohydrate into their simple sugars, thermophillic bacteria have been found to be useful. These bacteria have the ability to convert cellulose to glucose and hemicelluloses to xylose[7]. In recent years, thermophilic microorganisms have received great attention as they are active and do not die easily even at high temperatures[8, 9, 10]. There is, thus, an obvious attention on enzymes from these species as they resist chemical reagents and extreme pH values, apart from being thermophilic compared to the ones that are from mesophilic organisms. For commercial production of bioethanol, both hexose and pentose sugars present in the hydroysate should be fermented and should show sufficient tolerance to the inhibitors present in the hydrolysate[11, 12].

II. Materials and Method

A. Sample collection from hot springs

Water samples from various sites within hot springs located in two regions: Tatopani (**27°56'51"N, 85°57'6"E**) and Kharapani (**28°21'36"N, 83°57'36"E**) in Nepal were collected in sterile tubes. Altogether 36 samples were collected from these two areas. Similarly, 6 samples from various sections of ruminant's (goat) gut from a local slaughter house, 2 samples from hay spray from Praramva biotech in Kathmandu and 3 soil samples from different rotten wood around Kathmandu University were collected. All these samples were collected in 15 and 50 ml falcon tubes where freezing temperature was maintained by ice packs, until it reached the laboratory at Kathmandu University. All the samples were stored at 4 ^{0}C until further use.

B. Isolation of organisms

Serial dilutions of up to 10 $^{(-6)}$ of 1 ml of homogeneously mixed water samples was done in sterile water and plated on Nutrient Broth (NB) containing 0.2% (Carboxy methylcellulose)CMC (Qualigens) and 0.2% xylan (Sigma) and incubated at varying temperature ranging 40^0 C, 55^{0}C and 60^{0}C. A loop full of samples from rest of the gut and hay spray were mixed in sterile water and serially diluted as mentioned above. Finally one loop full of 10^{-6} diluted sample were then streaked in almost 15 Nutrient Agar plate and incubated at 37^{0}C. Microorganisms growing in presence of xylan and cellulose were isolated and stored as a stock at 4^{0}C for screening test of cellulolytic and xylanolytic activity later. Similarly samples from

rotten wood are also isolated using (Yeast extract (10g/l), peptone (20g/l) and dextrose (10g/l)) YEPD media and incubated at 25^0C for 2 days and stored at 4^0C for its fermentation test.

C. Preliminary screening of cellulolytic, xylanolytic and xylose utilizing microorganisms

The cellulose degrading capacity of microorganisms was screened on CMC media (K_2HPO4, 0.05% $MgSO_4$, 0.05% KCl, 1% CMC (Qualigens) sodium salt, 0.02% peptone, and 1.7% agar) Agar plate[18]. The spot plated microorganisms were then incubated at 55^0 C (thermophilic), 37^0 C (mesophilic) for 48 hours. The plate was then flooded with 0.1% Congo red solution for about 15-20 minutess followed by washing of plates with 1M NaCl for 15 minutes.

Similar method as that in the screening of cellulolytic microorganisms was used to test the xylanolytic activity. The only difference being media with 1% Birchwood Xylan as the screening media. Previously isolated microorganisms from rotten wood samples were inoculated in (0.3g peptone, 0.15g carbohydrate, 0.45g NaCl, 0.00054g phenol red and dissolved in 30 ml distilled water) media poured in 15ml test tube containing inverted durham's tube to check their carbohydrate fermenting ability. The so isolated and selected microorganism was named as HS1 (from hotspring), 1b (from ruminant's gut), h1 (from hay spray), 1i, 3i, 6ii (from rotten wood).

D. Morphological study

Morphological characteristics like colony characters and gram stain response were studied as follows:

1. Colony characteristics study

The colony characters like color, shape, surfaces, size, elevation, edges and opacity of all the isolated cultures of microorganism were observed.

2. Gram staining

Gram reaction of the isolated and screened microorganisms was carried out by preparing a thin smear of the cultures in a clean glass slide which was then air-dried. The smear was stained with crystal violet for 1 min followed by pouring of iodine and decolorization with alcohol. The slide was then rinsed, counterstained with Safranin for 2 mins and finally observed in oil emulsion lens under microscope.

E. Biochemical test

Different biochemical tests were performed as follows and the results were obtained.

