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Molecular Identification of Novel Yeast Isolate Associated with Xylitol Production and Its Physicochemical Parameters Optimization

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Xylitol, a natural sweetener, was produced by biotechnological methods using novel yeast which was isolated from sugarcane extracts of Sathyamangalam region, Tamilnadu, India. The yeast was identified based on its morphological, microscopical analysis and 18S rRNA molecular studies. As per the phylogenetic tree's suggestion, the yeast strain was named as *Candida tropicalis isolate Balki1*. The partial 18S rRNA sequence was submitted to NCBI Genbank and the accession number *KC415251* was assigned. Improved fermentation conditions of xylitol production were found at pH 5, Temperature 30°C & initial xylose concentration 10% (w/v) respectively. Xylose reductase was the key enzyme mediating the xylitol production. The kinetic parameters were estimated as 71.54 mM and 0.696 U/mg of protein for the enzyme xylose reductase. The study suggests that the first reported yeast isolate can be espoused for large scale xylitol production.

Key words: Xylitol, xylose reductase, *Candida tropicalis*, XR assay.

Currently in xylitol producing chemical industries, xylose-rich hydro lysate is hydrogenated at high temperature and pressure with the help of nickel as catalyst. It is relatively complex process and consumes more energy. 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. According to Hyvonen and Slotte, xylitol does not undergo Maillard reaction. Hence, it is used as a sole sweetener in the formulations of food products such as yogurts[2]. It is being used in confectionary products for infants and adults[3]. It is used solely or in combination with other sugar substitutes in the manufacture of sugarless chocolates, Chewing gums, hard caramels, licorice sweets, wafer fillings, pastilles, and other confectioneries for diabetics[4]. It is also used for preparation of jams, jellies, marmalades, desserts and relishes[5]. It is recommended for diabetic patients, as it causes only limited increase in glucose and insulin levels in blood[6]. Also xylitol finds its application in post-traumatic states or post-operative when efficient glucose utilization is inhibited, due to the induced resistance to insulin by excessively secreted stress hormones[7, 8].

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Candida yeasts are promising strains in fermenting about 40% D-xylose in 24-48 h to yield xylitol[9]. According to Gong *et al.*, out of ten yeast strains compared for xylose conversion, *C. tropicalis* HPX2 showed highest xylitol yield of 0.8 g/g of xylose[10]. The xylitol fermentation process can be influenced by the abiotic factors, initial substrate concentration and inoculum age, this affecting the metabolic activity and the viability of the cells[11]. Present investigation were undertaken to address the problem of xylitol production through biotechnological methods. It starts from the identification of novel yeast isolates to mass production of the natural sweetener for the benefit of biopharmaceutical and healthcare sectors. This first reported yeast strain can be optimized further to be espoused in large scale production.

MATERIALS AND METHODS

Microorganism and Culture Medium

The microorganism used in this study was isolated from sugarcane collected from Sathyamangalam, Tamil Nadu, India. *Candida tropicalis* isolate Balki1 (*KC415251*) was maintained on agar slant containing 3 g of yeast extract, 5 g of peptone 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[12]. Studies on process optimization and kinetic parameter estimation were carried out with MM medium described elsewhere. Initial screening was performed with standard PDA medium procured from Hi media Laboratories.

Molecular identification

Molecular identification of isolate was carried out based on 18S rRNA studies and the evolutionary relationship was found using phylogenetic analysis. 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 [14] and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted using the MEGA 4.0 software tool.

Fermentation Condition

Petridishes inoculated with the *Candida tropicalis* isolate Balki1 were incubated at 35^0C for 48 hours. MM medium was sterilized at 121°C for 20 minutes without xylose as described elsewhere[12]. After cooling the flasks to room temperature, the medium was inoculated with yeast isolates (0.5% v/v) following the aseptic addition of xylose in the medium. Fermentation was carried out in 250 ml Erlenmeyer flask at 30°C for 48 – 72 hours.

