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Bioethanol Production from Paper using marine Isolate *Bacillus pumilus*

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ABSTRACT

Bioethanol may serve both as an additive or complete replacement for petroleum-derived transportation fuels, particularly gasoline in spark ignition (SI) engines. Many useful products like biofuels, chemicals, improved animal feeds and human nutrients can be produced making use of these residues of plant biomass which has actually treated as waste. Lignin, cellulose and hemicellulose are the major constituents of lignocellulose which in turn contributes to the major component in the structure of woody and non woody plants and also a source of organic matter which can be regenerated and used for deriving the required substances. Marine microbes have a very significant role to play in our lives because of their abundancy and activity. They are remarkable because they have the ability to produce many commercially important bioactive compounds and have the ability to bring about remediation by enzymes produced by the microbes in the ocean. In the present study, bacteria were isolated from marine sources and screened for the ability to produce lignocellulases. One of the bacterial isolates which exhibited an extensive ability to breakdown lignocelluloses was identified as *Bacillus pumilus* and chosen for further study. Domestic, industrial and agro waste rich in lignocellulosic content were subjected to enzymatic hydrolysis by the isolate. Paper was found to be one of the substrates releasing sugars in abundant amounts. Physical and chemical parameters were optimised with paper to further increase the yield of sugars. The optimised conditions were used for enzymatic hydrolysis of the substrate to give reducing sugars in much higher quantity. These sugars were fermented to alcohol by *S.cerevisiae* forming 12.42% of alcohol which is close to that of the yields utilised at commercial levels.

Key words: Paper, *Bacillus pumilus*, Bioethanol, Optimisation.

INTRODUCTION

The increasing dependency on oil imports and the growing emissions of greenhouse gases are the two main concerns which justify the introduction of public policy incentives in Europe for developing lignocellulosic ethanol. According to International Energy Agency (2008) the total world demand for oil is projected to rise by 1% every year mostly due to increasing demand in emerging markets, especially India (3.9%/year) and China (3.5%/year). In view of continuously rising petroleum costs and dependence upon fossil fuel resources, considerable attention has been focused on alternative energy resources. Production of ethanol or ethyl alcohol (CH_3CH_2OH) from biomass is one way to reduce both the consumption of crude oil and environmental pollution (Lang *et al.,* 2001). Bioethanol represents one of the most prominent technical options due to the possibility of blending it with fossil fuels and using in the existing automobiles without significant adaptations. Unlike fossil fuels, ethanol is a renewable source produced through fermentation of sugars. Ethanol is widely used as a partial gasoline replacement in the USA. Fuel ethanol that is produced from corn has been used in gasohol or oxygenated fuels since the 1980s. These gasoline fuels contain up to 10% ethanol by volume.

Smaller scale production of bioethanol started more recently from lignocellulosic feedstock derived from agricultural residue. However, lignocellulosic biomass requires a more complicated hydrolysis stage. The

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reason for this is that cellulose in the wood contains carbohydrate polymers called cellulose. Cellulose is made up of long chains of glucose and a more complex set of enzymes are required to break the long chains. Therefore, lignocellulosic bioethanol is technically more demanding and thus more expensive. Thus, this study was carried out on production of bioethanol from paper at a less expensive cost.

The conversion of paper, a lignocellulosic biomass to bioethanol includes two processes: enzymatic hydrolysis of cellulose in the lignocellulosic materials to fermentable reducing sugars, and fermentation of the sugars to ethanol. The hydrolysis is usually catalyzed by cellulase enzymes, and the fermentation is carried out by yeasts or bacteria.

Marine microbes live in a biologically competitive environment with unique conditions of pH, temperature, pressure, oxygen, light, nutrients and salinity, which is especially rich in chlorine and bromine elements. There is no wonder that marine microbial metabolites exhibit special biological activities compared with 'terrestrial' bacteria. Thus, the conversion of lignocellulosic biomass to bioethanol was checked using marine microorganism as they exhibit a varied kind of secondary metabolites and enzymes.

MATERIALS AND METHODS

Isolation and Identification of Microorganism:

The microorganisms were isolated fromCuddalore marine bodies as the present study deals with the identification of the capacity of the marine microbes to produce ethanol from lignocellulosic biomass. The microbes isolated were identified using basic microbiological techniques (Brown *et al*., 1985) and molecular analysis. The microorganisms were checked for its cellulose (Cai, Buswell and Chang, 1994; Pointing *et al*., 1999a), lignin (Buswell*et al*, 1996.) and xylan(Biely, 2003. Cai*et al*, 1994) degrading capacity.*Bacillus pumilus* isolated from the sample showed maximal degradation. Lignocellulosic waste from agriculture, domestic and industry were checked for biodegradation by *Bacillus pumilus*, paper was found to be one of the substrates degraded to reducing sugars abundantly, it was selected for further optimisation and ethanol production.