1. IMVIC test

Indole Test: The isolated micro species cultures were incubated in sterile broth of tryptophan or tryptone media (Hi media) for 24-48 hrs 37 ^{o}C before performing the test. 5 drops of Kovac's reagent was added to the culture broth.

Methyl red test: Isolates were inoculated into a tube containing MR-VP broth (Hi media) with 5 drops of the pH indicator methyl red and incubated at 37°C for 2 days.

Voges proskauer reaction (VP) test: This test was done by inoculation of a loop full of culture in MR-VP broth and incubated at 37^{o}C for 48 hrs followed by addition of Barrit's reagent.

2. Catalase test

A loop full of culture was placed on the glass slide that contained Hydrogen peroxide (H_2O_2) liquid.

3. Triple sugar iron agar test

TSI test is done to determine carbohydrate fermentation and H_2S production. The sample was stabbed into the butt and streaked on the slant of TSI media (Hi media) with the help of sterile straight wire. Then the media was incubated at 30ºC for 18 hrs. Then the color of slant and butt was observed.

4. Oxidative fermentation test

Two tubes of medium were taken where one tube was heated in boiling water for 10 minutes to drive off the oxygen. It was then cooled and inoculated by inserting a straight wire vertically. One tube was incubated aerobically and the second tube was sealed at the surface with a layer of sterile liquid paraffin oil to create anaerobic conditions. Incubation was carried out at 37°C for 48 hrs. Tubes were examined daily for color change to determine the oxidative and fermentative capacity of microbes.

5. Citrate utilization test

To assess ability of an organism to utilize citrate as a nutrient, Simmons Citrate agar slant was prepared such that citrate was the only carbon source. The medium contained Bromthymol blue indicator. The slant was inoculated with straight wire and incubated for overnight at 37°C.

6. Pectinase test

It is done to check whether the organism can digest pectin or not by producing pectinase. The agar plates were prepared and streaked with the culture incubated at 37°C for 24 hrs. The plates were then washed with potassium iodide. Then the plates are observed whether clear zones were appeared.

F. Substrate choice and pre-treatment

Agricultural wastes including rice straw, sugarcane bagasse and corn stover collected from different available area were then dried and cut into small pieces.

G. Total solids and moisture content

The sample was weighed into weighing dish which was then placed in oven at 105°C till the weight is constant and allowed to cool in a dessicator.

H. Pretreatment followed by steam explosion

The organic substrates from section F were ground and added in 1N NaOH solution, 3.35% H_2SO_4, and H_2O_2 at 1:10 substrate to chemical ratio. After suspension in the NaOH, H_2SO_4 and H_2O_2, the samples were autoclaved at 121°C for one hour. The contents were then filtered through vacuum filter and the residue was neutralized further, so as to be used for hydrolysis experiments.

I. Cellulase assay

Total cellulase activity in the culture filtrate was determined according to the method of Mandels et al (1976).The reducing sugar released from filter paper per ml per min at 540nm was determined by Dinitro salicylic acid (DNS) method. One unit of total cellulase activity was defined as the amount of enzyme releasing 1µmole of reducing sugar per minute[19].

Optimization of production of cellulase enzyme: To select the suitable temperature for saccharification, the selected strains were cultivated with varying temperatures of 25℃-60℃, pH range 4-8, incubation period range of 5-6 days, by keeping all other parameters constant.

Partial purification of cellulose: All procedures of the cellulase purification were carried out at 4℃. The culture supernatant was separated by centrifugation process followed by fractional ammonium sulphate precipitation by adding solid ammonium sulphate to the culture filtrate to 80% saturation. The resulting precipitate was collected by centrifugation at 4,000 × g for 30 min and dissolved in sodium citrate buffer and dialysed overnight against three changes of the same buffer and stored at 4℃. Partially purified Enzyme was confirmed by SDS-PAGE with the stacking and resolving gel concentrations were 4% and 12% of polyacrylamide respectively. After the electrophoresis, the gels were stained with Coomassie blue for visualization of protein bands.

