RJC

RASĀYAN *J. Chem.*
Vol. 7 | No.3 |241 – 245 | July – September | 2014
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

MOLECULAR IDENTIFICATION AND FACTORIAL OPTIMIZATION OF MICROBIAL ISOLATES FOR POLYOL PRODUCTION

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ABSTRACT

Xylitol is a pentitol, a natural sweetener, found its application in food/ pharmaceutical industries, exploited especially for its anti-cariogenic properties. Xylitol is a valuable product with rising demands in market, it becomes significant to study and optimize the production with more of economic justification. In this work, xylitol obtained by biotechnological methods was based on fermentation by yeast isolated from sugarcane extracts collected from the Sathyamangalam, Tamilnadu, India. Yeast isolates were identified using conventional microbiological approach, modern metagenomic analysis and the gene sequence was deposited in NCBI repository. The growth of *Candida parapsilosis strain BKR1 (NCBI accession no.:KC462059)* andits xylitol production capability were assessed. The single factorial optimization revealed that pH, Temperature and initial substrate concentration was 4, 30ºC and 0.1 g/ml respectively.

Keywords: xylosemetabolism, xylitol, Candida sp., factorial optimization

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INTRODUCTION

In order to decrease the production cost and meet the increasing xylitol demand in market, the industry is actively developing a high yield but low energy-consumption alternative for xylitol production.[1]A comprehensive analysis made on the biotechnological production and future applications of this rare sugar alcohol reveals its commercial importance in the near future. It is being used in confectionary products for infants and adults.[2]since it doesn't cause hyperglycemia, it is recommended for diabetic patients.[3]

Debaryomyceshansenii gave a higher yield than several other selected pentose fermenting yeasts.[4] Candida mogii gave the highest yield of 0.7 g/g when compared with other yeast strains.[5]The fermentation process can be influenced also by the inoculum age. This property affects the metabolic activity and the viability of the cells.[6]

EXPERIMENTAL

Media, Microorganism and Identification

The microorganism used in this study was isolated from sugarcane collected from Sathyamangalam, Tamil Nadu India. Totally twenty four strains were isolated by primary screening from serially diluted sugarcane extract on xylose containing minimal medium. Among them, *Candida parapsilosis strain BKR1* (*KC462059*) was the high xylitol yielding yeast identified and used throughout the study. It was maintained on agar slant containing 3 g of malt extract, 3 g of yeast extract, 5 g of peptone, 10 g of xylose and 15 g of agar per litre at 37 °C for 48 hours. The modified minimal (MM)medium used contains:5g K_2HPO_4, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2.2H_2O$, 1g Yeast Extract, 20g Xylose per litre.[5]Studies on process optimization were carried out with MM medium described elsewhere.Molecular

identification of isolate was carried out based on 18S rRNA studies and the evolutionary relationship was found using phylogenetic analysis.

Fermentation Condition

Petridishes inoculated with the *Candida parapsilosis strain BKR1* were incubated at 30^0C for 48 hours. Fermentation was carried out in 250 ml Erlenmeyer flask at 35°C. MM medium was sterilized at 121°C for 20 min. After cooling the flasks to room temperature, the flasks were inoculated with grown culture broth.

Single Factorial Optimization

The yeast cultures were optimized for the following parameters: pH, Temperature and initial xylose concentration. The cultures were optimized for the pH (3 – 7), Temperature (25 - 45°C) and initial xylose concentration (2, 3, 5, 7, 10& 15 g/l).The broth samples were collected periodically to estimate biomass concentration.