Optimization of Physical parameters

pH:

Lignocellulosic biomass was ground into cut into small pieces and transferred to flasks containing water. Different pH 4.0-9.0 were set using 1N NaOH and 1N HCl(Pezsa and Ailer, 2011). The flasks were sterilised and inoculated with isolated culture of *Bacillus pumilus*and incubated at room temperature. The amount of reducing sugar was estimated using DNS (Miller, 1959) method and lignin degradation was carried out using veratryl alcohol assay. The DNS and veratryl alcohol assay readings were taken at an interval of 7 days for 8 consecutive weeks.

High temperatures:

Paper was cut into small pieces and was added in flasks containing water and kept at different temperatures i.e., 100^0C, 150^0C, 200^0C and 250^0C for 1hr (Brownell and Saddler, 1986). Once the pretreatment was carried out the flasks were inoculated with the strain of *Bacillus pumilus*. The reducing sugar was estimated using DNS (Miller, 1959) and lignin degradation assay using veratryl alcohol (Frederick, 1992).

Incubation Temperature:

Paper pieces added into flasks each containing 100ml of water, sterilised at 121^0C for 15 minutes at 15 psi and then inoculated with *Bacillus pumilus*. After inoculation, the flasks were incubated at 4 different temperatures to study the impact of the incubation temperature on degradation of lignocellulosic biomass. The temperatures at which the incubation was done were 25, 30, 37 and 40°C (Charitha Devi *et al.,* 2012). Reducing sugar was estimated using DNS (Miller 1959) and lignin degradation assay using veratryl alcohol (Frederick, 1992) to check the degradation of lignocellulosic biomass. The readings were taken at regular interval of 7 days for 8 consecutive weeks to check the impact of the incubation temperature.

Optimization of Chemical Parameters

Carbon source:

The substrate was cut into small pieces and was added into flasks containing water with different concentrations of maltose sources - 0.5%, 1.0%, 1.5% and 2.0%. The flasks was autoclaved at 121^0C for 15 minutes and then inoculated with *Bacillus pumilus*. Reading was taken for reducing sugar using DNS (Miller, 1959) and lignin degradation assay using veratryl alcohol. The readings were further taken weekly for 8 consecutive weeks.

Nitrogen source:

The lignocellulosic biomasssubstrate was cut into small pieces and was added into flasks containing water with different concentrations of ammonium nitrate sources - 0.5%, 1.0%, 1.5% and 2.0%. The flasks were inoculated with *Bacillus pumilus*. Reading was taken for reducing sugar using DNS and lignin degradation assay using veratryl alcohol (Frederick, 1992). The readings were taken at a regular of 7 days for 8 consecutive weeks.

Acid Treatment:

Paper was cut into small pieces and was added into flasks containing different concentrations of acid 0.1%, 0.3%, 0.5%, 0.7%, 0.9% and 1% (Leenakul and Tippayawong, 2010, Nutawan*et al*., 2010). The flasks were incubated at room temperature for 24 hours. The

substrates were then neutralized (Umbrin*et al*, 2011) and sterilised at 121^{0}C for 15 minutes at 15 psi. Once the flasks had cooled, they were inoculated with *Bacillus pumilus*. Reading was taken for reducing sugar using DNS (Miller, 1959) and lignin degradation assay using veratryl alcohol. The readings were taken weekly for 8 consecutive weeks.

Alkali Treatment:

The lignocellulosic biomass paper was cut into small pieces and was added into flasks containing different concentrations of alkali - 0.1%, 0.3%, 0.5%, 0.7%, 0.9% and 1% (Ashishvyas*et al,.* 2005). The flasks were incubated at room temperature for 24 h. The substrates were then neutralized and autoclaved and inoculated with *Bacillus pumilus*. Reading was taken for reducing sugar using DNS and lignin degradation assay using veratryl alcohol assay.

Solid State Fermentation:

Paper was cut into small pieces and suspended in water. Pretreatment was carried out with Acid overnight with optimal of 0.3% acid, high temperature of 200^{0}C. The substrate was suspended in 1000ml of distilled water and the flasks were autoclaved. The flasks were allowed to cool to room temperature. Once flasks were cooled, maltose- 2% and ammonium nitrate- 0.5% sources were added.

The pretreated flasks were inoculated with *Bacilluspumilus,* the pH was set at 4and incubated at30^{0}C for 30 days. The incubated substrates were taken and filtered with 0.45 micron Whatmann filter paper by using filtration unit (Fatma, 2010). The filtrate was collected separately for each organism in a sterile Erlenmeyer flask and inoculated with 3% *Saccharomyces cerevisiae*. The inoculated flasks were incubated at 30°C for 40 days.