J. Enzymatic Saccharification

Weighed out pretreated biomass sample equivalent to 0.1 g of cellulose. To each flask, add 5.0 mL 0.1 M, pH 4.8 sodium citrate buffer. Distilled water was added to bring the total volume in each flask to 10.00 ml. The content of each vial was brought to 50°C by warming in the incubator set at 50°C ± 1°C. To each flask was added an appropriate volume of the crude cellulase enzyme, xylanase unit (0.022U/ml) and cellulase activity (<0.37 U/ml). A reaction blank was prepared for the substrate. The substrate blank contains buffer, water, and the identical amount of substrate in 10 ml volume. Closed the flasks tightly and were place in shaking incubator or fixed speed rotator that has been placed in the incubator. Set the temperature to 50°C and incubate with shaking or rotation sufficient to keep solids in constant suspension for a period of 72 to 168 hours or until the release of soluble sugars from the sample(s) becomes negligible.1 ml aliquot was removed at each predetermined time interval after the flasks contents have been well mixed by shaking and reducing sugar was checked by DNS method [20].

K. Fermentation

The flask, in which enzymatic process is carried out, is used for fermentation process. In the flask, the broth culture of the microorganism (3i) used for fermentation process was added. The broth culture should be 10% of the final volume to be adjusted. Some amount of distilled autoclaved water was added in the fermentation flask. The flasks were then kept in the incubator with shaker at the temperature of 25°C. Every 24 hrs reducing sugar was checked by DNS method to check if sugar is decreasing or not. Every 24 hrs 10 ml of the fermented liquid was stored in the tubes for the estimation of alcohol later.

L. Ethanol estimation by potassium dichromate method

Standard ethanol solution of 3.95 mg/ml stock was prepared by using distilled water. Solution was then diluted in the range of 7.9mg/ml to 39.5 mg/ml. 1 ml of each solution and each fermented sample was taken in different test tubes along with 1 ml potassium dichromate solution followed by 5 ml of conc. H_2SO_4 acid. Test tubes were boiled for 10 mins. Absorbance was taken at 600 nm in spectrophotometer.

III. Results and Discussion

The temperature and pH of the sampling site Tatopani was around 42° C and 7.3 respectively where we collected 16 samples. But only two distinct isolates could be derived using NB (0.2% CMC, 0.2% xylan) as the primary isolation media. 20 samples have been collected from Kharapani site where the temperature and pH of the sampling site were 52° C and 7.7 respectively. About 13 samples of microorganism from this location had been taken into consideration. We were also able to isolate 5 different microorganisms from ruminant's gut, 2 form hay and 6 from rotten wood. Among 28 isolated microbes, HS1(from hotspring), 1b (from ruminant's gut) and 1i, 3i, 6ii (from rotten wood) that represented each of the sample sources

with highest cellulolytic, xylanolytic or xylose utilizing activities were selected for further round of tests before saccharification and fermentation process.

Almost all these selected isolates were found to be Gram negative, long as well as short rods. Only one Gram positive isolate was observed i.e. 1b under light microscope.

Morphology of microorganism was studied in order to determine its size, color, shape, elevation, opacity etc. Color, elevation and consistency were found similar in case of HS1 and 1b whereas shape, size and all other characters varied from each other as shown in Table 1. Biochemical test for the identification of various metabolic properties of different bacterial species showed (HS_1) to be positive to congo-red test, Voges–Proskauer (VP) test and catalase test and negative to indole test, methyl red test, citrate utilization test, triple sugar iron test and pectinase test. Similarly, microorganisms from goat's gut showed positive congored, indole, citrate utilization test and catalase test whereas negative to methyl red, Vp, triple sugar iron and pectinase test as in Table 2.Oxidative fermentation test showed that HS_1 can metabolize fructose, sucrose, cellulose and xylan both aerobically and anaerobically whereas it can metabolize glucose in anaerobic condition only. Similarly, GG_1 can metabolize fructose, sucrose, cellulose and xylan in anaerobic condition only. Though HS1 and 1b showed good result of biochemical test but it is difficult to link with other related microorganism before genetic characterization. These two organisms were subjected for saccharification where 1b showed better results of brix and reducing sugar in all three substrates when done thrice whereas HS1 was unable to saccharify any of them. Comparing with 1i and 6ii, 3i was able to ferment glucose and xylose separately and in combined form with gas production as shown in Fig 4.