Factorial Optimization

The yeast cultures optimized for the physicochemical parameters such as pH (3 – 7), Temperature (25 - 45°C) and initial xylose concentration (2, 3, 5, 7, 10 & 12 g/100 ml) (w/v) respectively. The broth samples were collected periodically to estimate biomass concentration, xylitol concentration and xylose reductase activity.

Preparation of Crude Enzyme Extract

Cells were harvested by centrifugation at 6000 rpm, and washed in phosphate buffer (50 mM, pH 7.2) and the cell pellets were stored in refrigerator (4°C). For enzyme assays, cell extracts were thawed and disrupted by sonicator (SONICS, USA) using ultrasound waves at 4°C for 20 min. Cell homogenates were centrifuged at 10000 rpm for 15 min (4°C) and the supernatant solution was used for enzymatic assays.

Purification of Xylitol

After initial centrifugation, the cell free supernatant was filtered through 0.45µm membrane (Millipore). Further, permeate was extracted against ethyl acetate (1:3). The aqueous fraction was analysed for xylitol using High Performance Liquid Chromatography.

Enzyme assay & Kinetic parameter estimation

Xylose reductase activity was determined spectrophotometrically (Perkin Elmer ë 35) at 340 nm with a reaction mixture (600 µl) containing 350 µl 70 mM Tris HCl buffer (pH 7.2), 50 µl 1.2 mM NADPH, 50 µl 2 M xylose and 150 µl crude enzyme (modified) at room temperature[15]. One unit of XR was defined as amount of enzyme required to produce 1ìM of xylitol in one minute[16]. Specific activities were expressed as U/mg of protein based on protein determinations according to the Lowry's method using bovine serum albumin as the standard[17].

Michalies Menton kinetic parameters were calculated from Double-reciprocal plot

(Lineweaver-Burk plot). Enzyme activity was measured with various concentrations of NADPH. The experiments were performed in triplicates and average values are taken.

Analytical Methods

The sugars and sugar alcohols were determined by high performance liquid chromatography using an ion moderated partition chromatography column SHODEX SC 1011 sugar column (300 X 7.8 mm). Samples are eluted with using deionized HPLC grade water at a flow rate of 0.5 ml/min at 80°C and detected with a differential refractometer (WATERS 410)[18].

RESULTS AND DISCUSSION

Among 27 strains isolated from PDA medium, five yeasts could able to survive in xylose assimilation test. (Datas not provided). High xylitol yielding strain was isolated using HPLC and deposited in the NCBI gene bank with accession number *KC415251* after metagenomic analysis.

Identification of Microorganism

The isolate was morphologically and microscopically confirmed as yeast genera. (Fig.1a & 1b) It was named as *Candida tropicalis isolate Balki1* based on 18s rRNA sequence similarities. Phylogeny was performed using Neighbour joining method. The optimal tree with the sum of branch length = 0.65150691 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches[14]. Using Maximum composite likelihood method, all positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 287 positions in the final dataset.

Fermentation behaviour of yeast isolate

The cell absorbance was plotted against time. Also the substrate and product concentration were measured using HPLC. The concentration of xylose was decreased to 0.96 mg/ml from initial 20 g/l after 120 hours. The concentration of xylitol increases from 0.2 mg/ml to 4.25 mg/ml for 72 hours and declined to 2.19 mg/ml for 120 hours.

Single Factorial Optimization

Effect of pH on xylitol production

To determine the optimum pH for xylitol production with *C. tropicalis isolate Balki1*, batch cultivations were carried out in 250 ml Erlenmeyer flasks varying pH 4 to 7. The biomass and xylitol production in terms of yield coefficients ($Y_{p/s}$ and $Y_{x/s}$). The biomass production rate was higher at pH 6. But the xylitol yield was higher at the pH 5. Thus, optimal pH found for the xylitol production was preferably achieved at pH 5. First the yeast consumed xylose for its cellular metabolism and maintenance (~16 hours). Later the xylose might have been induced xylose reductase in presence of cofactor to produce xylitol at aerobic condition (~48 hrs). Xylose consumption and conversion to xylitol by the yeast was maximal at pH 5. Therefore, xylitol concentration and xylose consumption rate was superior at optimal pH 5. However it is interesting that Yahashi *et al.*, found the optimum