About 200ml of each sample were drawn on 10th, 20th, 30th and 40th day and distillation was carried out. The alcohol estimation was carried out by potassium dichromate method (William, 1950) and the amount of alcohol was calculated.

RESULTS AND DISCUSSION

*Bacillus pumilus*exhibited maximal degradation of cellulose Paper at pH 4 in the 5th week (Fig. 1), incubation temperature at 30°C in the 5th week(Fig. 2), acid hydrolysis 0.3% in the 3rd week (Fig. 4), alkali hydrolysis 0.1%in the 3rd week (Fig. 5), high temperature treatment at 200°C in the 2nd week(Fig. 3), ammonium nitrate being the best nitrogen source at 1.5% in the 2nd week (Fig. 6) and the variation of maltose source at 2% concentration in the 4th week (Fig. 7) having no increased impact in degradation.

Bacillus pumilus exhibited maximal degradation of lignin Paper at pH 7 in the 1st week (Fig. 1), incubation temperature at 37°C in the 7th week (Fig. 2), acid hydrolysis 0.9% in the 3rd week (Fig. 4), alkali hydrolysis 0.5%in the 8th week(Fig. 5),high temperature treatment at 100°C in the 4th week (Fig. 3), ammonium nitrate at 1.5% in the initial reading(Fig. 6) and the variation of maltose source at 1.0% concentration in the 8th week (Fig. 7) having no increased impact in degradation.

According to results, the substrate degradation was a direct function of enzyme activities. cellulose was degraded more than hemicellulose and it could again be correlated to higher cellulase activity than xylanase activity, with low ligninase activity. This finding was a variant from the observations of Madhu Choudhary*et al,.* (2009).

Figure-1. Optimization of pH indicates that maximum reducing sugar and lignin oxidation is at pH 4 in 5th week and pH 7 in the 1st week.

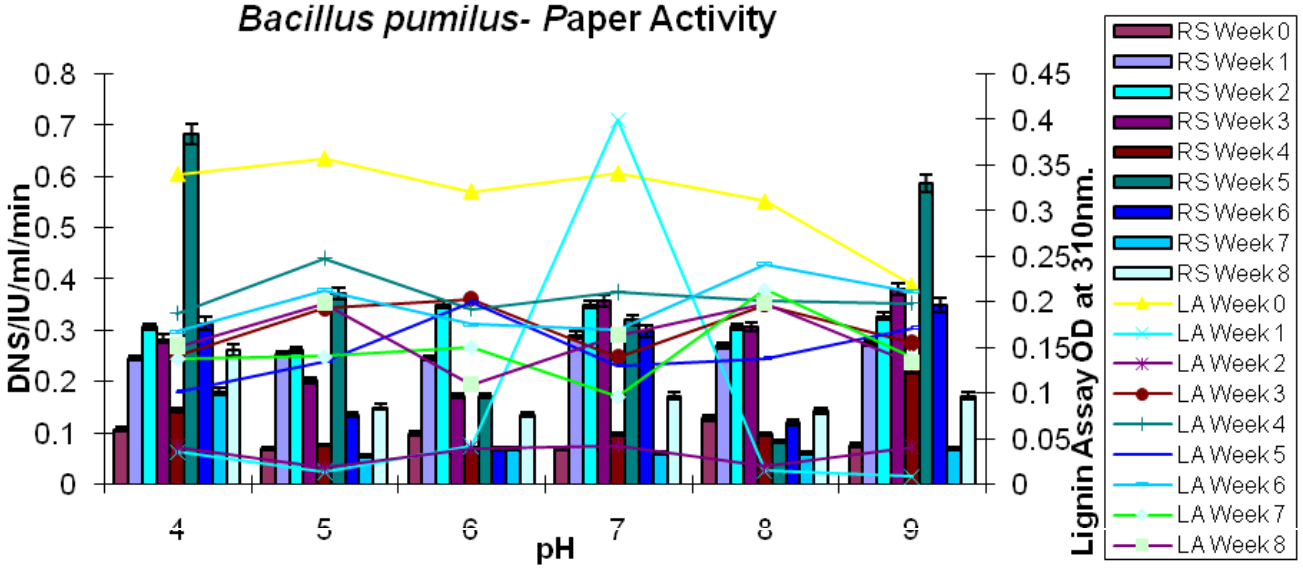


Figure-2. Optimization of Incubation Temperature indicates that maximum reducing sugar and lignin oxidation is at 30^{0}C in 5th week and 37^{0}C in the 7th week.

