IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203

Human Journals
Research Article
September 2016 Vol.:7, Issue:2
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Production of Bioethanol by Bacterial Co-Culture from Wheat Straw through Simultaneous Saccharification and Co-Fermentation

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Submission: 25 August 2016
Accepted: 1 September 2016
Published: 25 September 2016

www.ijppr.humanjournals.com

Keywords: Bioethanol, Co-fermentation, Wheat straw, Simultaneous Saccharification, Cellulase

ABSTRACT

In this study, the production of bioethanol by co-culture of *Bacillus licheniformis and Sacchromyces cerevisiae* from wheat straw through simultaneous scarification and co-fermentation was investigated. Simultaneous sacchrification and co-fermentation allows wheat straw hydrolysis by cellulase enzyme, which is produced by *Bacillus licheniformis* and subsequent conversion of produced reducing sugar into ethanol by *Sacchromyces cerevisiae*. In the current work, the pre-treatment of wheat straw and optimization of parameters like, co-inoculation time, pH of the medium, substrate concentrations and nitrogen source concentration increases the yield of ethanol to 5.77 % (v/v). Scale up of optimized medium to fermentor level of 1.5 liter resulted in significant enhancement of bioethanol production up to 18.635% (v/v).

1. INTRODUCTION

In the present scenario, world's requirement for fuel is increasing at a high rate;in contrast, the fossil fuels are decreasing on much higher speed. When ethanol is used as fuel, it acts as an environment-friendly fuel as compared to fossil fuel, which causes huge greenhouse gas emission. It is now being used with a mixture of biodiesel to increase its energy yield in the automobile industry. Production of fuel using waste products can provide a possibility of cost effectiveness. Ethanol is now being used as a fuel in many countries [1]. Apart from this, ethanol is a major solvent used in pharmaceutical industry [2]. With the increase in demand for ethanol and the high cost of production as traditional substrates like molasses and malt are inadequate and costly there remains a need for cost effective and abundant source of ethanol [4]. A process is required which can easily convert substrate to ethanol with less complexity.

Production of ethanol from waste lignocellulosic agricultural products is possible [3]. As the lignocellulosic substrate is one of the most abundant renewables and low-cost substrate [4]. Wheat straw is excessively available for ethanol production and contains 35-45% cellulose. Pretreatment of the lignocellulosic substrate increases the available substrate for glucose production and hence leads to increased ethanol yield [5]. In past decades bioethanol is being produced by addition of cellulolytic enzymes produced in another system and adding them to degrade lignocelluloses and then further microbes are used to produce ethanol. Genetically engineered strains are also available which can both degrade and convert degraded glucose into ethanol [8]. Immobilization is another technique used for the production of ethanol where one strain is immobilized and lignocelluloses degraded substrate is added for ethanol yield. Co-culture is also an additional technique being used where the combination of two ethanol-producing strain [7]. But again the cost of using two different systems for separate enzyme production and the instability of genetically engineered strains is a major problem being faced by the industry.

In this study, theco-culture technique is used to produce ethanol in one system simultaneously to produce ethanol from wheat straw. All the conditions for ethanol production are optimized to get maximum yield of ethanol. *Bacillus licheniformis* is used as lignocelluloses degrading bacteria and *Saccharomyces cerevisiae* is used for ethanol production. So, simultaneous saccharification and co-fermentation take place in one complete mixture where bacteria and yeast grow together but yeast's growth totally is dependent on glucose produced by the other

microbe. Once a sustainable amount of glucose is available to yeast, it triggers the ethanol production.

2. MATERIAL AND METHODS

2.1 Materials

Wheat straw was heat dried and milled. Soluble starch, sodium thiosulphate, phenol, and sulphuric acid were purchased from LobaChemiePvt. Ltd. Potassium dichromate and peptone were purchased from Central Drug House, India. Yeast extract, agar, and ammonium nitrate were purchased from Titan Pvt. Ltd., India. Dextrose, 3,5-dinitrosalicylic acid, and sodium metabisulphite were purchased from MolychemPvt. Ltd. and potassium iodide and potassium hydroxide were purchased from Thomas Baker (Chemicals) Pvt. Ltd., India. Rochelle salt was obtained from QualikemsPvt. Ltd., India.