Moisture content of rice, bagasse and corn was found to be 10.6%, 9.72%, 10.8%. The substrates were made easily accessible for enzymes through pretreatment process using alkali, acid, hydrogen peroxide and water followed by steam explosion and was found best in acid and alkali pretreatment as shown in Fig 1. The choice of pretreatment may vary depending on the substrate. Fermentation was hindered by acid pretreatment of substrate may be due to the production of inhibitors after thermolysis so alkali pretreatment were prefered and neutralized before sacharification. Before saccharification substrates were neutralized, washed and brix was measured as 1° (rice), 1° (corn), and 4.5° (bagasse). Brix measured before and after alkali pretreatment/steam explosion was found to be 8° (rice), 9.2° (corn), 9.6° (bagasse) and 15.3° (rice), 12.5° (corn), 11.4° (bagasse).

The degree Brix of the sample before saccharification, during and after fermentation media, was also measured to estimate the amount of total sugar present in the sample. Brix was high during and after saccharification but reduced during fermentation as shown in Fig 5. This was an expected result as the total monosaccharide sugars formed after saccharification by hydrolysis of the complex polysaccharides would be high. And thus during fermentation 3i utilized the total fermentable sugar resulting in the decrease of such monosaccharides in the medium to convert into alcohol.

Different organisms that were isolated were subjected to saccharification of the pretreated substrate, and among them 1b showed best activity, because of the increased absorbance, relating to increased formation of soluble sugars (Fig 2).

Enzyme assay of crude enzyme obtained from 1b was performed and was found 0.022U/ml xylanase activity. Filter Paper assay was also done where 0.5 ml of crude enzyme was able to release 1.411 mg/ml glucose. Partial Purification of this enzyme was carried out through ammonium sulphate precipitation and dialysis. The activity of the purified enzyme was measured before and after purification. After precipitation, the zone of inhibition was compared against the unprecipitated supernatant. Hydrolyzing capacity of crude enzyme before purification was 3.5cm (xylan media), 3.3cm (CMC media) in

case of 1b and 1.875cm (CMC media) in case of HS1.Similarly, hydrolyzing capacity after purification was 5cm (xylan media), 5.6cm (CMC media) in case of 1b and 2.85cm (CMC media) in case of HS1. HS1 did not show any xylanase activity before and after purification. After purification and congo-red test 1b was selected and further subjected for saccharification.

Enzymatic saccharification optimization experiments showed the best condition of saccharification in pH 5, temperature 55°C, 4 days incubation period. The best media was found to be 2X media (xylan/CMC:0.1g/l, yeast extract: 6.0 g/l, ammonium sulphate: 0.1 g/l, sodium chloride: 0.3g/l, magnesium sulphate 0.1g/l, calcium carbonate: 0.02g/l) which has yeast extract other than all other media. Organism in this media liberated highest amount of reducing sugar as estimated by DNS method. Therefore we can say organism grown in this media showed highest enzymatic activity in comparision to all other media as shown in Fig 3.

Saccharification was carried out at optimized pH, temperature, media and with different innoculum ratio (organism grown separately using cellulose and xylan in different flask for the enhancement of specific production of enzyme). Highest glucose (10.1552 mg/ml) was released in 40:60 inoculum ratios in 4 days incubation. Further alcohol was also estimated and found to be 5.567mg/ml when 3i was subjected for fermentation.

Conclusion

Microorganism residing in the nature and inside various herbivorous animals degraded agricultural and various other organic wastes,additionally converted them into ethanol.This indicates the organic wastes could be a suitable source of renewable energy source like bioethanol and biofuel,if effective enrichment methods could be found out for the optimum production of the so recognized microorganisms. The result of this study would be more significant if genetic characterization and genetic improvement is done.

Acknowledgement

The authors gratefully acknowledge the contributions of Renewable Nepal team for their support, research motivation and research grant.

References

[1] Puppan D. Environmental evaluation of biofuels, Priodica Polytechnica Series -Social and Management Sciences, 2002; 10(1.): 95–116.

[2] US Congress, Energy Independence and Security, Act of 2007[online]
Available from: http://www.gpo.gov/fdsys/pkg/BILLS-110hr6enr/pdf/BILLS-110hr6enr.pdf
[Accessed 4th January 2007].

[3] Sun Y and Cheng J. Hydrolysis of lignocellulosic materials for ethanol production: a review, Bioresource Technology, 2001; 83: 1–11.

[4] Hahn- Hägerdal B, Galbe M, Gorwa-Grauslund MF, et al. Bio-ethanol-the fuel of tomorrow from the residues of today, Trends Biotechnol, 2006; 24:549-56.

