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COMPARISON OF CELLULOSIC ETHANOL PRODUCTION OF *SCHIZOSACCHAROMYCES POMBE* AND *SACCHAROMYCES CEREVISIAE* FROM GLUCOSE HYDROLYSATE

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ABSTRACT

The yeast strains *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* are known best for their ethanol production capability at the industrial level which produces about 12% of ethanol from glucose through fermentation process. The substrate utilization of these strains is limited and so it fails to utilize cellulose. As an attempt to produce cellulosic ethanol from *S. pombe* and *S. cerevisiae* from pretreated lignocellulosic biomass which was hydrolyzed separately with crude cellulase system of *E. cloacae* JV and *B. licheniformis* for the conversion of cellulose to glucose. Then the fermentation was carried out with the glucose present in the hydrolysate as substrate. The ethanol produced by *S. pombe* and *S. cerevisiae* from microbial hydrolysis of 4% NaOH treated rice straw was 7 % and 7-9% respectively.

Keywords: *S. pombe*, *S. cerevisiae*, Ethanol, Cellulase, Cellulose

INTRODUCTION

The yeast strains *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are known for their well-studied genetic and physiological background (Forsburg, 2005). *S. cerevisiae* is commonly called as Baker's yeast is so familiar for ethanol production at industrial level for many decades. It is believed that it was originally isolated from the skin of grapes. *S. cerevisiae* cells are round to ovoid, 5–10 micrometer's in diameter. It reproduces by budding and it is the microorganism behind the most common type of fermentation because it can grow aerobically on glucose, maltose and trehalose. Galactose and fructose are khown to be two of the best fermenting sugars. The ability of yeasts to use different sugars can differ depending on whether they are grown aerobically or anaerobically. Some strains cannot grow anaerobically on sucrose and trehalose (Costanzo *et al.*, 2010).

Compared to *S. cerevisiae*, *S. pombe* is also known for the ethanol production. The major difference between both strains in terms of growth is *S. pombe* divides by fission method. It is a unicellular eukaryote with rod shaped cells 3-4 micrometers in diameter. On evaluating the ethanol production capability of *S. pombe* it was concluded that the application *S. pombe* in controlled and optimized conditions would increase ethanol production at industrial level due to its high tolerance against glucose and ethanol (Abubaker *et al*., 2012).

Many works concentrate on ethanol production using bacteria, yeast and recombinant strains. All these researchers are only for making people intoxicated and financial security. Pollution from the current fossil fuels affects the green house and their demand and rise in cost put many ways for the search of alternate fuel. Ethanol is less toxic and flammable if it is produced with low cost there will be terrible change in the world economy. Annual predictions of global oil production declare that there will be a decline from the current 25 billion barrels to approximately 5 billion barrels in 2050. Therefore, there is a great interest in exploring alternative energy sources (Sun and Cheng, 2002). In 2010, a genetically engineered yeast strain was developed that produces its own cellulose-digesting enzymes. Assuming the same technology can be scaled to industrial levels; it would eliminate one or more steps of cellulolysis, reducing both the time required and costs of production (Tokuda, 2007).

Extensive research has been completed on conversion of lignocellulosic materials to ethanol in the last two decades (Bjerre, 1996). The conversion includes two processes: hydrolysis of cellulose in the lignocellulosic materials to fermentable reducing sugars which is usually catalyzed by cellulase enzymes. The factors that have been identified to affect the hydrolysis of cellulose include porosity (accessible surface area) of the waste materials, cellulose fiber crystallinity, and lignin and hemicellulose content (McMillan, 1994). Secondly, the fermentation process for the conversion of sugars to ethanol with the help of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). The hydrolysis is usually catalyzed by cellulase enzymes, and the fermentation is carried out by yeasts or bacteria.

Even the enzymes are performing sufficient role in converting cellulose to glucose, the availability of microorganisms for efficient saccharification and ethanol production from cellulose is unavailable. In the Present study an attempt has been made to hydrolyse the chemically pretreated rice straw using bacterial cellulase system

and saccharification of the released sugar in the hydrolysate for ethanol production using *S. cerevisiae* and *S. pombe* fermentation system.