2.2 Preparation of wheat straw

The selected variety of wheat straw was dried in a hot air oven and grinded to proper size using a blender. Wheat straw (2.5mg/ml) and ammonium nitrate (2.5mg/ml) were added in 100ml of double distilled water and it was then autoclaved. At the same time, a separate pretreated wheat straw media was also prepared by treatment of wheat straw with 1% potassium hydroxide, followed by incubating the treated wheat straw overnight at room temperature and further steam pretreated [*9,10,11*].

2.4 Simultaneous saccharification and Fermentation (SSF)

In a typical reaction, the nascent and pretreated wheat straw medium was inoculated with *Bacillus licheniformis (*10^6-10^8 CFU/ml) and incubated under shaking conditions at 30°C. After 48 hrs of incubation, the bacterium inoculated wheat straw medium was co-cultured with*Saccharomyces cerevisiae* (10^6-10^8 CFU/ml) and incubated under shaking conditions at 30°C for 120 hrs. Then, the effect of different parameters on production of ethanol was optimized by changing the co-inoculation time (24, 48, 72 hrs), substrate concentration (2.5, 5, 7.5 and 10 mg/ml), pH of medium (pH 5, 6, 7, 8) and concentration of nitrogen source (0.03, 0.06, 0.09 and 0.12 M). Samples were withdrawn at every 24hr interval from each flask for estimation of ethanol using potassium dichromate redox titration [13]and gas chromatography.

The optimized reaction condition was scaled up from 100ml to 1.5liter in a pre-sterilized fermenter. The fermenter conditions were initially set to 30°C and agitation speed at 121 rpm [12]. Aeration was given for cell growth followed by anaerobic condition [14].

2.5 Scale up of SSF

The Bio-age fermenter of 3liter capacity was filled with 2liter distilled water and autoclaved. Then autoclaving along with the fermentation medium was done after adjusting the medium pH to 7 and it was allowed to reach room temperature. *Bacillus licheniformis* was inoculated in 1.5liter of fermentation medium. After 72 hrs, of incubation,*Saccharomyces cerevisiae* was inoculated. For determining the concentration of ethanol the medium was centrifuged and the supernatant was collected separately and the sample was analyzed by GC [15].

2.5 Characterization techniques

Ethanol in the fermentation medium was estimated by gas chromatography. Agilent 6890 gas chromatograph equipped with Flame Ionization Detector (FID) was used for the separation and quantification of ethanol. A Zebron column was fitted into the instrument. The detector and injector temperature was maintained at 260°C. The gas chromatograph was connected to an integrator and computer system to determine the area of ethanol and internal standard peak.

3. RESULT AND DISCUSSION

The production of bioethanol from wheat straw by simultaneous saccharification and co-fermentation was estimated and optimized using various process parameters. The alkali treatment of wheat straw increased the production of glucose from 0.184 mg/ml to 0.223mg/ml when compared to untreated wheat straw after 24 hrs of incubation (Figure 1a). The increase in production of glucose thereby increased the production of ethanol from 0.34%to 0.49% (Figure 1b). The alkali treatment allows the removal of lignin content of wheat straw due to their solubility in an alkaline medium that hampers the structural support and integrity of plant cell wall and exposing the cellulose fibers for hydrolysis[17, 18].

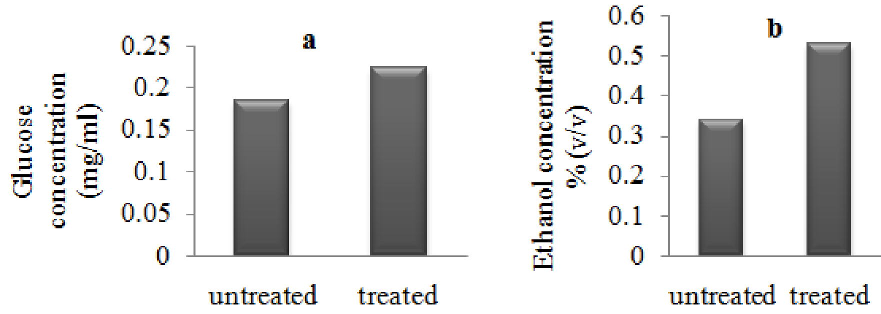

Figure 1: Comparison of glucose concentration (a) and bioethanol concentration (b) of untreated and treated wheat straw

