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Optimization of ethanol production using pretreated corn cob and sugarcane bagasse hydrolysate by *Candida parapsilosis strain* BKR1

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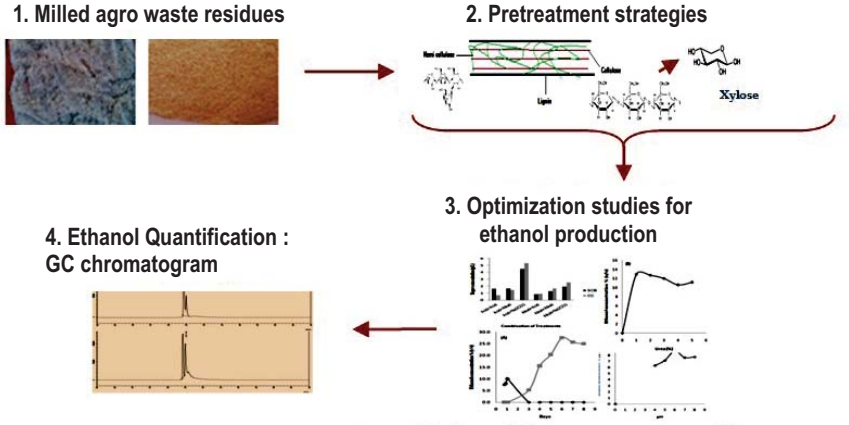
Abstract

Aim : Bioethanol is a suitable and best alternative to the replenishing fossil fuel in the fast growing world. To meet the rising demand of ethanol in the global market, there needs to be lot of improvisation in the existing process. One such method is to utilize more pentose sugar along with hexose in the substrate for better yield. In the present study, the efficiency of *Candida parapsilosis BKR1* species for the production of ethanol was studied in corncobs and sugarcane bagasse.

Methodology : The sugarcane bagasse and corn cobs were collected from local market, Coimbatore and *Candida parapsilosis BKR1 was isolated from sugar cane extract.* The lignocellulosic materials were treated by various combination of pretreatment (acid and alkali pretreatment) and hydrolysis (acid, alkaline and sodium carbonate hydrolysis) methods to maximize the fermentable sugars in the substrate. The fermentation conditions such as pH, inoculum age, nitrogen source and fermentation time for ethanol production were optimized and the fermented media of both substrates were subjected to distillation to find ethanol percentage by gas chromatography.

Results : Acid pretreatment with sodium carbonate hydrolysis produced higher sugar yield for both the lignocellulosic materials. The studies on optimization showed maximum production using 24th hr inoculum, with 1% nitrogen source at pH 6, during 6th day of fermentation @ 120 rpm for corn cobs and sugarcane bagasse. The results revealed that ethanol concentration in sugarcane bagasse and corn cobs were 27.93 g l^{-1} and 20.78 g l^{-1}, respectively.

Interpretation : The *Candida parapsilosis BKR1* strain efficiently utilized sugarcane bagasse than corn cobs and produced high concentration of ethanol.



Production & Optimization of Ethanol from agro waste residues by *Candida parapsilosis BKR1*

Introduction

The demand for alternative fuel is increasing day by day due to the depletion of fossil fuels and high CO_2 emission. Ethanol, a major component of worldwide gasoline market is a key for the above considered problems. Although energy equivalent of ethanol is lesser than petroleum fuel, recent trends in the technologies raises the demand for ethanol production (Mussatto *et al.,* 2010).

Lignocellulosic biomass, such as sugarcane bagasse/ corn cob, can be economical and easily available source used for the biofuel production. Bioethanol production from lignocellulosic biomass rich in hemicellulose comprises the hydrolysis of complex sugar followed by reduced sugar fermentation. The production of ethanol from renewable biomass such as sugarcane beets, corn, cassava, potatoes, paper and agricultural waste, converting the carbohydrate enriched lignocellulosic materials into ethanol by various micro organisms (Lin *et al.,* 2006; Ray *et al.,* 2008).