Analytical Methods

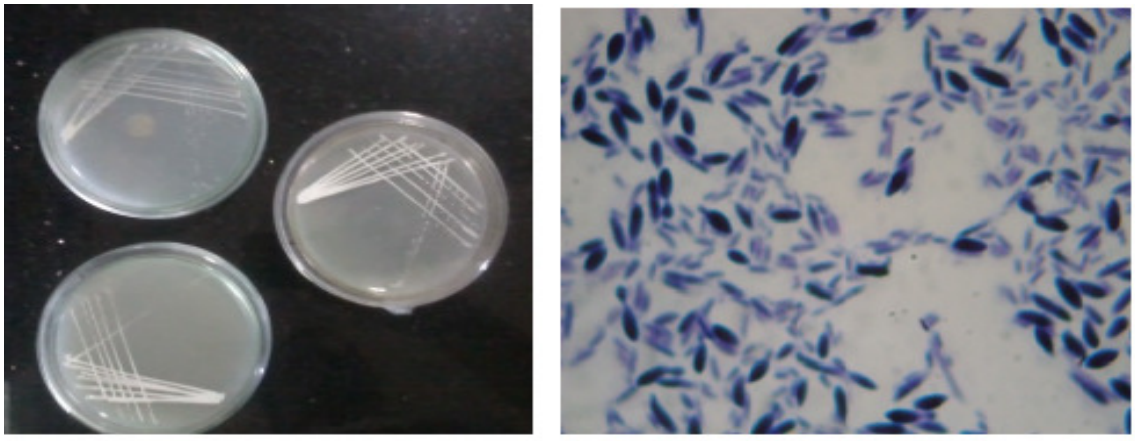
The sugars and sugar alcohols were determined by high performance liquid chromatography using an ion moderated partition chromatography column SHODEX SC (8 mm X 300 mm) 1011 sugar column (300 X 7.8 mm). Samples are eluted with deionized HPLC grade water at a flow rate of 0.5 ml/min at 80°C and detected with a differential refractometer (WATERS 410).[7]Fourier Transform IR spectroscopy was also performed for the samples in the range of 1000 – 4000 cm^{-1}.

RESULTS AND DISCUSSION

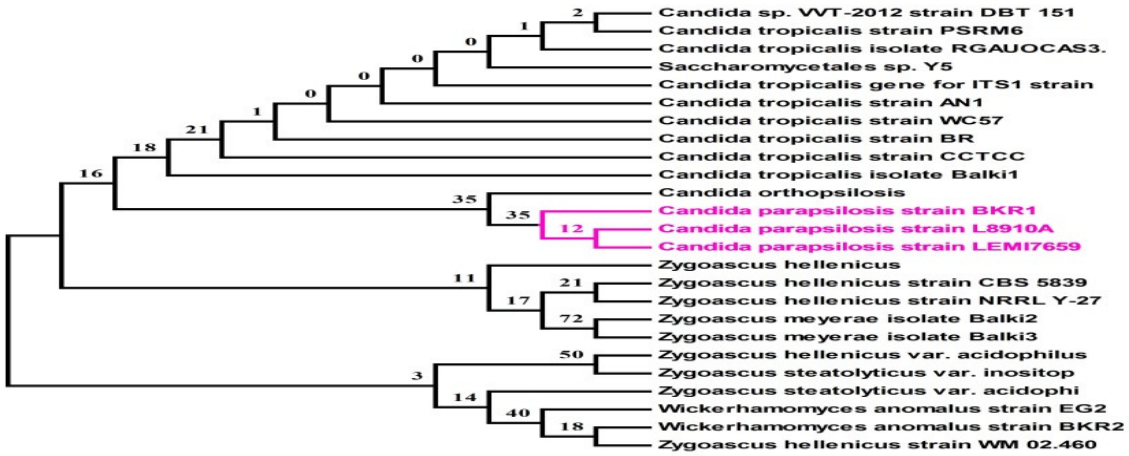
Isolation and Identification of yeasts

Among 27 strains isolated from PDA medium, five yeasts could able to survive in xylose assimilation test. (Data not shown)High xylitol yielding strain was identified using metagenomic analysis and deposited in the NCBI gene bank with accession number KC462059. It was identified and named as *Candida parapsilosis strain BKR1*based on 18s rRNA sequence similarities. The isolate was morphologically and microscopically confirmed as yeast genera (Fig.-1).

The evolutionary history was inferred using the Neighbour-Joining method[13]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[6] and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted using the MEGA 4.0 software tool (Fig.-1).



(a) (b)



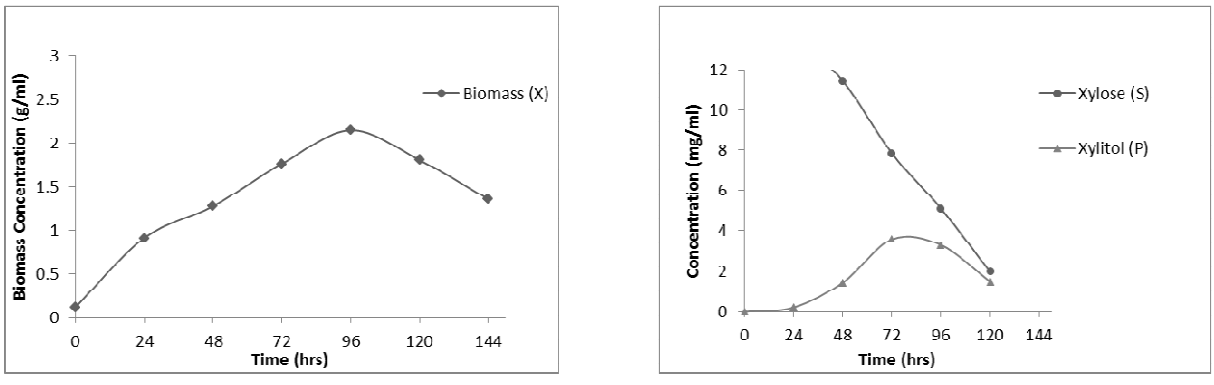
(c)