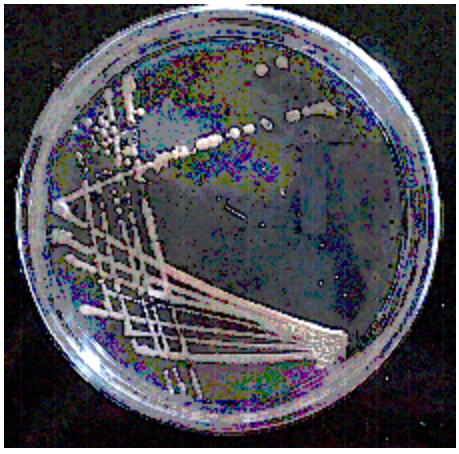
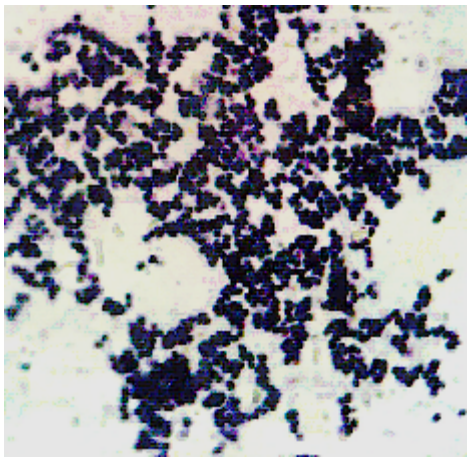
(a)  (b) 

Fig. 1. Morphological (a) and microscopic (b) appearance of *C.tropicalis* isolate balki1

pH for the xylitol production and growth of *C. tropicalis* were pH 4.0. The physiological factors play a major role in triggering xylose reductase gene[19].

Effect of Temperature on xylitol production

In the xylose fermentation, temperature is a critical factor and has astute influence on metabolic activities of yeast. However from the graph, it is observed that temperature above 35°C tends to reduce the xylitol productivity. The result is reassuring the invention of Silva and Afshar that the yield of xylitol decreases dramatically when temperature rises above 37°C[20]. The suitable temperature for maximal xylitol production and growth of *C. tropicalis* yeast is 30°C. This is in accordance with Cao *et al.*, who found that the optimum temperature for the growth of *C. tropicalis* was 28-30°C[21].

Effect of Initial Xylose Concentration in xylitol production

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 are 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 in later phase[11]. Thus the optimized xylose concentration was found to be 10 g/ 100 ml of Liquid broth. This corroborates the findings of Silva and Afschar who also emphasis that for *C. tropicalis*, the optimum xylose concentration was 100 mg/ ml[20]. Also the increase in concentration beyond this initial concentration favours the cell growth but the xylitol production capability is affected initially. This peculiar strain was of much significance in initial xylose concentration uptake as 100 mg/ml of medium. Further increase in xylose concentration reduces the xylitol production and forwards the pathway to xylulose production (datas not shown) based on HPLC results.

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 inoculum age and oxygen transport alters the substrate utilization rate. Even slight variation in the xylose uptake triggers the xylitol dehydrogenase and converts the accumulated xylitol[22].

Kinetic Parameters

The values obtained for kinetic parameters Michelis Menten constant (K_m) and maximal reaction velocity (V_m) for xylose reductase

Table 1. Enzyme activities of xylose reductase in various yeasts with D-xylose as a substrate[22]

Microorganism	XR Specific activity (U/g protein)	
	NADPH	NADH
Candida boidinii (Kloeckera sp.,) no. 2201	0.055	0.288
C.boidinii NRRL Y – 17213	0.019	0.112
C.guilliermondii NRC 5578	0.5210	0.050
C.guilliermondii NRC 5578	1.191	n.d
C.mogii ATCC 18364	0.160	0.060
C. *parapsilosis* ATCC *28474*	0.416	0.161
C. shehatae ATCC 22984	0.333	0.1
C. *shehatae* CBS 5813	0.480	0.210
C. *tropicalis* IF0 *06 18*	10.640	1.720
C. utilis CBS 621	0.075	n.d*
Debaryomyces hansenii DTIA-77	0.091	n.r*
Pachysolen tannophilus CBS 4044	0.220	0.009
P. tannophilus NRRL Y-2460	0.033	0.008
P. tannophilus U-U-27 (mutant)	0.173	0.118