Most typical microorganisms used for the production of bioethanol are *Saccharomyces cerevisiae*, *Zymomonas mobilis* and *Escherichia coli* (Lin *et al.,* 2006) which are capable of fermenting hexose sugars/ sucrose. Most of the ethanol fermenting organism source is limited to hexose sugars. Pentose fermenting organisms are restricted to *Pichiastipitis, Candida shehatae, Pachysolen tannophilus* (Martiniano *et al.,* 2013). Finding new microbial isolates from the natural sources can enable effective fermentation of pentose sugars to yield ethanol (Mussatto *et al.,* 2010). The consistent results in optimization studies are essential to develop commercially significant process with sustainable solution. The different types of ethanol producing strains such as *Saccharomyces cerevisiae, Zygosaccharomyces sp., Saccharomyces ellipsoids, Schizosaccharomyces pombe* and *Schizosaccharomyces mallaeri* were optimized for maximum ethanol production (Osman *et al.,* 2011).

The strain *Candida parapsilosis BKR1* used in the present study *was isolated from sugar cane extract* and studied for the ability of utilizing sugarcane bagasse and corn cobs for the production of ethanol. The substrates used in the present investigation are agricultural waste residues obtained from sugarcane processing plant and corn fields.

Materials and Methods

Sample collection :*Candida parapsilosis strain BKR1, isolated from sugar cane extracts at Sathyamangalam, Tamilnadu, was used for ethanol production.* Biomass such as sugarcane bagasse (SCB) and corn cobs (CC) required for fermentation were collected from the local market, Coimbatore, Tamil Nadu and all other chemicals were of analytical grade.

Microbial inoculum preparation : Isolated *Candida parapsilosis strain BKR1* was sub cultured in xylose rich medium containing 20 g of xylose supplemented with Yeast extract (5 g) and Peptone (10 g). The culture was inoculated into 150 ml conical flask containing 10 ml of medium and agitated at 200 rpm at 30°C for 24 hr. After 24 hrs of incubation, culture was centrifuged at 2200 g for 15 min. The collected cells were washed with sterile distilled water.A suspension was prepared with the cell mass in sterile distilled water and utilized as inoculum.

Pretreatment of lignocellulosic materials : Sugarcane bagasse (SCB) and corn cobs (CC) were washed, shadow dried and their size were reduced. Further treatment involved delignification and hydrolysis of SCB and CC which were done separately by acid and alkali.

Dried and chopped sugarcane bagasse and corn cobs were immersed in sulfuric acid in 1:10 (w/v) ratio and next to this process, biomass were similarly subjected to alkaline treatment with 1N NaOH solution in 1:10 (w/v) ratio. These mixtures were hydrolysed at 121°C for 15 min. The obtained hydrolysates were pressed through cheese cloth and solid biomass was taken for further hydrolysis.

These pretreated samples were further hydrolysed by 0.5% H_2SO_4 solution, 0.5 N NaOH and 1N Na_2CO_3 separately at 121°C for 20 min. The hydrolysates were analyzed for reducing sugar concentration. Among these samples, hydrolysates with highest sugar concentration were selected for fermentation process.

Analysis of sugars and ethanol : The concentration of reducing sugar was determined by 3,5-dinitrosalicylic method (Miller, 1959). The ethanol concentration was determined by potassium dichromate method and gas chromatography method using Rtx-5 column (30 m, 0.32 ID) and nitrogen carrier gas (53.9 ml min^{-1}). Injection and FID temperatures were 150°C and 200°C, respectively. The temperature of oven was maintained at 325°C.

The concentration of ethanol was calculated by the following formula (Cachet, 2011):

$$Ca=[Cstd *As] / Astd$$

Where, C_a is the concentration of analyte in the sample; C_{std} is the concentration of analyte in standard; A_a is the area of analyte from the sample chromatogram and A_{std} is the area of analyte from the standard chromatogram.