The effect of the time duration between culturing and co-culturing of respective microorganism on bioethanol production from wheat straw was also estimated. The production of ethanol was increased from 0.27% to 0.53% as the duration between culturing and co-culturing was extended from 24 to 72 hrs.The increase in production of bioethanol may be due to the increase in production of cellulases with time [19]. Further, the ethanol production was estimated to be 0.53%, 0.88%, 0.92 %, 1.34% and 1.12% (v/v) after 24, 48, 72, 96 and 120 hrs, respectively of co-culturing (Figure 2). In this study, the maximum ethanol production was observed after 96 hrs of co-culturing after which the ethanol production dropped. This is due to the penetration of ethanol into the microbial cell wall after a significant quantity of ethanol has been produced, which tend to act lethal to the microorganism, thereby decreasing the ethanol production [20].

From the above results, it was observed that the appropriate time for co-culturing *Saccharomyces cerevisiae* was 72 hrs after culturing wheat straw with *Bacillus licheniformis* and the further process parameters were optimized taking it into consideration. Figure 2 elucidates the optimum pH for the production of bioethanol from wheat straw by simultaneous saccharification and co-fermentation. After 96 hrs of co-culturing, the production of bioethanol increased from 0.935 % to 2.6% (v/v),when pH was increased from 5 to 7 with a sudden decrease to 2.2% when the pH was changed to 8. The similar results were observed for the production of bioethanol after 24 hours, 48 hours, 72 hours and 120 hours of co-culturing. In which, the maximum ethanol production was estimated after 96 hours of co-culturing and at pH 7. The maximum amount of glucose available for fermentation by *Saccharomyces cerevisiae* is produced at pH 7 as it is the optimum pH for cellulase activity [21].

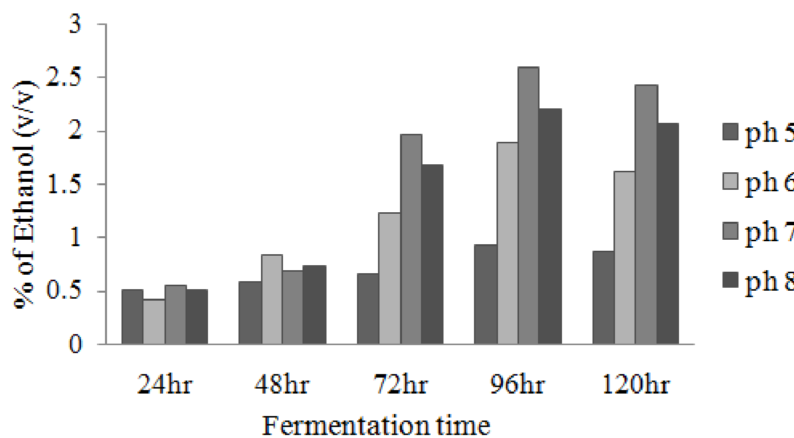

Figure 2: Effect of pH on bioethanol production by co-culturing of *Bacillus licheniformis* and *Saccharomyces cerevisiae.*

The effect of initial wheat straw concentration on production of bioethanol by co-culturing technique was optimized. Figure 3showsan increase in bioethanol production from 2.91% to 4.1% (v/v) as the substrate concentration was increased from 2.5mg/ml to 5mg/ml after 96 hrs of co-culturing. A sudden decrease in production of bioethanol from 3.8% to 3.26% (v/v) was observed, when the initial wheat straw concentration was increased to 7.5mg/ml and 10mg/ml, respectively. The similar results were observed for the production of bioethanol after 24 hrs, 48 hrs, 72 hrs and 120 hrs of co-culturing. The increase in initial wheat straw concentration above an optimum value disturbs the process of simultaneous saccharification and co-fermentation due to the phenomena of catabolite repression that tend to lower the yield of glucose, thereby, lowering the ethanol production [22].