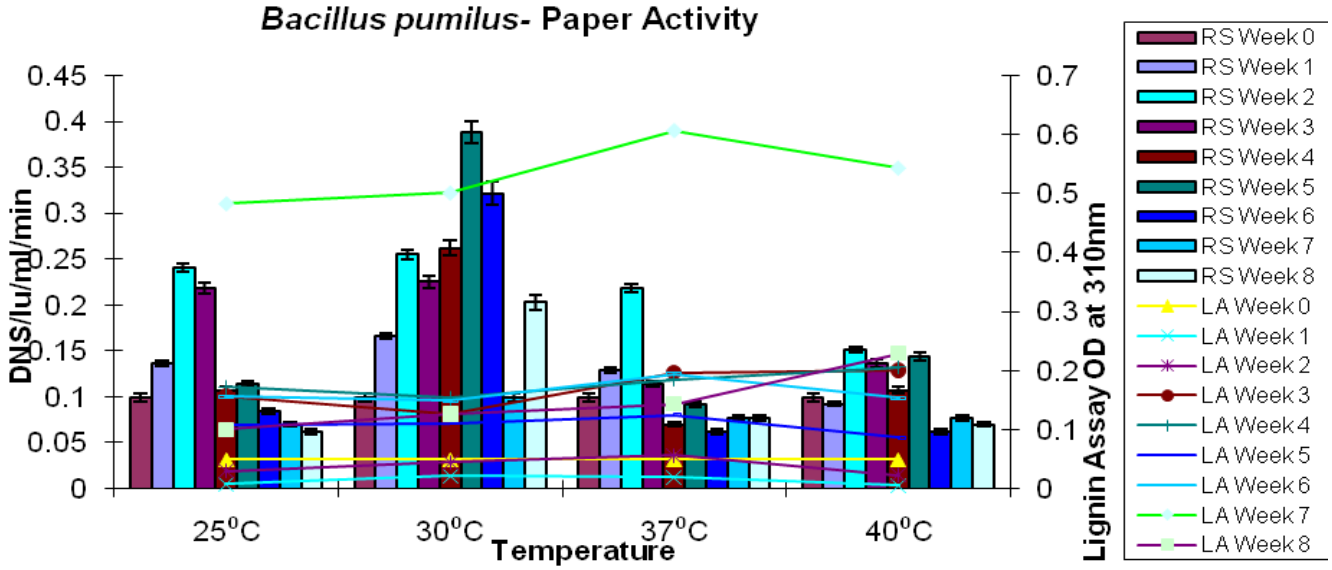
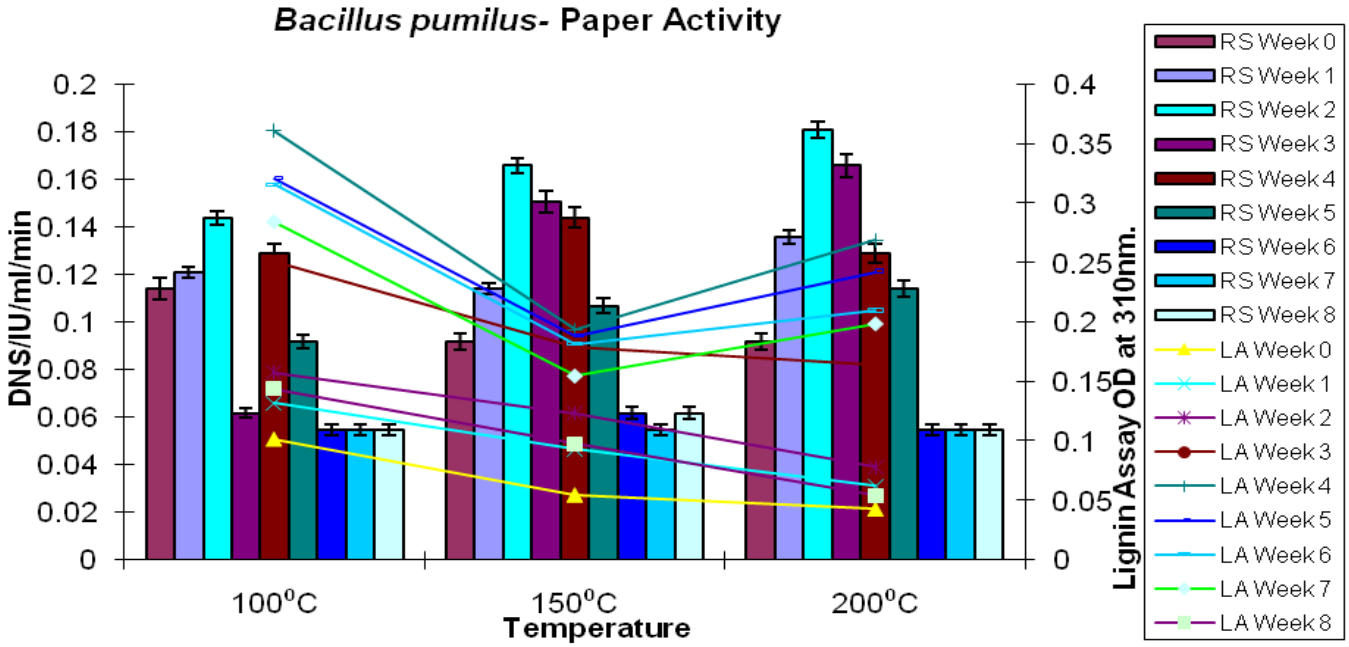