[5] Bothast RJ, Saha BC. Ethanol production from agricultural biomass substrates, Adv. Appl. Microbiol., 1997; 44: 261-286.

[6] Joshi B, Bhatt MR, Sharma D, et al. Lignocellulosic ethanol production: Current practices and recent developments, Biotechnology and Molecular Biology Review, 2011; Vol. 6(8):172-182.

[7] Sonnleitner B, Fiechter A. Advantages of using thermophiles in biotechnological processes: expectations and reality, Trends Biotechnol., 1983; 1: 74-80.

[8] Becker P, Abu-Reesh I, Markossian S. Determination of the kinetic parameters during continuous cultivation of the lipase producing thermopile Bacillus sp. IHI-91 on olive oil, App. Microbiol. Biotechnol., 1997; 48: 184-190.

[9] Beg QK, Bhushan B, Kapoor M, et al. Production and characterization of thermostable xylanase and pectinase from Streptomyces sp, QG-11-3. J Ind. Microbiol. Biotechnol., 2000; 24: 396-402.

[10] Lee DW, Koh YS, Kim KJ, et al. Isolation and characterization of a thermophilic lipase from Bacillus thermoleovorans ID-1, FEMS Microbiol. Lett., 1999; 179: 393-400.

[11] Hahn-Hägerdal B, Karhumaa K, Fonseca C, et al. Towards industrial pentose-fermenting yeast strains. Applied Microbiology and Biotechnology, 2007; 74: 937-953.

[12] Almeida JRM, Modig T, Petersson A, et al. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae, Journal of Chemical Technology & Biotechnology, 2007; 82:340-349.

[13] Hussin N. Akmar I. Asma B, et al. Identification of appropriate sample and culture method for isolation of new thermophilic bacteria from hot spring, African Journal of Microbiology Research, 2011; 5(3): 217-221.

[14] Matsumura Y, Minowa T and Yamamota H. Amount, Availability, and potential use of rice straw (agriculture residue) biomass as an energy resource in Japan, Biomass and Bioenergy, 2005; 29:347-354.

[15] Zhu S D, Wu Y X, Yu ZN, et al. Pretreatment by microwave/alkali of rice straw and its enzymic hydrolysis, Process Biochem, 2005;40: 3082-3086.

[16] Brownell HH and Saddler JN. Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis, Biotechnol Bioeng., 1987; 29(1):228–35.

[17] Bisaria VS. Bioprocessing of agro-residues to value added products. In: Martin A M (Ed.).Bioconversion of Waste Materials to Industrial Products. 2nd ed. UK: Chapman and Hall; 1998.p. 197-246.

[18] Kasana RC, Salwan R, Dhar H,et al. A Rapid and Easy Method for the Detection of Microbial Cellulases on Agar, 2008; 57(5):503-507.

[19] NREL. Measurement of cellulase activities. Laboratory Analytical Procedure. Golden, CO: NREL; 2008.

[20] NREL. Enzymatic Saccharification of Lignocellulosic Biomass. Laboratory Analytical Procedure.Golden, CO: NREL;2008.

Figures and Table

Table 1: Morphology of the microorganisms

	HS.$_1$	**1b**
Color	Cream	Cream
Shape	Circular	Irregular
Surface	Smooth	Wavy
Size	3-4mm	<1mm
Elevation	Low convex	Low convex
Edge	Entire	Lobate
Opacity	Translucent	Opaque
Consistency	Mucoid	Mucoid