2. MATERIALS AND METHODS

2.1 Microorganisms: *Enterobacter cloacae* JV (NCBI FJ799063.1) was isolated and characterized in the laboratory from the gut of termite (*Heterotermes indicola*). *Bacillus licheniformis* (MTCC 1520), *Shizosaccharomyces pombe* (MTCC 191) and *Saccharomyces cerevisiae* (MTCC 202) were obtained from Microbial Type Culture Collection, Chandigarh, India. *E. cloacae* JV and *B. licheniformis* were grown in nutrient broth. The yeast strains were cultured in the Yeast Peptone Dextrose (YPD) medium containing Yeast extract (0.3 g), Peptone (1 g), Dextrose (2 g) in 100ml of distilled water.

2.2 Congored overlay method: To visualize the cellulolytic activity of the collected microorganisms, *E. cloacae* (20 µl) and *B. licheniformis* (20 µl) were inoculated on luria agar supplemented with 0.6% CMC (Vasan *et al.*, 2011) and incubated overnight at 37ºC. After incubation, congo red solution was overlayed for 20 min. The surface of the agar was washed with 1M NaCl for identification of yellow color clearing zones around the wells (Wood, 1985). After the qualitative analysis through agar diffusion method, quantitative analysis of cellulase enzymes was measured spectrophotometrically.

2.3 Quantitative analysis of celluase activity: Endoglucanase, exoglucanase and cellobiase activity of the bacterial strains were measured spectrophotrmetrically by determining the amount of glucose release after reacting with substrates by DNS method. Endoglucanase activity was determined by adding 1 ml of 0.6% CMC to 1 ml of supernatant of *E. cloacae* and *B. licheniformis* separately and incubated at 50ºC for 30 min. Three milliliter of DNS (Miller, 1959) was added to stop the reaction and the mixtures were kept in a boiling water bath for 10 min and the absorbance was noted at 540 nm. Enzyme activities were calculated from the glucose standard graph (Ghose, 1987). Similarly the exoglucanase and cellobiase activity was carried out with 1x6 cm of Whitman filter paper (wt 50 mg) and 15 mM Cellobiose respectively.

2.4 Pyruvate decarboxylase (PDC) and Alcohol dehydrogenase (ADH) assay: PDC activity were measured by monitoring the pyruvic acid-dependent oxidation of NADH with ADH as a coupling enzyme whereas assay of ADH was measured by the ethanol-dependent reduction of NAD, in which the conversion of NAD to NADH (Millimolar extinction coefficient of ß-NADH at 340 nm is 6.22) spectrophotometricaly. After 48 hrs, the culture was centrifuged at 7000 rpm for 10 min and the supernatant was collected. For determining PDC activity, the reaction mixture (3 ml) consisting 2.7 ml of citrate buffer , 50 µl of NADH, 100 µl of Sodium pyruvate with 100 µl of culture supernatant of *S. pombe* incubated at 25ºC for 5 min and the absorbance was measured at 340 nm (Gounaris *et al*.,1971). For ADH activity, reaction mixture (3 ml) consisting 2.4 ml of Tris HCl , 100 µl of NAD, 300 µl of isopropanol with 200 µl of culture supernatant of *S. pombe* was incubated at 40ºC for 5 min and the absorbance was measured at 340 nm (Fibla *et al.*,1993).

2.5 Ethanol production of yeast strains:

2.5.1. Preparation of starter culture: Starter cultures were prepared by inoculating single colony of yeast strains grown on YEPD agar plate in 3 ml of YEPD medium. The culture was incubated at 30ºC in a shaker at 150 rpm. After 2 -3 days, the aerobically grown culture was sub cultured in 25 ml of YEPD medium and incubated for 2 days under shaking condition. After incubation the well grown cultures were aseptically transferred (10 ml) to the fermentation media (100 ml) for ethanol production.