Figure-3. Pre Treatment with High Temperature indicated highest yield of reducing sugar at 200^{0}C in 2nd week and lignin oxidation at 100^{0}C in 4th week.



Pre-treatment of various substrate with 1% (1N NaOH) Alkali proved to be sufficient to obtain maximum delignification, enhancing lignocellulase activity which varies with the results obtained by Acharya *et al.* (2008) where they pretreated saw dust with 2N NaOH and obtained maximum cellulase activity at 9.6% concentration, but is on par with the results of Keshwani (2009) carried out on switch grass with the usage of 1% and 3% NaOH.

The addition of various carbon sources increased the sugar yield and enzymatic hydrolysis in our study which is opposite to the result obtained by Moussa *et al.* (2007) whose findings reported the repression of cellulases on addition of carbon source.

The present study has given low concentration of acid in some substrates yielding maximum sugars which is in contrast to the finding of Bowen *et al.* (2010) which states the use high concentration of acids for increased sugar yields.

Alcohol Percentage:

The alcohol fermentation was carried out using the optimised conditions and maximum alcohol percentage was obtained in Paper by *Bacillus pumilus*was12.42%on 20th day (Fig. 8) followed by 11.71% on 10th day. The least alcohol percentage was seen on 40th day with a percentage of 4.33%.

The maximum Bioethanol yield was found on the 20th day which varies with the findings of Naresh Sharma *et*

Figure-4. Pre Treatment with Acid indicates highest reducing sugar at 0.3 % and lignin oxidation at 0.9 % in the 3rd week.