Fig.-1: (a) Morphological; (b) microscopic appearance and (c) Phylogenetic relationship of *C.parapsilosisstrain BKR1*

Xylitol Production and Optimization

The biomass absorbance was plotted against time which is shown in Fig.-2. Also the substrate concentration and product concentration was measured using HPLC. The concentration of xylose was decreased to 1.96 mg/ml from initial 20 g/lafter 120 hours. The concentration of xylitol increases from 0.2 mg/ml to 1.47 mg/ml. Thus the approximate yield of xylitolwas 0.0735 g/g in 120 hours.

To determine the optimum pH for biomass and xylitol production with *Candida parapsilosis strain BKR1*, batch cultivations were carried out in 250 ml Erlenmeyer flasks varying pH 3 to 7. The results were summarized in Fig.3, for the biomass and xylitol production in terms of yield coefficients ($Y_{p/s}$ and $Y_{x/s}$). The biomass production was slightly higher at pH 5 rather than at pH 4. But the xylitol production was higher at the pH 4. Thus, optimal pH found for the xylitol production was preferably achieved at pH 4.First the yeast consumed xylose for its cellular metabolism and maintenance (~16 hours). Later the xylose substrate might have induced xylose reductase to produce xylitol at aerobic condition (~48 hrs). Xylose consumption and conversion to xylitol by the yeast was maximal at pH 4. (Fig 3) Therefore xylitol concentration and xylose consumption rate was superior at optimal pH value. However it is interesting that Deok-Kun Oh., found the optimum pH for the xylitol production and growth of *C.parapsilosis* was pH 4.0.[8]The physiological factors play a major role in triggering xylose reductase gene.



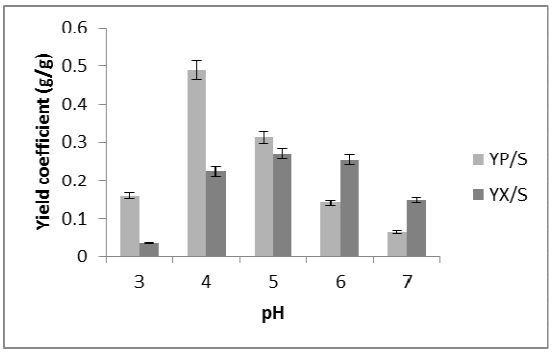
(a) (b)

Fig.-2: (a) Profiles of *Candida parapsilosis strain BKR1*growth *;* (b) Xylose depletion and Xylitol Production in the minimal modified medium containing 20g/l initial xylose concentration

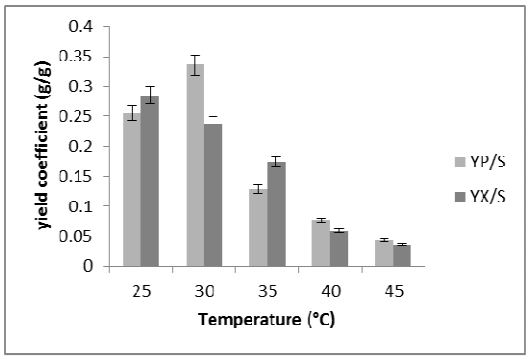
In the xylose fermentation, temperature is a critical factor and has perceptive influence on metabolic activities of yeast. However from the graph, Fig.4, it is observed that temperature above 35°C tends to reduce the xylitol productivity. The result is reassuring the invention of Deok-Kun Oh that the yield of xylitol is maximum at optimum 30ºC.[8]The suitable temperature for maximal xylitol production and growth of *C.parapsilosis* is 30°C. This is in accordance with the earlier published results of authors who worked with *Candida parapsilosis*.

It was proved experimentally that initial xylose concentration can influence the xylitol production. At the same time initial rise in the concentration of xylose enhances the oxygen level, therefore avoiding the inhibition of microbial growth. The results shown in Fig.5 is strongly supporting the findings of Ghindea et al (2010) who stated that at high substrate concentrations, a significant cellular growth takes place at the beginning of the fermentation process, and the xylitol production rate is considerably improved later for the *Candida sp.,*.[6]Thus the optimized xylose concentration was found to be0.1 g/ ml.All the experiments were carried out three times for checking consistency. Further increase in xylose concentration reduces the xylitol production and disrupts the pathway to xylulose production (datas not shown).