2.5.2. Batch Fermentation of *S. cerevisiae* and *S. pombe* using glucose: Fermentation media was prepared by dissolving 10 g of glucose and 0.175 g of Na_2HPO_4 in 100 ml of distilled water. After transfer of inoculum into the fermentation media, the fermentation experiment was carried out under anaerobic condition at different time intervals to calculate the optimum fermentation time for *S. cerevisiae* and *S. pombe* for maximum conversion of glucose to ethanol. The fermentation was carried out at four different time intervals (24, 48, 72, and 98 hrs). The fermentation was carried out at pH-6 under the stirring condition. After the fermentation process, ethanol produced by *S. cerevisiae* was collected by simple distillation process at 78ºC. The collected ethanol was quantitatively measured by potassium dichromate method. In this method, 4.5 ml of potassium dichromate was mixed with 0.5 ml of collected ethanol and it was heated at 60ºC for 10 min. Then the absorbance was measured at 600 nm and percentage of ethanol was calculated with the ethanol standard graph.

2.5.3. Cellulosic ethanol production by Separate Hydrolysis Fermentation of rice straw: The 4% NaOH treated rice straw was hydrolysed by secretion of cellulase enzyme of *E. cloacae* and *B. licheniformis* separately (Sangkharak *et al*., 2011). One ml of 1 OD culture of by *E. cloacae* and *B. licheniformis* was inoculated in the luria broth containing 0.1% of 4% NaOH treated rice straw separately for 24 hrs in the shaker. After 24 hrs the culture was centrifuged at

7000 rpm for 10 min and filter sterilized. The sterile filtrate of both *E. cloacae* and *B. licheniformis* was used as fermentation medium for the yeast strains. The amount of glucose in the hydrolysate was estimated by DNS method.

2.5.4. Ethanol production of yeast from rice straw hydrolysate: The starter culture of yeast strains were inoculated to the fermentation media (filter sterilized supernatant of *E. cloacae* and *B. licheniformis* separately) supplemented with Na_2HPO_4. The fermentation process was carried out for 72 hours at pH- 6 under anaerobic condition with constant stirring. After fermentation, the glucose in the fermentation media was measured by centrifuging the fermentation media at 10,000 rpm for 10 min. One ml of supernatant was mixed with 3 ml of DNS reagent and it was kept in a boiling water bath for 10 min and the absorbance was measured at 540 nm. The ethanol produced from the rice straw hydrolysate by the yeast strains was collected by simple distillation process at 78˚C. The collected ethanol was quantitatively measured by potassium di chromate method (Kiransree *et al.,* 2000).

3. RESULTS

3.1 Cellulolytic zone: Cellulase activity of *E. cloacae* and *B. licheniformis* was preliminarily confirmed using the congo red overlay method. *E. cloacae* showed the highest ratio of clear zone than the *B. licheniformis*. The formation of yellow zone indicates that the strong interaction of Congo red with β-(1,4), β-(1,3)-d-glucans of the cellulase produced by *E. cloacae* and *B. licheniformis.* The cellulolytic zone of *E. cloacae* was highly intense and larger zone compared to *B. licheniformis* (Fig.1).

3.2 Cellulase enzyme activity: The endoglucanase, exoglucanase and cellobiose enzyme activities of *E. cloacae* and *B. licheniformis* were determined by DNS method (Fig.2a and 2b). The specific enzyme activities were calculated from estimating the amount of glucose released using the glucose standard graph. The formula used for calculating enzyme activities are, For Filter paper: mg of glucose released × 0.185; For CMC: mg of glucose released × 0.37

For Cellobiose: mg of glucose released × 0.0926. From the enzyme activity graph it was clear that the endoglucanase activity was higher in both *E. cloacae* and *B. licheniformis.*

3.3 ADH and PDC activity of *S. pombe* and S. *cerevisiae*: The rate of ethanol production of *S. pombe* and *S. cerevisiae* will vary with respect to ADH and PDC activity. ADH and PDC activity of *S. pombe* and *S. cerevisiae* was 0.745 and 0.139 IU/ml and 0.605 and 0.131 IU/ml respectively (Fig. 3a and 3b).