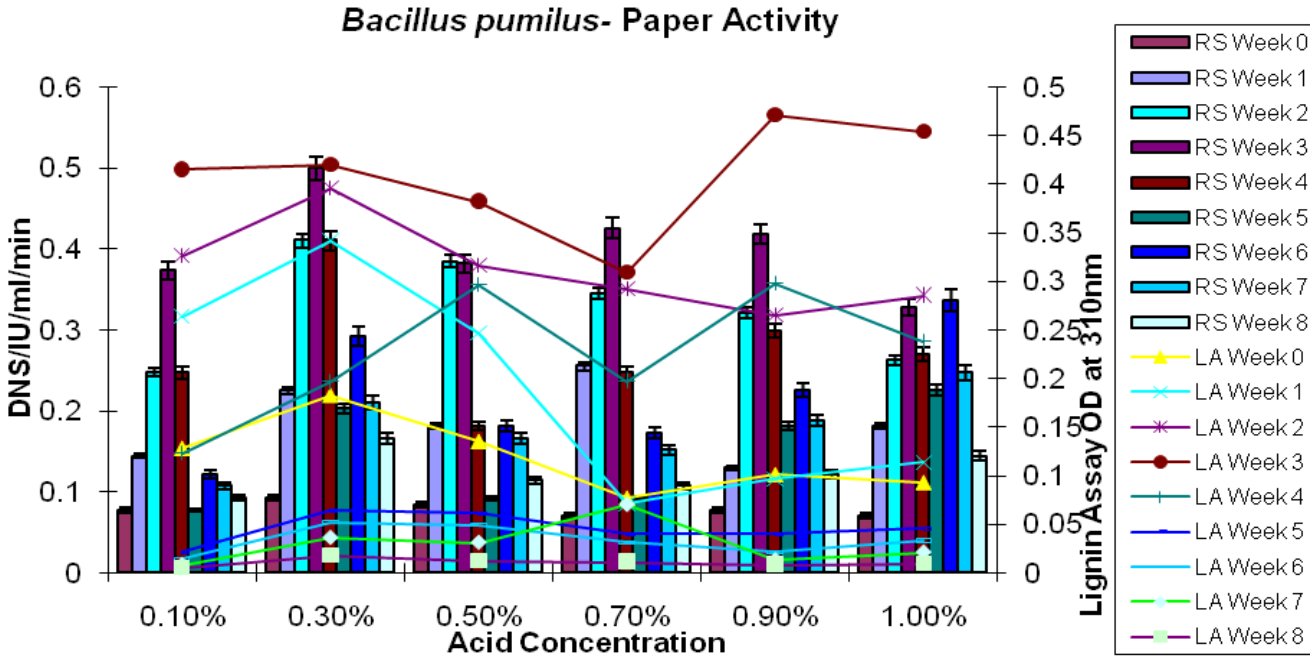
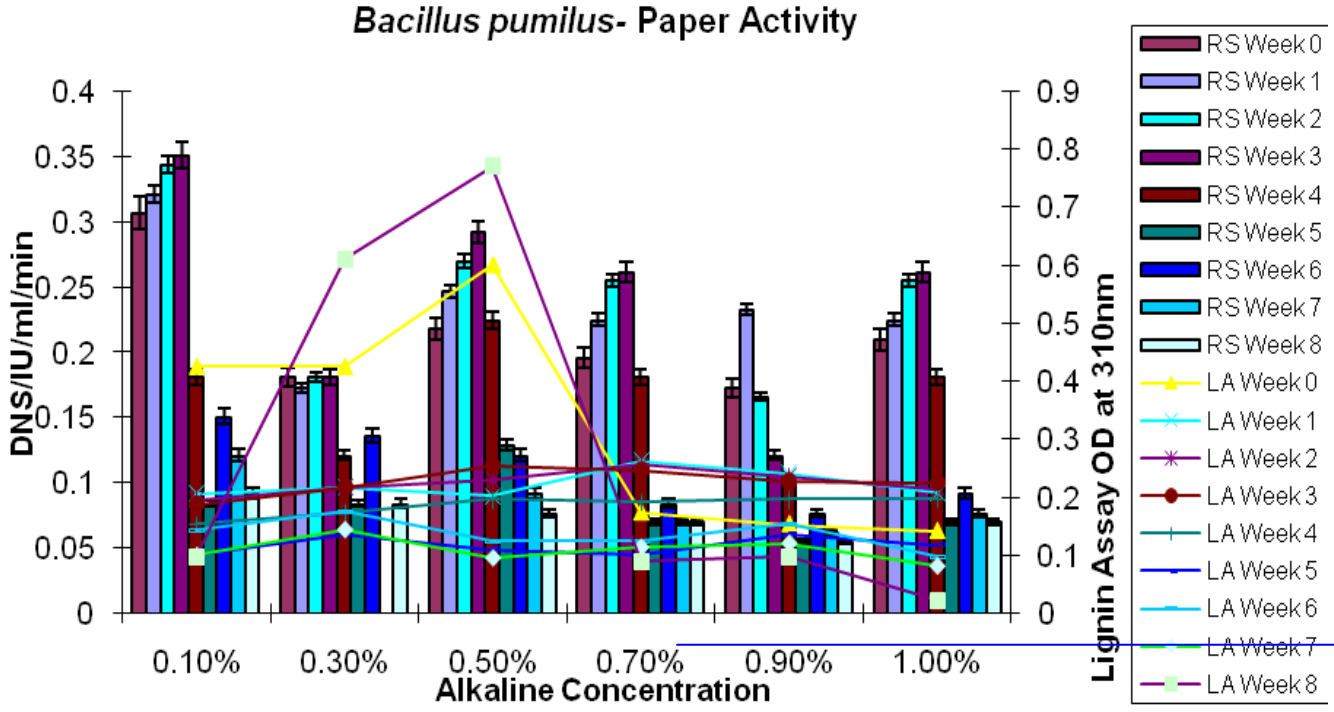


Figure-5. Treatment with Alkali indicates highest reducing sugar at 0.1 % in 3rd week and lignin oxidation at 0.5 % in the 8th week.



al. (2007) where the maximum yield was found on 2nd day with a combination of two waste.

Alcohol Concentration (g/l):

The amount of alcohol was maximally seen on the 20th day. The highest amount of alcohol was found to be 98.0g/l. The least amount was 34.23g/l and found be produced on 40th day of fermentation (Fig. 9).

CONCLUSION

Development and growing industrialisation have a great impact on growth of a country, but this has also lead to rapid decline of renewable sources of energy. In such an era the microorganism which has the ability to

Figure-6. Optimization with nitrogen source: Ammonium nitrate indicates highest yield of reducing sugar at 1.5 % in 2nd week and lignin oxidation at 1.5 % in the initial reading.