Oxygen represents an important factor in xylose degradation by yeasts. Xylitol is the intermediary product which was readily acted by xylitol dehydrogenase and converted to xylulose. It is significant to note that the gas transport alters the xylose uptake rate. Even slight variation in the substrate utilization triggers the xylitol dehydrogenase and converts the accumulated xylitol.[9]



(a)



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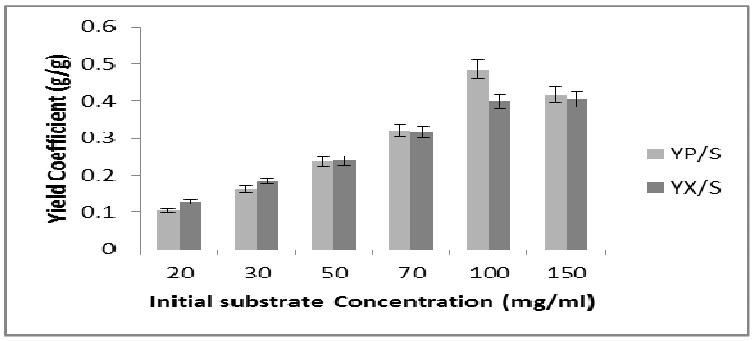


(c)

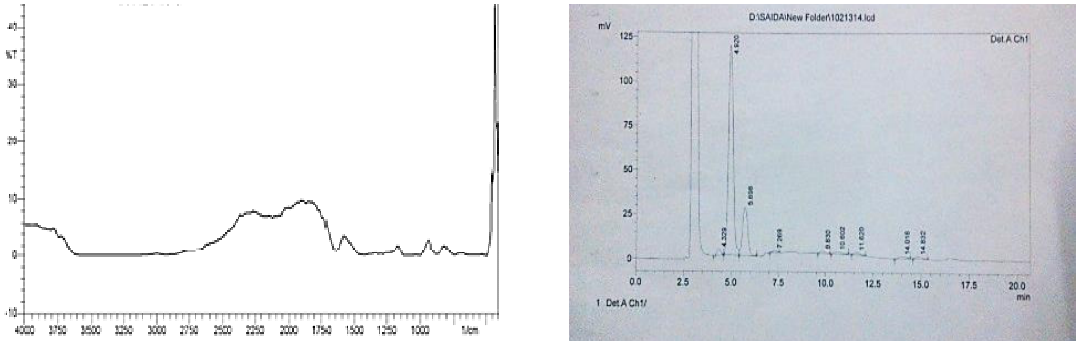
Fig.-3: (a) Effect of pH, (b) Temperature and (c) Initial xylose concentration on *candida parapsilosisstrain BKR1* growth and Xylitol production with standard error bars.

Product validation

The cell free fermentation broth were decolorized and extracted for xylitol using ethyl acetate manually. The samples were analysed in both FTIR and HPLC. The peaks obtained from the FTIR suggest that there is more C –OH; C-H and aldehyde bonds are present in the sample. This ascertains that the poly alcohol

might be present in the aqueous extract with impurities such as hemicellulose, xylose, xylulose and furfural.

The HPLC results showed the presence of xylitol based on the retention time (4.9 min) of standard pentitol procured. According to Cruz et al., the hydrolyzates purification by solvent extraction yields a phenolic-rich extract, and consequently, the hydrolyzate is discoloured.[10]The liquid-liquid extraction using ethyl acetate is a promising solvent to extract xylitol. However for commercial scale a standard separation method should be developed (Fig.-4).



(a) (b)

Fig.-4: (a) Xylitol Characterization by Fourier Transform Infrared Spectroscopy and (b) High Performance Liquid Chromatography

CONCLUSION

Candida parapsilosis strain BKR1 was isolated and it could be effectively employed in large scale processing of xylose containing lignocellulosic materials to meet the market demand for the xylitol. Conversely, modification in growth medium, optimization and downstream process is inevitable to implement pilot scale plant. Also, the xylitol yield was found moderate during the optimization studies (~0.075 g/g). Though, the previous report for *Candida sp.,* was more than the observed yield, the newly isolated strain has more potential in terms of its viability. It is noteworthy to mention that single factorial optimization will be helpful in performing the statistical optimizations in future.

ACKNOWLEDGEMENT

Authors thanks to The Principal and Management of Bannari Amman Institute of Technology, Sathyamangalam for their laboratory facility

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[RJC-1153/2014]