Optimization of fermentation conditions : Many strategies are involved in order to maximize ethanol production. These strategies include both pretreatment methods and the fermentation conditions. The present study focused on the fermentation to obtain maximum concentration of ethanol - some of these conditions include age of inoculum, fermentation duration, nitrogen source and pH of fermentation medium. The

Table 1 : Various combinations of pretreatments processed on the raw materials –Sugarcane Bagasse (SCB) and Corn Cops (CC)

Process	Raw Material	Delignification	Hydrolysis
Run 1	SCB	Acid	Acid
Run 2	SCB	Acid	NaOH
Run 3	SCB	Acid	Sodium Carbonate
Run 4	SCB	Alkali	Acid
Run 5	SCB	Alkali	NaOH
Run 6	SCB	Alkali	Sodium carbonate
Run 7	CC	Acid	Acid
Run 8	CC	Acid	NaOH
Run 9	CC	Acid	Sodium Carbonate
Run 10	CC	Alkali	Acid
Run 11	CC	Alkali	NaOH
Run 12	CC	Alkali	Sodium carbonate

optimized studies were done in pure xylose substrate and the optimized conditions were taken for raw material fermentation.

Effect of inoculum age : *Candida parapsilosis BKR1 strain was taken in xylose supplemented medium and kept* at 200 rpm, 30°C. The effect of age of inoculum was studied by taking cultures at various time intervals such as 18, 20, 22, 24 and 26 hrs and inoculated in xylose containing medium. Samples were taken at regular intervals and analyzed for ethanol concentration.

Effect of pH : The pH of xylose medium was set from 4 to 8 by adjusting with 1N HCl and 1N NaOH. The optimized age of inoculum culture was taken and inoculated for all medium ranging from pH 4 to pH 8 and samples were analyzed for ethanol concentration at regular intervals.

Effect of fermentation time : The xylose containing medium with optimized pH and age of inoculum was taken for fermentation. The ethanol concentration was analyzed at regular interval from 0-6 days.

Effect of nitrogen source : The effect of urea in ethanol production was studied by varying the percentage of urea (1%, 2%, 3%, 4% and 5% nitrogen source (10:1 v/v) in the fermentation medium.

Substrate fermentation : The supernatant from treated hydrolyzates were used as fermentation medium and optimized conditions from the optimization studies were taken for further process. 1 % (v/v) of optimized inoculum was added and the medium was kept at 37°C with 120 rpm and fermented for a period of 6 days. After fermentation, the cells were separated by centrifuging and supernatant taken for distillation process. The distillates were analyzed for ethanol concentration by Gas Chromatography.

Results and Discussion

Treatment of lignocelluloses materials : The lignocellulosic materials are composed of cellulose, hemicellulose, lignin, extractives and several inorganic materials. Among these the cellulose and hemicelluloses which is made up of sugars has to be separated and hydrolysed for further fermentation process. To achieve this, the lignocellulosic materials were degraded and delignified to release cellulose and hemicelluloses components, which were further hydrolysed to release the reducing sugars. The results of pretreatments are shown in Fig. 1. Acid along with Na_2CO_3 treatment of lignocellulosic materials was found to release maximum sugar concentration as compared to other treatment procedures. The maximum sugar substrate release was found for acid with Na_2CO_3 treatment combination. Size reduction of raw material decreased the polymerization of cellulosic content and also increased the accessible surface area of the material for further degradation by physiological and