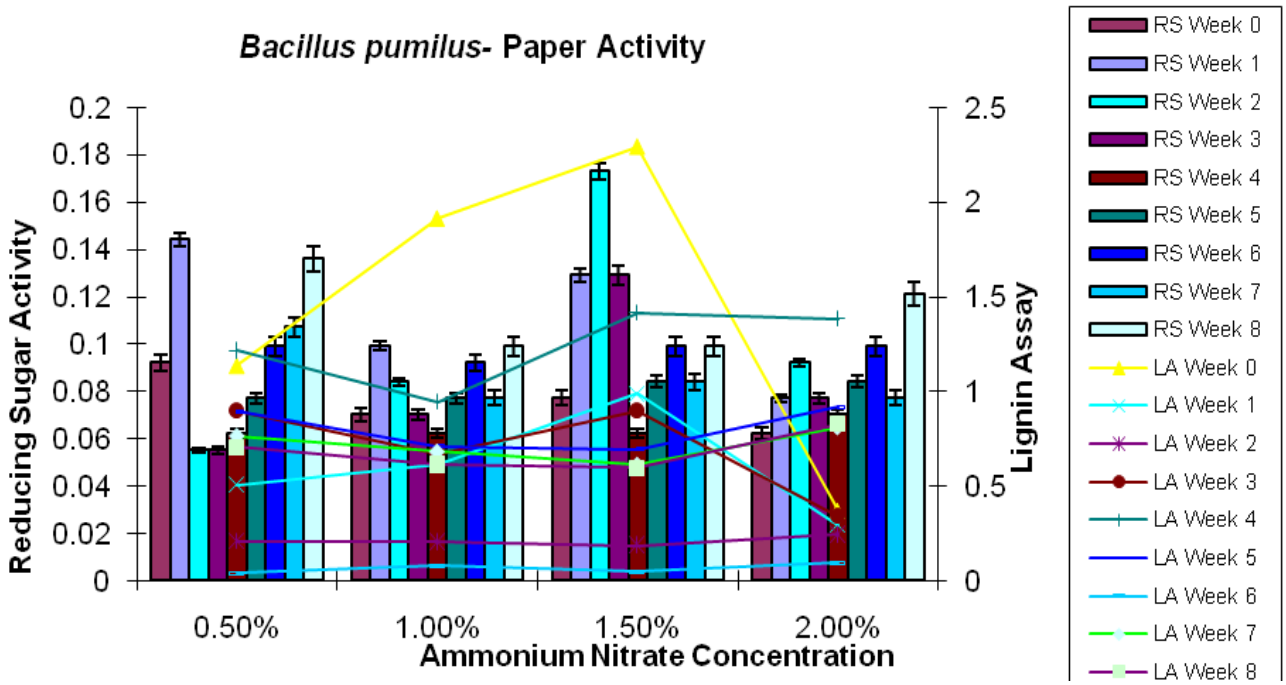


Figure-7. Optimization with Carbon source: Maltose indicates highest yield of reducing sugar at 2.0% in 4th week and lignin oxidation at 1.0% in the 8th week.

