

Original Research Article

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Hydrolysis of Hemicellulosic Feed Stocks by *Enterobacter* Species in the Production of Bio-Ethanol

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ABSTRACT

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Biomass have gained much attention as a new sustainable energy source alternative to petroleum-based fuels. The second-generation bioethanol from lignocellulosic materials became a promising approach since the lingo-celluloses are not directly in competition with food sources. The present study focused on screening the *Enterobacter* species isolated from environment for hemicellulytic activity on beech wood, hard wood, bagasse and wheat straw. The optimization of pH, Temperature, Ratio of feedstock to water and feedstock to microbe concentration was analyzed. Optimum temperature, pH and substrate concentration for hydrolysis for feed stocks was found to be 32°C, 7.4 pH, 8.0 g and 250 ml feedstock to water. An ethanol yield of 2.3% v/v for beech wood, 2.1% v/v for hard wood, 1.92 % v/v for bagasse & 1.7% v/v for wheat straw was obtained for 8.0% of the feedstock.

Introduction

In recent years, environmental concerns related to increased average temperatures and rising atmospheric carbon dioxide levels have prompted much debate on the use of fossil fuels. About 25 % of global carbon dioxide emissions come from the transportation sector, with three quarters of that being attributed to road transport (IEA, 2009) (IEA, 2009). Additionally, there is a striking difference in the use of vehicles around the world.

The expected increase in global population to an estimated 8.3 billion by 2030 (Arundel, 2009), means energy needs for transportation could double by 2050 (IEA, 2009). The need for a more sustainable and renewable fuel is thus evident and imperative.

There is a variety of potential biofuels that could be produced and used to replace petroleum based fuels, including bioethanol,

biogas, biodiesel and bio-butanol. Each fuel clearly has its own virtues and disadvantages and a combination of them will likely be needed in order to efficiently replace oil.

Almost all fuel ethanol today is produced from sugar (sugar cane) or starch (mainly corn), through '1st generation or conventional' bioethanol processes. The production of conventional bioethanol has been increasingly questioned for competing for agricultural land with food and feed production. Therefore, the issue is no longer only how to replace fossil fuels, but how to do this in a sustainable manner (Goldemberg, 2006). This has shifted focus away from using conventional agricultural raw materials to using lignocellulosic biomass, also known as '2nd generation' or advanced biofuels.

There are two processes options envisaged to release fermentable sugars from lignocellulosic feedstocks of biomass. One approach is acid or alkali hydrolysis to break down the complex structure long chain monosaccharide's or polysaccharides into simple sugars. The second or an alternative method is enzymatic hydrolysis with pretreated process to reduce the size of the feedstock which is accessible for hydrolysis, after employing the pretreated lignocellulosic feedstock biomass to fermentable sugars, finally the microbial fermentation to produce bioethanol and carbon dioxide (Borjesson, 2009). The bottleneck for the use of lignocellulosic biomass as raw material for the production of biofuels is the lack of technology for the efficient conversion of biomass. The limiting factor is the low cost processing technologies for the conversion of this feedstock to biofuel does not exist as on date. Of all the steps described earlier, it is the biomass hydrolysis step which requires special attention. Any process which has an

efficient and low cost hydrolysis step will emerge as the best process for the conversion of biomass to biofuel (Borjesson, 2009). Significant research is going on for finding the efficient and low cost hydrolysis route for the lignocellulosic feedstock conversion. This research work is an attempt on similar lines to find an innovative method of hydrolysis of hemicellulose biomass using bacteria obtained from the environment.

Materials and Methods

In the present study, four different lignocellulosic biomaterials from which the hemicellulose can be extracted have been considered and utilized. They are industrial hardwood effluent, wheat straw, sugarcane bagasse and beech wood as Xylan. The hemicellulose was obtained by pretreatment and by alkali hydrolysis.

Isolation of bacteria

Soil samples were collected from various places of Karnataka during the monsoon season. The samples were collected in sterile polyethylene bags and bottles, which were preserved in the refrigerator for further investigation. Standard microbiological methods were followed for the purpose of isolation (Brown, 1985). One ml of the desired dilution was transferred aseptically into MacConkey's agar, blood agar and Nutrient agar for the isolation of bacteria. Plates were incubated for 24- 48 hrs for bacterial fungal growth. The isolates thus obtained were characterized.