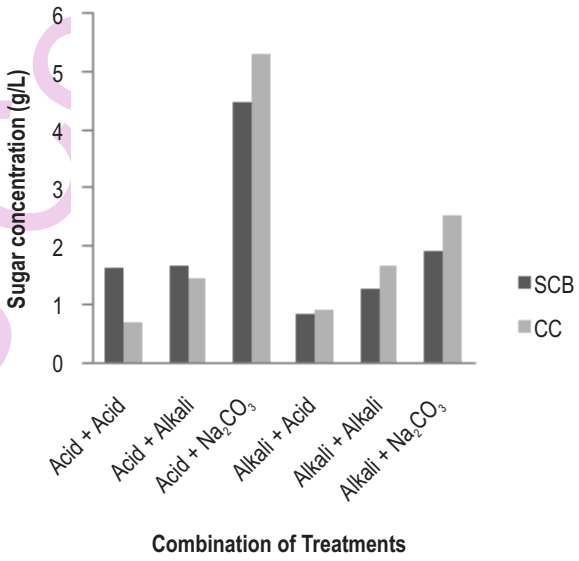


Fig. 1 : Reducing sugar concentration of pretreated and hydrolyzed samples of sugarcane bagasse (SCB) and corn cobs (CC)

microbial reactions.

In sugarcane bagasse (SCB) residual pretreatment, run 1, 2 and 3 had high sugar yield when compared with that of than 4, 5 and 6 which indicates that acid pretreatment gave high sugar yield than alkali treatment but the results were contrast in corncob (CC) residues, as both acid and alkali treatment gave more similar amount of reducing sugars. These pretreated samples were further hydrolyzed by acid and alkali (Sodium hydroxide and Sodium carbonate). Among the methods tried, acid pretreated with sodium carbonate hydrolysis (Run 3 & 9) yielded high reducing sugars, followed by alkali with sodium carbonate (Run 6 & 12) than other process. It suggests that the acid pretreatment might completely solubilize hemicelluloses and gives better hydrolysable substrates. Verardi et al. (2016) showed that the yields of monosaccharide's from a lignocellulosic biomass is complex but economical for monosaccharides such as xylose,