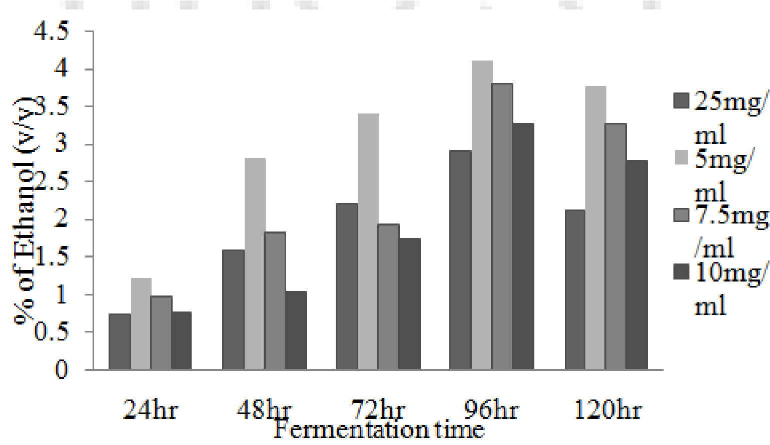

Figure 3: Effect of substrate concentration on bioethanol production by co-culturing of *Bacillus licheniformis* and *Saccharomyces cerevisiae.*

Finally, the effect of nitrogen source (ammonium nitrate) concentration on the bioethanol production from wheat straw was carried out. In which, the production of bioethanol initially

increased from 3.99% to 5.77% (v/v) as the concentration of ammonium nitrate was increased from 0.03 to 0.06M and a significant decrease from 4.52%to 3.3% (v/v), when the concentration of ammonium nitrate was increased to 0.09M and 0.12M, respectively (Figure 4).The similar results were obtained for the production of bioethanol after 24 hrs, 48 hrs, 72 hrs and 120 hrs of co-culturing. The increase in nitrogen concentration leads to increase in cellulase synthesis which in turn increases glucose production causing catabolite repression, this may be the reason attributed to decrease in bioethanol concentration at higher nitrogen concentrations in this process [23].

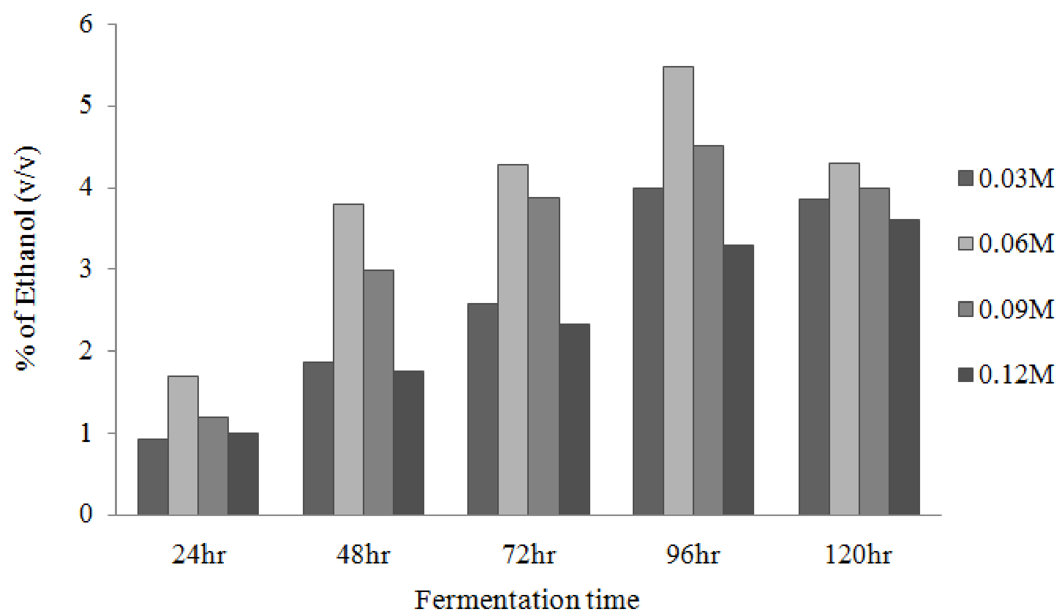

Figure 4: Effect of nitrogen source concentration on bioethanol production by co-culturing of *Bacillus licheniformis* and *Saccharomyces cerevisiae.*

From the above results, it was observed that the optimum bioethanol production from wheat straw by simultaneous saccharification and co-fermentation is obtained after 96 hrs, when the time duration between culturing and co-culturing is 72 hrs and other process parameters are adjusted to pH 7, initial wheat straw concentration at 5mg/ml and ammonium nitrate concentration of 0.06M. When scale up was done from shake flask to fermenter scale of 1.5 liter and then, the ethanol production was found to 18% by gas chromatography for the supernatant collected after 96hrs of co-culturing. As shown in Figure 5, the ethanol production increased from 5.77% to 18 %. It has been already shown that on scale up, which leads to increased cellulase production as well as the ethanol yield [24].