3.4 Batch fermentation of *S. pombe* and *S. cerevisiae*: The batch fermentation of *S. pombe* and *S. cerevisiae* for ethanol production was carried out with 10% of glucose under anaerobic condition at different time intervals. The fermentation period of *S. pombe* for 72 hr produces higher percentage of ethanol. The production of ethanol proportionally increased as the fermentation time increased till 72 hrs. After 96 hr, the ethanol production rate was decreased (Fig. 4a and 4b).

3.5 Microbial cellulase enzyme hydrolysis of rice straw: The cellulolysis process for conversion of cellulose in the 4% NaOH pretreated rice straw to glucose was performed by the extracellular cellulase produced by *E. cloacae* and *B. licheniformis* in the media. The cellulase enzyme activity of these strains was measured by DNS method. The rate of cellulose conversion in *E. cloacae* was higher than the gram positive *B. licheniformis.*

3.6 Ethanol production from cellulose hydrolysate: The ethanol produced by *S. pombe* and *S. cerevisiae* from enzyme hydrolysed rice straw was measured spectrophotometerically by potassium dichromate method. The percentage of ethanol produced from the cellulose hydrolysate *B. licheniformis* was higher than the ethanol produced from the cellulose hydrolysate of *E. cloacae* (Fig. 5a and 5b).

3.7 Estimation of glucose during separate hydrolysis and fermentation: The amount of glucose released before and after fermentation in the hydrolysate of *E. cloacae* and *B. licheniformis* was 0.58, 0.52, 0.36 and 0.20 mg/ml respectively. The amount of glucose released during hydrolysis process was more in *E. cloacae* whereas the glucose consumption rate for ethanol production was lower in the *E. cloacae* cellulase hydrolyzed rice straw. Compared to both cellulase hydrolysates, the percentage yield of ethanol was higher in *B. licheniformis* (Table 1.1 and 1.2).