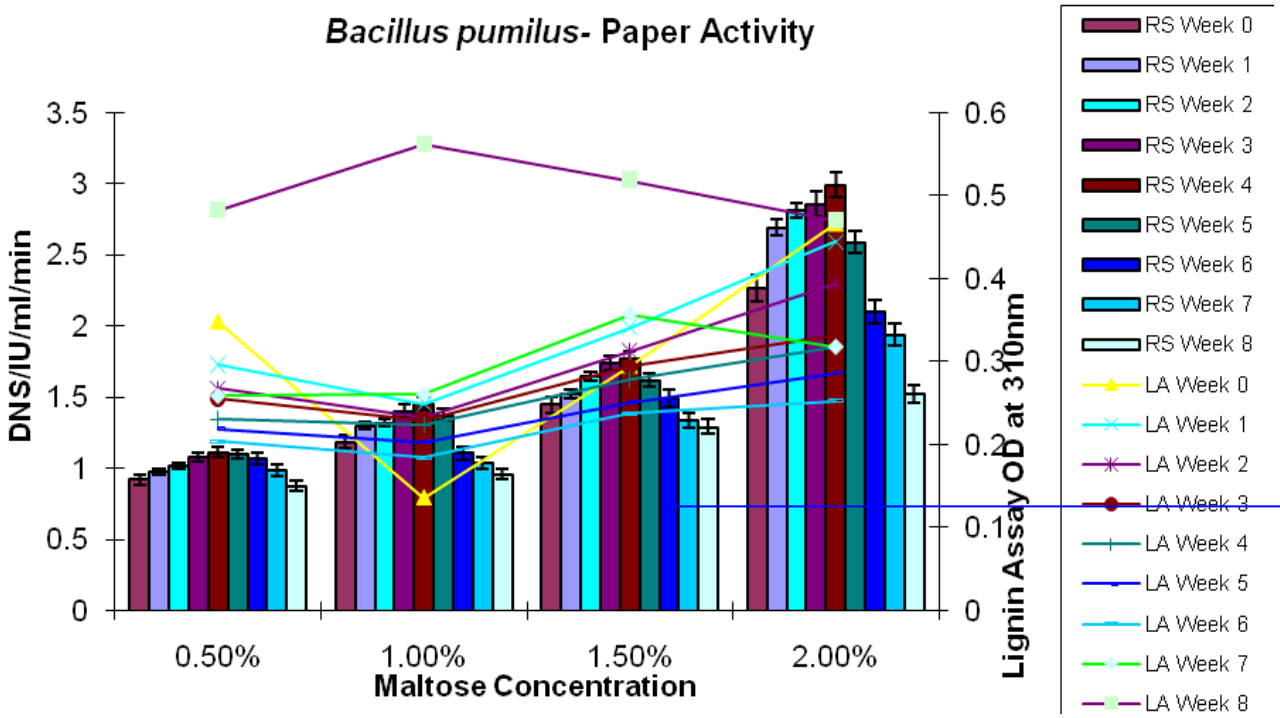


Figure-8. Alcohol Percentage for *Bacillus pumilus* against Paper.

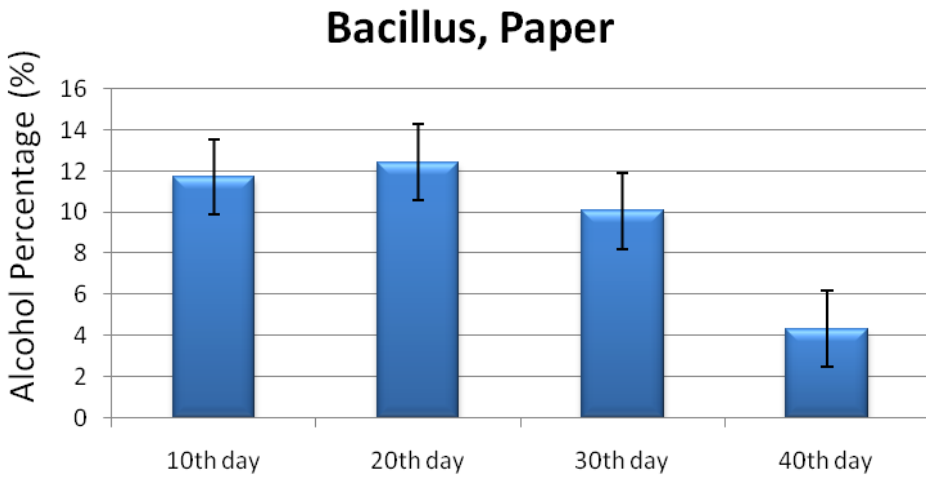
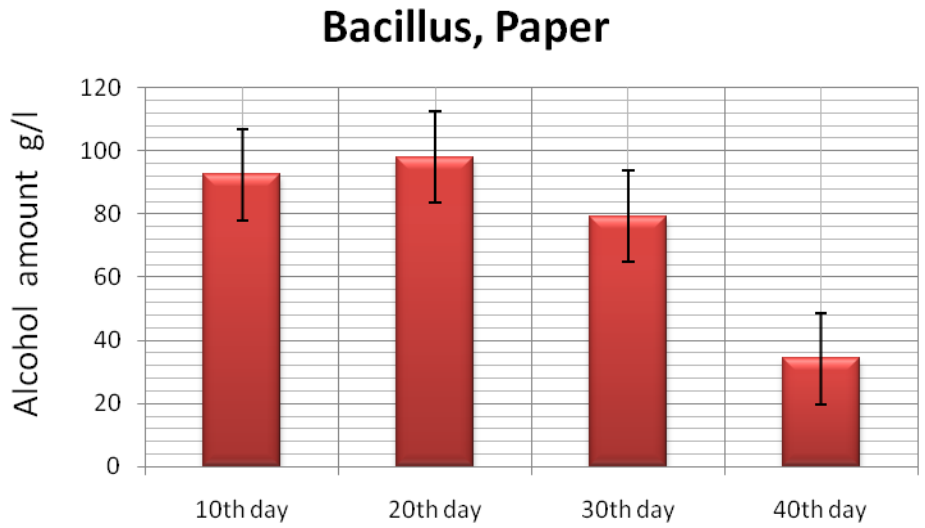


Figure-9. Alcohol amount for *Bacillus pumilus* against Paper.



break down the substrate which can generate huge amount of sugars to convert to alcohol by the standard known organism *S. cerevisae* can definitely contribute to the thought of producing alcohol at the commercial level. This can be used as an alternate source of fuel or biofuel for motor vehicles.

Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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