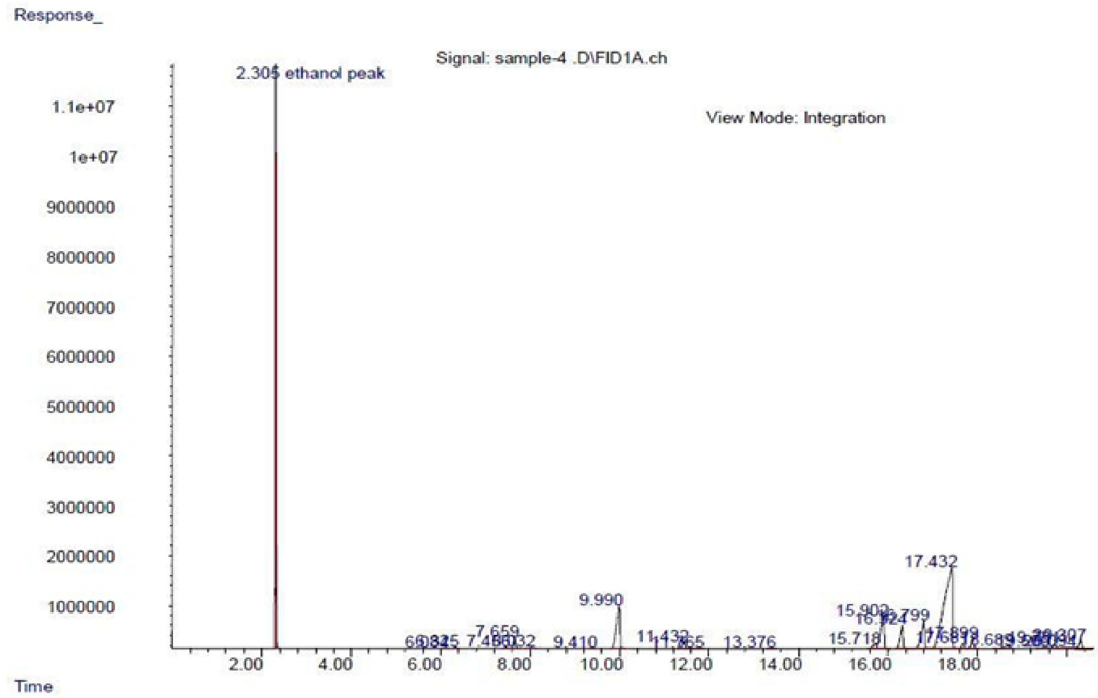

Figure 5: Gas chromatogram of bioethanol produced by co-culturing of *Bacillus licheniformis* and *Saccharomyces cerevisiae* at fermentor scale.

4. CONCLUSION

The cultural conditions (substrate loading, inoculum concentration, pH, and temperature and nitrogen source) of the bacterial culture media used were optimized to enhance the enzyme production. It was observed that cellulase activity was enhanced by optimizing cultural parameters in the fermentation media.

The pre-treatment of wheat straw with 1% Potassium hydroxide enhanced the production of bioethanol. The optimization of different process parameters shows that the highest ethanol production by co-culturing of *Bacillus licheniformis* and *Saccharomyces cerevisiae* is achieved after the third day of co-culturing at a pH of 7, with Nitrogen source and substrate concentration 0.06M and 5 mg/ml respectively. It was concluded that. Bioethanol production was finally characterized by Gas chromatography which comes 18.635% (v/v)

Acknowledgements

The financial assistance received from the is deeply acknowledged. The authors are very grateful to School of Biosciences and Biotechnology, Lovely Professional University (LPU.), Punjab, India. We are thankful to Allele life sciences (P) Ltd, India for providing the facility of Gas chromatography for characterization.

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