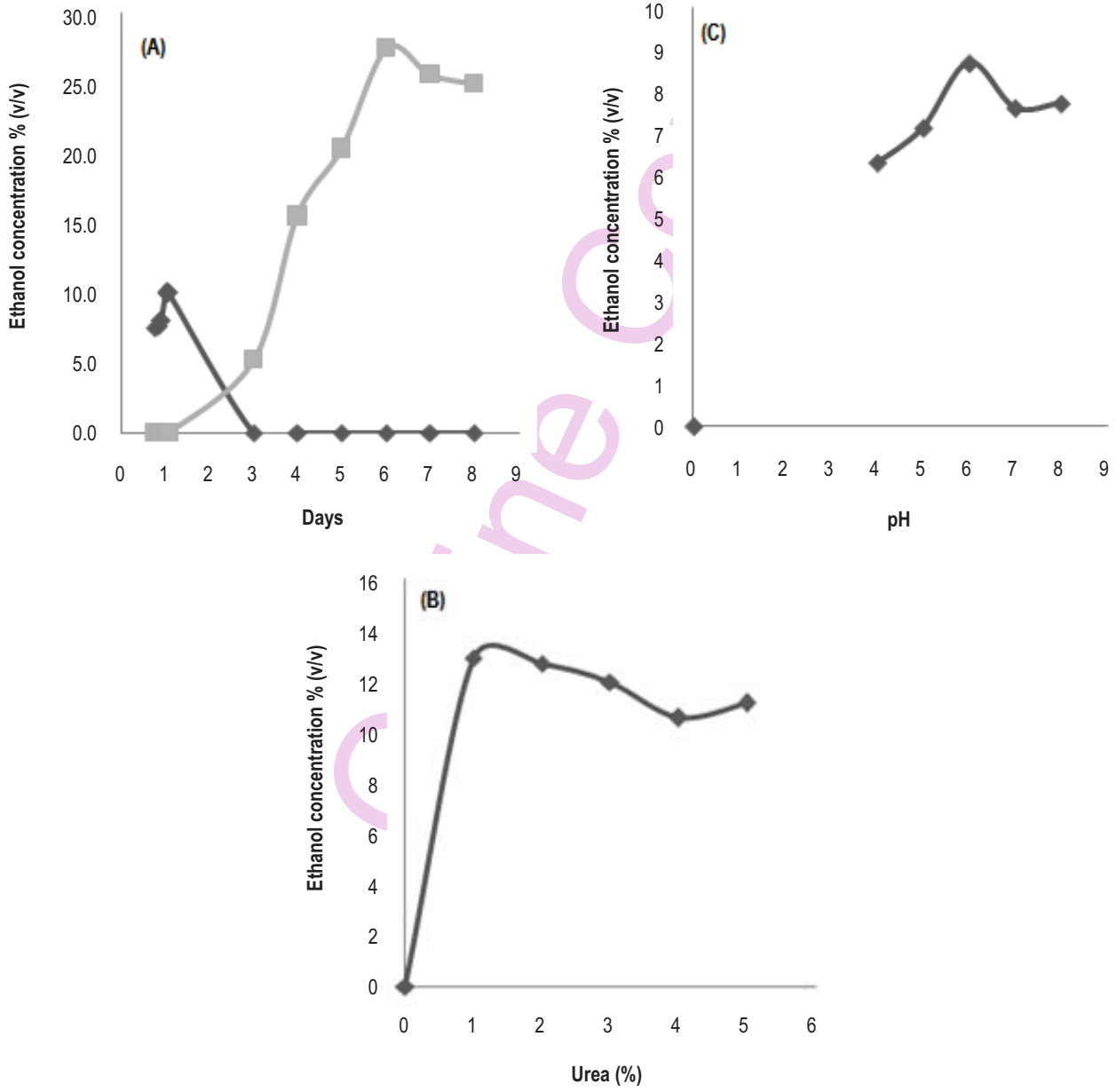


Fig. 2 : Effect of parameters on ethanol production from xylose (A) fermentation time (■) and age of inoculums (♦); (B) pH; (C) nitrogen source

arabinose, glucose and mannose.

Sodium carbonate hydrolysis yields more sugar than other hydrolysis methods mainly due to hydrophilic acid dissolvable nature of sodium carbonate. The results are contrary to **Bjerre** *et al.,* (1996) who stated that addition of sodium carbonate dint has much effect on hemicellulose conversion. These might be due to inhibition of degradation of sugars to byproducts like furfural which will decrease the sugar concentration. Due to the above mentioned reasons, the interference between undesired byproducts and enhances the hemicellulose conversion from agro waste residues.Sodium carbonate might act as detoxifier to prevent degradation of products and also hydrolyze the product. Also the sugar concentration was found to be maximum (>5 gl^{-1}) for sugarcane bagasse compared to corncob residue probably due to interference effect of furfural derivatives in CC residues.

Optimization Studies

Effect of inoculum age : The growth of *Candida parapsilosis BKR1* and simultaneous production of ethanol mainly depends on the production of sugars from CC and SCB residues as substrate. The inoculum age of *Candida parapsilosis BKR1* varied from 18 to 26 hrs (0.75 to 1.08 days). Fig.2A shows the effect of inoculum age on ethanol production. A decrease in the inoculum age leads to decreased production and the 24 hrs slant showed higher production of ethanol. Hence, it is recommended to use 24 hrs slant culture of *Candida parapsilosis BKR1* for economical production of ethanol. The optimized 24 hr microbial culture was able to yield maximum ethanol production due to the availability of majority of active viable microbes in their log phase. These log phase cultures ensures rapid growth and enhance the ethanol productivity. Further rise in the inoculum age also resulted in same ethanol yield of 10%.