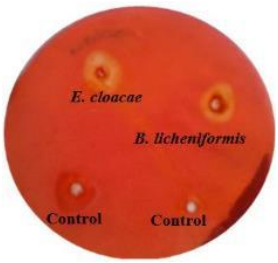

Fig.1.Cellulolytic zone of *E. cloacae* and *B. licheniformis*

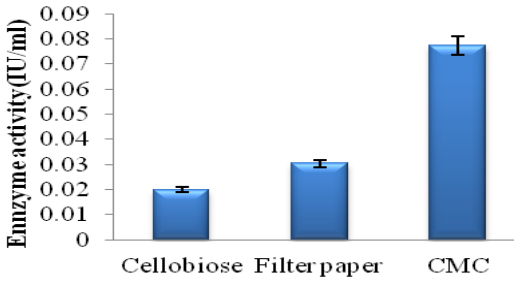

Fig.2a.Cellulase enzyme activity of *E. cloacae*

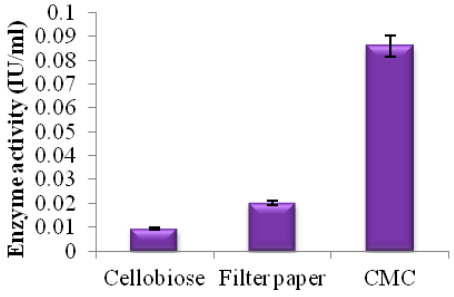

Fig.2b.Cellulase enzyme activity of *B. licheniformis*

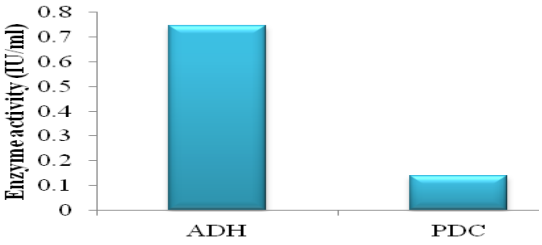

Fig. 3a. ADH and PDC activity of *S. pombe*

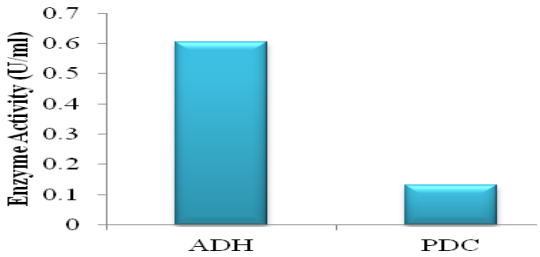

Fig. 3b. ADH and PDC activity of *S. cerevisiae*

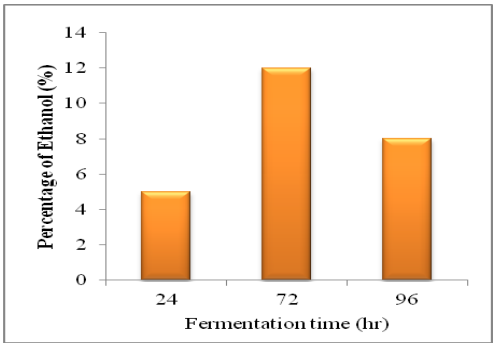

Fig.4a.Percentage of ethanol production by *S. pombe* at various time intervals

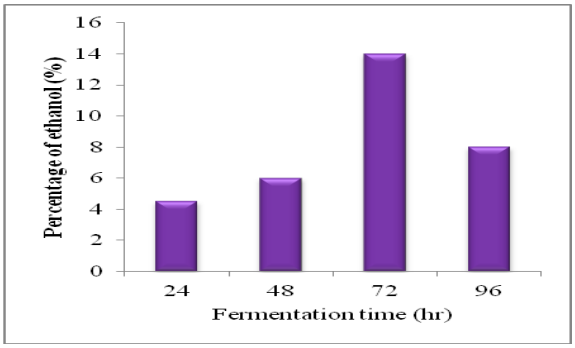

Fig.4b.Percentage of ethanol production by *S. cerevisiae* at various time intervals

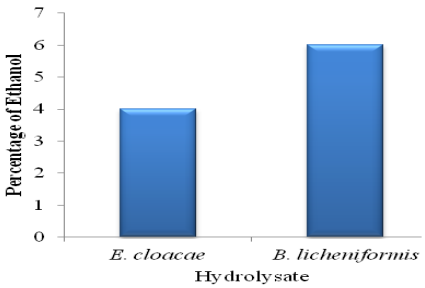

Fig. 5a Ethanol production *S. pombe* from hydrolysate

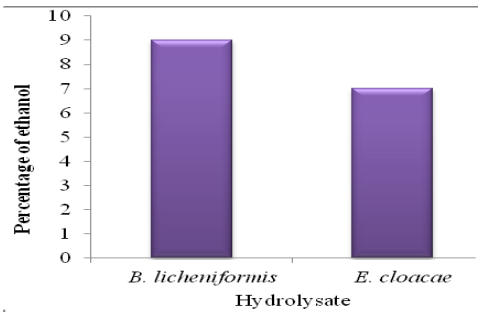

Fig. 5b Ethanol production *S. cerevisiae* from hydrolysate

Table.1.1 Estimation of glucose during separate hydrolysis and fermentation of *S. pombe*

Microorganisms	Glucose (mg/ml)		Glucose consumption rate (%)
	Before Fermentation	After fermentation	
E. cloacae	0.58	0.36	22
B. licheniformis	0.52	0.20	32

Table 1.2 Estimation of Glucose during separate hydrolysis and fermentation S. *cerevisiae*

Microorganisms	Glucose (mg/ml)		Glucose consumption rate (%)
	Before fermentation	After fermentation	
B. licheniformis	0.58	0.44	14
E. cloacae	0.52	0.29	20

4. DISCUSSION

The rate limiting step in the conversion of cellulose to fuels at industrial level is the hydrolysis process, especially the initial attack on the highly ordered insoluble structure of crystalline cellulose to glucose. Cellulase produced is the chief enzyme chiefly produced by many microorganisms such as fungi, bacteria and protozoan that catalyze cellulolysis (hydrolysis of cellulose). Cellulases are also produced by a few other types of organisms, such as some termites and the microbial intestinal symbionts of other termites. Several different kinds of cellulases are known, which differ in structure and mechanism (Bayer, 1998). *Proteus vulgaris, Klebsiella pneumoniae* and *Citrobacter freundii* were the gut microbes of *Bombyx mori* which were both cellulolytic and xylanolytic (Anand *et al*., 2010). The cellulolytic activity of *E. cloacae* was more efficient as the microorganism was isolated from the hind gut of termite which majorly feeds on the wood. The insect gut micro biota are playing the role in the digestion and are highly active (Vasan, 2011).