Hemicellulolytic (Xylanolytic) enzyme assay

Relatively little attention has been given to qualitative assays for Xylan utilization and only a few assay procedures have been

described. Actively growing culture was used to inoculate each assay medium and incubated for 24-72hrs for optimization study (Jørgensen *et al.*, 2003).

Dye staining of Xylan agar

The Xylanolysis basal medium was prepared by incorporating with 8% w/v hemicellulose obtained from the feedstocks and 1.6% w/v of agar and autoclaved. The sterile media were aseptically transferred to the sterile petri dishes and inoculated with the test organism individually. The plates were then incubated at room temperature for 48 h for bacteria. The media after the growth was flooded with iodine stain (0.25% w/v aqueous I₂ and KI) for 5 min, the stain was removed and the agar surface washed with distilled water. Xylan degradation around the colonies appeared as yellow-opaque area against a blue/reddish purple color for un-degraded (Biely, 1985; Cai *et al.*, 1995).

Experimentation

The behaviour of variables like temperature, pH and substrate on hydrolysis was investigated through systematic experimentation.

Optimum variable conditions for hydrolysis

The effect of temperature on hydrolysis of hemicellulose was determined by incubating the reaction mixture at different temperature ranging from 27 to 40°C. The behaviour of pH was determined by adjusting the pH of the reaction mixture from 1 to 10. The substrate concentration on hydrolysis was determined by preparing substrate suspensions 1 to 10% as suggested by some of the investigators (Sluiter 2008, Templeton, 2008, Surender 2010). Determination of total sugars, pentose

sugars and enzyme activities was analysed by liquid chromatography mass spectrometry (HYM BROTHER ANALYTICAL SOLUTIONS P. LTD) methods. All the required standard chemicals and sugar standards are procured from Himedia.

Statistics

All the tests were laid in completely randomized design and each treatment was tested for five times. ANOVA analyses were carried out with Assisat 7.5 beta.

Results and Discussion

Estimation of Enzymes

The total enzyme activities of Enterobacter species for beech wood, hard wood, bagasse and wheat straw is depicted in Table 1 and Fig. 1. Hemi-cellulase is an inducible enzyme and its production increases with increase in bacterial biomass over the incubation period and as the sugars in the substrate diminished, the production of the enzyme decreases. Enterobacter species showed highest enzyme activity for beech wood (94.7IU/ml) followed by hardwood (92.4 IU/ml), bagasse (90.43 IU/ml) and wheat straw (89.32 IU/ml). The highest concentration of enzymes produced in 48 hours of incubation and further starts decreasing.

Substrate/ feedstock concentration

The different concentration of feedstocks was inoculated with the predetermined fixed volume of the bacteria and the mean sugar concentration was recorded. The effect is shown in Table 2 and Fig.2.

It can be observed that for 2% of beech wood, 49.28mg/ml mean sugar

concentration were measured. Similarly for hard wood 40.46mg/ml mean sugar concentration was measured and for 37.30 46mg/ml for bagasse and 27.72 46mg/ml for wheat straw.

For the feedstock of concentration 6%, Highest mean sugar concentration of 738.83 mg/ml for beech wood, 698.05 mg/ml for hardwood, 564.27 mg/ml for bagasse and 428.73 mg/ml for wheat straw.

For the feedstock of concentration 8%, Highest mean sugar concentration of 1296.37 mg/ml for beech wood, 1139.37 mg/ml for hardwood, 1070.70 mg/ml for bagasse and 948.03 mg/ml for wheat straw.

At the feedstock concentration of 10% the mean sugar concentration started decreasing; highest mean sugar concentration of 520.70 mg/ml for beech wood, 448.05 mg/ml for hardwood, 367.04 mg/ml for bagasse and 312.75 mg/ml for wheat straw.

The optimum concentration of substrate that could be easily hydrolyzed by *Enterobacter* species is 8%, beyond that bacteria was unable to efficiently degrade the hemicellulose of the selected feedstock. The effect of hydrolysis by *Enterobacterspp* on substrate concentration sugar concentration for beech wood, hard wood effluent, Bagasse and wheat straw are presented table 2 and fig. 2

Temperature

The effect of temperature on hydrolysis of hemicellulose by *Enterobacter* species is shown in the Figure 3 and Table 3.