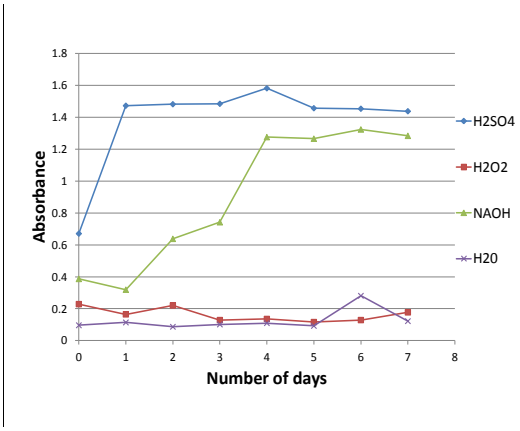


Fig.1: Pretreatment of substrate

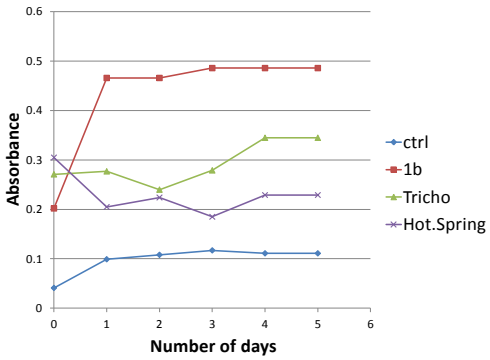
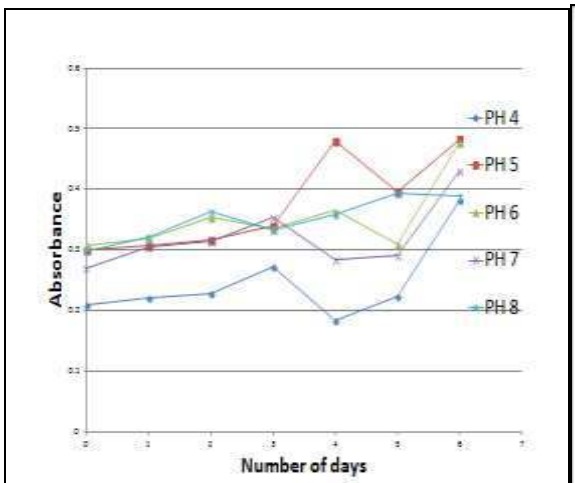
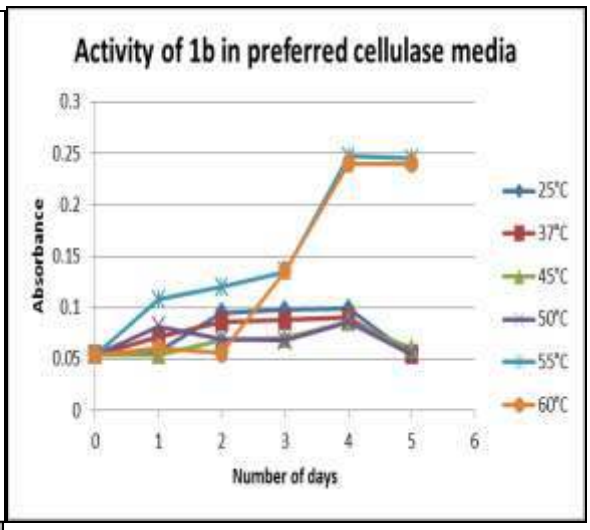


Fig. 2: Choice of best organism





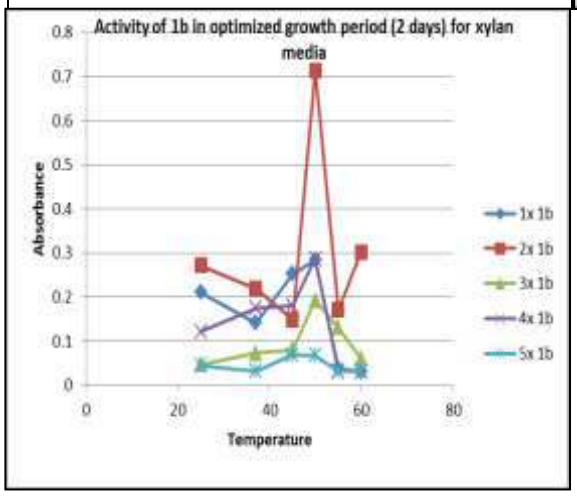


Fig. 3: Optimization of pH, incubation time, temperature and media for saccharification

Fig. 4: Fermentation test using Durham's tube

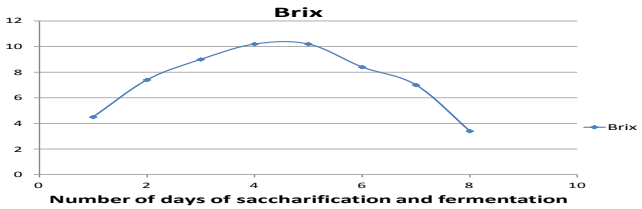


Fig. 5: Brix measurement during saccharification and fermentation