Effect of pH : Among the various pH of fermentation medium tried, the ethanol production was observed between pH 4-8 (Fig.2B). The maximum ethanol was obtained at pH 6 and no production of ethanol was observed below pH 4 (data not shown). These results suggested that maintaining intracellular pH is most vital for the proper functioning of glycolytic enzymes in yeast. Variation in the mid pH level creates ionic imbalance across the microbial cell wall which builds up stress and further inhibits yeast metabolic pathway for ethanol production. High acidic condition of the solution due to organic acids tends to reduce the microbial growth. Naturally by increasing the pH of fermentation medium, the microbial growth is inhibited and eventually the broth is flooded with more of organic acids (Graves *et al.,* 2006; Izmirlioglu and Demirci, 2012; Turhan *et al.,* 2010; Osman *et al.,* 2011). However these results were in contrast to Du preez *et al.,* (1986) who concluded that *Candida* was insensitive to pH range of 2.5 -6.5 for the production of ethanol. The ability of the *Candida* species to produce ethanol was reported at pH > 6 as 8.69 % (v/v). In the neutral and alkaline condition the ethanol yield were drastically reduced might be due to formation of salt when an unstable sugar reacts with alkali content in excess (Graves *et al.,* 2006).

Effect of fermentation time : The ethanol concentrations were analyzed from zero hour and there was no ethanol production for first 48 hrs (data not shown). This might be due to the carbon utilization for the cellular maintenance and production of xylitol based on their metabolic pathways. The production of xylitol was initiated at 48 hrs and reached maximum at 72 hrs. After 72 hrs, the xylitol concentration decreased with initiation of ethanol production. The concentration of ethanol started increasing from 72 hrs and gave maximum yield at 144 hrs (Fig.2A). The optimum ethanol yield was found to be 27.5% (v/v) on 6^{th} day. The probable reason for producing ethanol after 144 hrs is due to delayed metabolic pathways and accumulation of xylitol in the previous stages. The rate limiting step in the pentose phosphate pathway may be overcome by enhancing the redox stress induced during xylitol formation inside the microbial cell. Itelima *et al.,* (2013), concludes that the accumulation of xylitol enhances the redox potential inside the microbial cell which ultimately determines the rate of ethanol production, which is in line with the present investigation results.

Effect of nitrogen source : The organic nitrogen plays a major role in the growth of yeast cells and production of ethanol (Jeffries, 1985). Among the various sources and concentrations selected, 1% of urea in fermentation medium gave high ethanol concentration of 13% (v/v), which is depicted in Fig.2C. However, Nofemele *et al.,* (2012) and Chan-u-tit *et al.,* (2013) reported that the increasing percentage of urea decreased the production of ethanol. The reason for the reduction in ethanol production is probably due to the inhibition/ partial blockage of oxidoreductive enzymes involved in the pentose phosphate pathway. The ratio between NAD/ NADPH – linked xylose reductase plays a major role in determining the utilization of nitrogen source, supplied along with the microbial medium supplement. It is assumed that the optimum concentration of nitrogen source rapidly demands for more xylitol dehydrogenase in the metabolic pathway, which in turn enhances ethanol production.

Substrate fermentation : The optimized conditions taken for fermentation of hydrolyzates of SCB and CC along with 1% urea were taken as fermentation medium. The ethanol produced by fermentation of SCB and CC is shown in Fig. 3A-3C. The concentration of ethanol produced by SCB and CC was 27.93 and 20.78 g l^{-1}. These results are significantly similar to the results of Patle and Lal (2007) and Latif and Rajoka (2001) who reported ethanol production by mixed cultures using agricultural wastes. The interference of intermediate compounds such as HMF, furfural in corn cob based agro-waste residue, when compared with sugarcane bagasse residues which automatically reduces the ethanol yield. However, according to Zhao et al. (2015),