At 20°C mean sugar concentration obtained was 32.5µg/ml for beech wood, 29.7µg/ml for hardwood, 22.4µg/ml for bagasse and 19.0µg/ml for wheat straw.

At 30°C mean sugar concentration obtained was 735.5µg/ml for beech wood, 700.4µg/ml for hardwood, 563.0µg/ml for bagasse and 522.4µg/ml for wheat straw.

At 32°C mean sugar concentrations obtained was 1298.4µg/ml for beech wood, 1130.1µg/ml for hardwood, 1082.0µg/ml for bagasse and 950.4µg/ml for wheat straw.

SAAt 34°C mean sugar concentration obtained was 769.8µg/ml for beech wood, 692.2µg/ml for hardwood, 597.1µg/ml for bagasse and 439.8µg/ml for wheat straw.

At 38°C mean sugar concentrations was very low, indicating the temperature is not optimum for the growth of the bacteria and the hydrolysis of the hemicellulose. The mean sugar level estimated at 38°C was 102.2µg/ml for beech wood, 86.4µg/ml for hardwood, 55.0µg/ml for bagasse and 32.0µg/ml for wheat straw.

Hence the optimum temperature for *Enterobacter* species for the hydrolysis of hemicellulose from different feedstock is 32°C.

Effect of pH

Effect of pH on bacteria for hydrolysis of hemicellulose from different feedstocks is shown in the figure 4 and tables 4. The sugars were not detected in the pH of 1-4 and 11-14. The bacteria could not be cultured at the pH below 4 and above 11. The hydrolysis of hemicellulose increased from pH 5 and reached highest at the pH 7.4 and gradually decreased at the pH 7.4 till 10.

At pH 7.0, at temperature 32°C mean sugar concentration obtained was 847.4 µg/ml for beech wood, 598.4 µg/ml for hardwood, 455.9µg/ml for bagasse and 309.5µg/ml for wheat straw. At pH7.4, mean sugar

concentration obtained was 1773.7 µg/ml for beech wood, 1355.0µg/ml for hardwood, 1009.7µg/ml for bagasse and 928.3µg/ml for wheat straw.

At pH8, mean sugar concentration obtained was 948.3µg/ml for beech wood, 876.3 µg/ml for hardwood, 648.3µg/ml for bagasse and 608.7µg/ml for wheat straw.

Table.1 Enzyme activity (µmole/ml) of *Enterobacter* spp for different feed stocks

Time in Hours	Beech wood	Hard wood	Bagasse	Wheat Straw
	Enzyme activity µmole/ml or IU			
4	0.89	0.71	0.64	0.56
8	2.7	1.95	1.56	1.32
12	9.4	7.63	6.37	6.03
16	20.0	20	16.83	15.22
20	30.39	29.92	24.75	22.84
24	39.08	37.24	35.59	35.06
28	50.89	43.85	40.61	42.78
32	65.32	58.56	53.4	52.83
36	78.05	76.32	72.93	71.29
40	85.44	82.53	81.62	80.03
44	90.31	89.036	85.71	83.52
48	94.702	92.365	90.43	89.32
49	90.09	87.701	83.79	80.9
50	85.21	82.025	77.26	70.98

Table.2 Effect of Hydrolysis by *Enterobacter* spp on substrate concentration

Substrate (g)	Beech wood	Hard wood	Bagasse	Wheat Straw
	Pentose sugar concentration in µg/mL			
1	30.270	26.824	21.279	14.209
2	49.283	40.468	37.306	27.728
3	190.732	170.000	120.052	48.000
4	258.045	298.895	192.780	130.753
5	369.357	339.679	295.031	268.521
6	738.835	698.053	564.274	428.732
7	1008.037	926.284	802.224	782.036
8	1296.378	1139.374	1070.709	948.032
9	1107.905	915.276	883.378	775.235
10	520.703	448.053	367.048	312.753
11	173.335	126.582	98.428	90.028