Most of this work has emphasized the biochemistry, genetics and process development of fungi coupled to the further conversion of sugars produced to ethanol by yeast *S. cerevisiae* (Demain *et al.*, 2005). The diversity of microbial sugar metabolism is especially evident in the further metabolism of pyruvate. During fermentative growth, pyruvate may be converted into a multitude of compounds, including molecular hydrogen, carbon dioxide and many organic metabolites. Alternatively, respiratory dissimilation of pyruvate via Tri Carboxylic Acid (TCA) cycle leads to its complete oxidation to carbon dioxide and water. In view of the staggering diversity of sugar metabolism in the microbial world, a surprising unity exists among 700 yeasts species that are currently recognized, all strains investigated seems to be predominantly use the Embden-Meyerhof pathway for conversion of hexose phosphates to pyruvate (Pronk, 1996).

Yeast PDC is activated by the pyruvate released in the glycolysis process whereas inorganic phosphate is the inhibitor (Urk, 1989). However, a transient exposure to glucose leads to an immediate reduction of the intracellular phosphate concentration. These properties of PDC indicate that pyruvate in Crab tree-positive yeasts was metabolised *via* pyruvate decarboxylase during exposure to the excess of glucose. The PDC activity of *S. cerevisiae* and *S. Pombe* was 0.131 IU/ml and 0.139 IU/ml.

Physiologically, the ADH reaction in *S. cerevisiae* and in related species plays a dual and quite critical role in sugar metabolism. Almost all of the carbohydrates were used during fermentation process, a specific ADH isozyme serves to regenerate the glycolytic NAD^+ through the reduction of acetaldehyde to ethanol. Under aerobic conditions, respiration of the accumulated ethanol occurs after depletion of the fermentable sugar, again by the action of specific ADH isozymes. Thus, in *S. cerevisiae*, the ADH reaction links fermentative and respiratory (oxidative) carbon metabolism, allowing the optimal use of the sugar carbon (Smidt, 2008). The ADH activity of *S. cerevisiae* and *S. pombe* was 0.605 and 0.745 IU/ml.

Fermentation of the yeast strains were carried out at strict anaerobic because in the absence of oxygen, cells must rely exclusively on glycolysis to produce ATP as there is no aerobic metabolism. In yeast, the molecule that accepts hydrogen from NADH was pyruvate, the end product of glycolysis where the yeast enzymes remove a terminal CO_2 group from pyruvate through decarboxylation, producing a two-carbon molecule called acetaldehyde. The acetaldehyde accepts a hydrogen atom from NADH, producing NAD+ and ethanol (ethyl alcohol). This particular type of fermentation is of great interest to humans, since it is the source of the ethanol in wine and beer. Ethanol and CO_2 are byproducts of fermentation (Glasson, 1997).

S. cerevisiae is used extensively in batch fermentation to convert sugars to ethanol for the production of beverages and biofuels. *S. cerevisiae* is capable of very rapid rates of glycolysis and ethanol production under optimal conditions, producing over 50 mmol of ethanol per h per g of cell protein (Ingram *et al*., 1987). In the hydrolysis process even the glucose released by *E. cloacae* was more but only 22% of glucose was converted to ethanol by fermentation process. This is because a wide range of substrate utilization of *E. cloacae* (glucose, cellobiose, mannose, lactose etc.) led to the formation of many byproducts such as ethanol, acetic acid, furfural, lactic acid etc. So all these works reflects that the presence of furfural, acetic acid etc., during ethanol fermentation affect the ethanol yield.

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