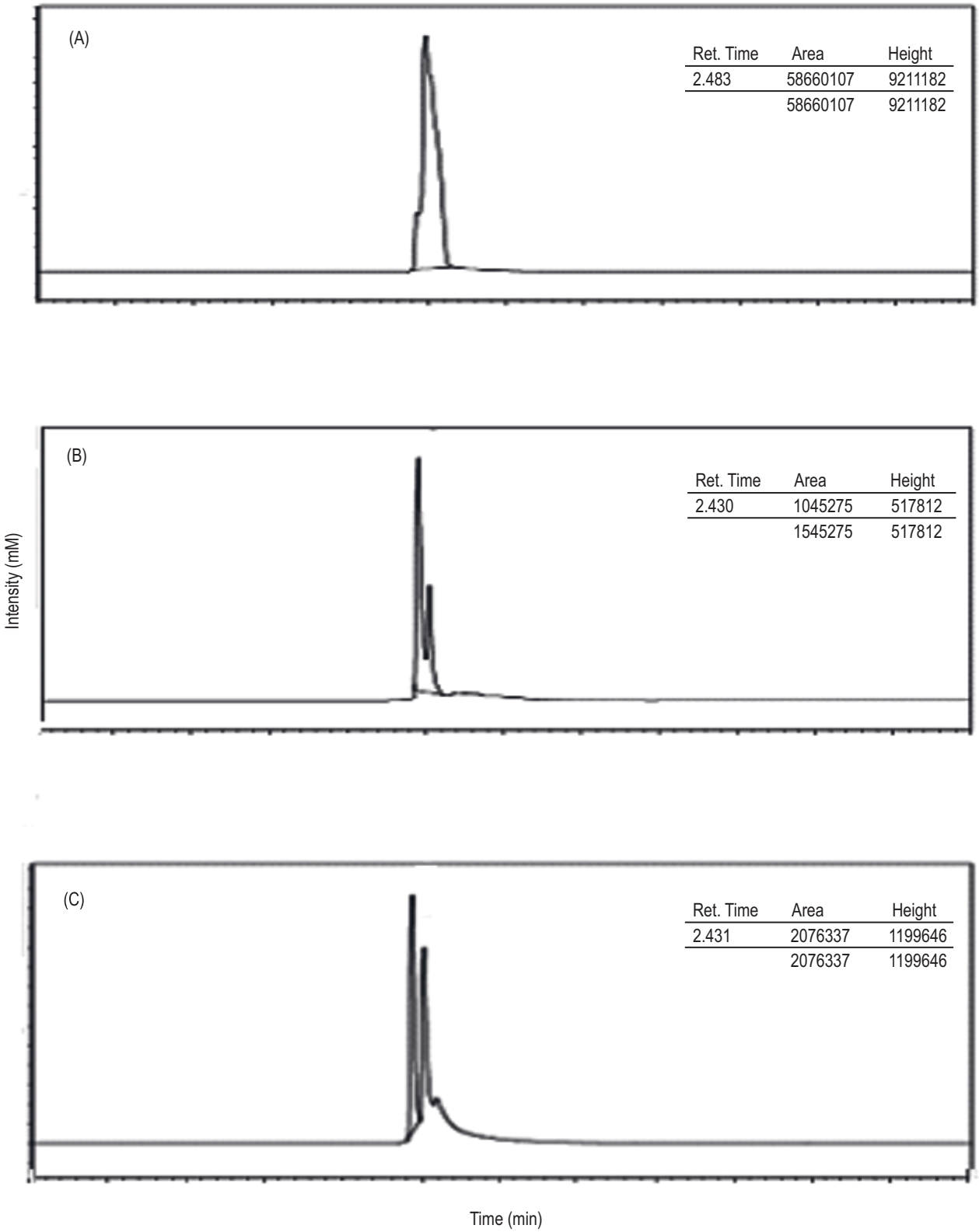


Fig. 3 : Gas Chromatogram showing the presence of ethanol in (A) reference standard; (B) corn cob and (C) sugarcane bagasse hydrolysate fermentation medium

regardless of existing technologies for lignocellulosic biomass, bioethanol is not competent with fossil fuels when economic feasibility is considered.

The present study envisaged the possibilities of Bioethanol production from the agrowaste residues using indigenous yeast isolate *Candida parapsilosis BKR1.* The ethanol produced was found to be maximum (27.93 g l^{-1}) for SCB pretreated agro residue than for CC pretreated agro residue (20.78 g l^{-1}) at optimum conditions.

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