Table.3 Effect of Temperature on *Enterobacter* spp for hydrolysis of feedstocks

Temperature °C	Beech wood	Hard wood	Bagasse	Wheat Straw
	Pentose sugar concentration in µg/mL			
20	32.5	29.7	22.4	19.0
22	57.9	41.6	37.3	28.2
24	98.1	74.9	52.8	40.0
26	197.8	170.0	120.7	97.1
28	286.4	209.1	197.6	143.8
29	529.0	411.7	337.4	295.1
30	735.5	700.4	563.0	522.4
31	1007.8	828.3	801.5	783.2
32	1298.4	1130.1	1082.0	950.4
33	1110.8	916.2	883.1	773.1
34	769.8	692.2	597.1	439.8
35	521.4	448.5	368.3	298.4
36	306.9	229.0	199.0	145.3
37	173.2	125.5	103.3	90.1
38	102.2	86.4	55.0	32.0

Table.4 Effect of pH on *Enterobacter* spp for hydrolysis of feedstocks

pH	Beech wood	Hard wood	Bagasse	Wheat Straw
	Pentose sugar concentration in µg/mL			
2	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	0.0
4	0.0	0.0	0	0.0
5	1.4	1.0	0.7	0.6
6	7.8	6.9	3.5	1.2
6.5	298.8	168.3	142.8	137.0
7	847.4	598.4	455.9	309.5
7.2	1438.5	990.7	805.1	710.8
7.4	1773.7	1355.0	1009.7	928.3
7.6	1604.8	1477.6	1280.3	1106.0
7.8	1324.3	1186.0	926.7	896.3
8	948.3	876.3	648.3	608.7
8.5	506.7	399.1	305.0	300.0
9	265.0	205.2	190.7	165.0
10	156.9	122.4	107.2	98.7

Fig.1 Enzyme activity ($\mu\text{mol}/\text{ml}$) of *Enterobacter* spp for different feed stocks

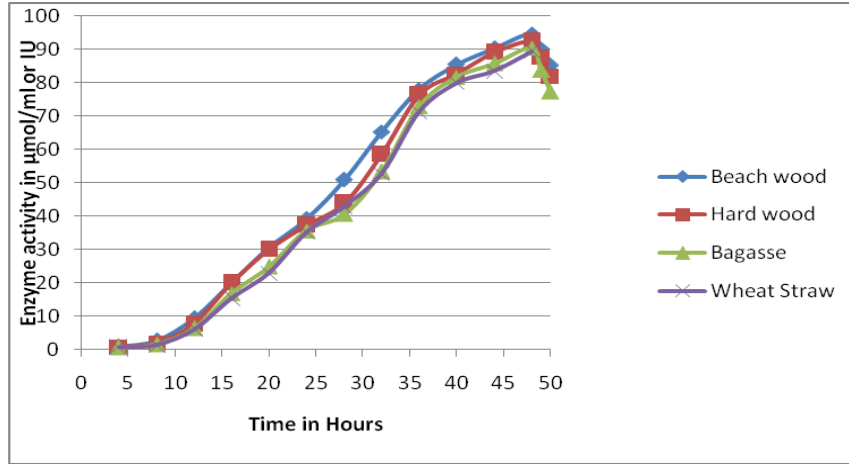


Fig.2 Effect of Hydrolysis by *Enterobacter* spp on substrate concentration

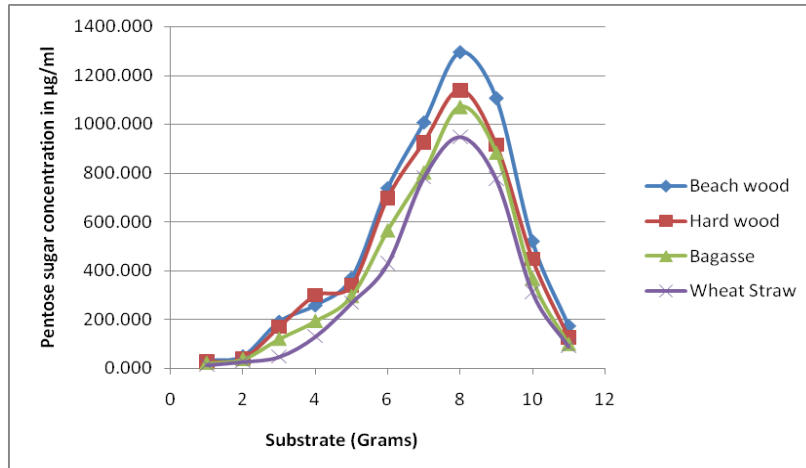


Fig.3 Effect of Temperature on *Enterobacter* spp for hydrolysis of feedstocks

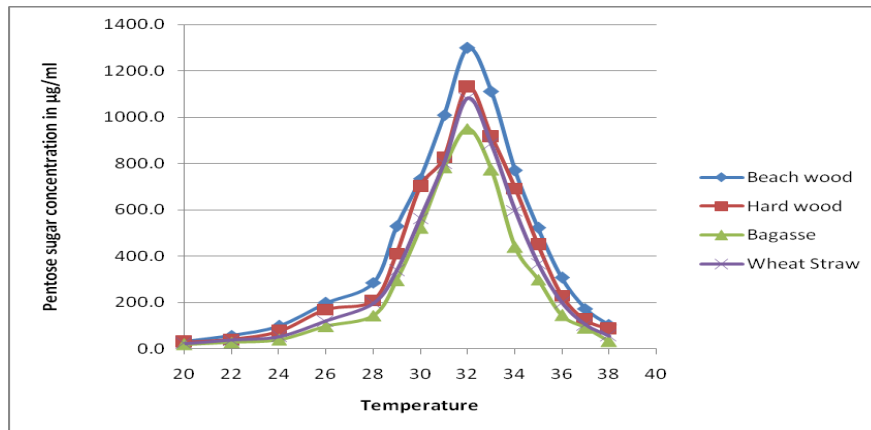
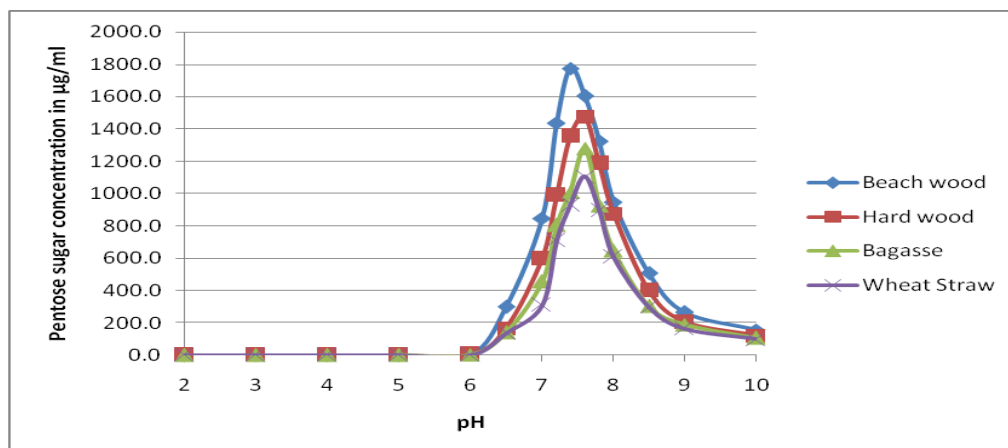


Fig.4 Effect of pH on *Enterobacter* spp for hydrolysis of feedstocks



The optimum pH for *Enterobacter* species for hydrolysis of hemicellulose feedstock is 7.4. At this pH highest amount of sugar was released by the enzymatic activity.

Ethanol production

Fermentation

The hydrolyzed products are subjected to fermentation for the production of ethanol. *E. coli* strain was inoculated into the culture filtrate and allowed for fermentation for 36h (Templeton, 2008).

After fermentation, it was filtered and subjected to distillation for ethanol at 80°C (Ghose, 1987, Milne 1992).

Ethanol estimation by high pressure liquid chromatography

The injected volume was 1 µl and the retention time was 25 min. Identification and quantification were based on direct comparison of the high performance liquid chromatogram (Waters Columns SH 1821 & SH 1811) response to ethanol standards.

Ethanol yield was 2.3% v/v for beech wood, 2.1% v/v for hard wood, 1.92 % v/v for bagasse & 1.7% v/v for wheat straw was

obtained.

In conclusion, the aim of this study was to gain a deeper understanding of how to process pre-treated hemicelluloses to get commercial end products. The results of the work have contributed to expanding the body of knowledge within this increasingly important field. The optimization experiments have shown that the organism isolated is an efficient hemicellulolytic hydrolyser. The organism effectively hydrolyzed hemicellulose to pentose sugars and this sugar was converted to bio-ethanol

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