*n.d. – not detected; n.r. – not reported

of *C. tropicalis isolate Balki1*, showed the following characteristics: the apparent K_m & V_m values were found to be 71.54 µM and 0.770 U/ml respectively. The specific activity of the xylose reductase was calculated to be 0.696 U/mg of protein. From the table.1, it is obvious that the *C. tropicalis isolate Balki1* deserves to be ranked as commercially significant strain due to its relatively higher specific activity for the crude extract. Except the two strains (*C. guilliermondii* NRC 5578 and C. *tropicalis* IF0 *0618)*, the isolated strain showed higher activity. The decrease in the K_m values represents the increased substrate affinity and increase in V_m reveals the highest enzyme activity. From the earlier investigations of Sirisansaneeyakul et al., the specific activity of Xylose Reductase from *Candida mogii* was found to be 0.12 U/mg of Protein[12]. It is much interesting to denote the xylitol production capability increases by enhancing specific activity of Xylose reductase. According to Cortez et al., if the enzyme molecules are partially disrupted in structure, the affinity for the substrate decreases and that of coenzyme increase[23]. Hence it is important to consider the kinetic constants might have been affected for purified enzymes. It is noteworthy to discuss the findings of Guo et al., that the *candida sp.*, isolated from them could yield a specific activity 0.59 U/mg of protein[24]. The cofactor NADPH assures better xylitol yield compared to another cofactor NADH. According to Furlan et al., the oxygen limitation leads to the increase in specific xylitol production rate possibly due to the action of xylose reductase enzyme[25]. According to Bruinenberg et al., NADPH is mainly regenerated by the enzymes glucose-6-phosphate and 6-phosphogluconate dehydrogenase in almost all xylose consuming yeast[26]. Thus oxygen limiting period allows the yeast to create imbalance in NAD^+/NADH redox system and xylose reduction is fully dependent on NADPH. Ultimately the NADH/ NADPH concentration increases, which favours the xylitol accumulation. However the present investigation only deals with NADPH cofactor.

Xylitol Purification

The cell free fermentation broth were decolorized and extracted for xylitol using ethyl acetate. The samples were analysed in HPLC. The HPLC results showed the presence of xylitol based on the retention time of standard. According to Cruz et al., & Hierro et al., the hydrolyzates purification by solvent extraction yields a phenolic-rich extract, and consequently, the hydrolyzate is discoloured [27,28]. The liquid-liquid extraction using ethyl acetate is a promising solvent to extract xylitol and it can't be employed for large scale extraction due to its techno-economic considerations. The chromatogram shows the presence of xylitol based on its retention time and quantified was found from the area under the peak.

CONCLUSION

In this study, *Candida tropicalis isolate Balki1* was isolated and identified. The gene sequence was deposited in NCBI, USA (accession number: KC415251). This organism could be effectively employed in large scale processing of xylose containing lignocellulosic materials to meet the market demand for the xylitol. However, reactor studies should be carried out to espouse unique xylitol separation strategy from fermentation broths. Conversely, modification in growth medium, optimization and downstream process is inevitable to implement pilot scale plant. Evaluation of kinetic parameters of xylose reductase revealed that the moderate affinity of the Xylose reductase enzyme towards co-substrate (~70 mM) plays a significant role in utilizing sugarcane bagasse or corn hydrolyzates. The relatively high specific activity (0.696 U/mg of protein) for Xylose reductase enzyme from *Candida tropicalis isolate Balki1 (KC415251)* strain was first reported. Also, the xylitol yield was found to be high during the optimization studies (~0.45 g/g) which could be improved during scale up studies. The findings of the present study corroborates with most of the earlier investigations which were made on the same species. *Candida tropicalis isolate Balki1,* a novel yeast strain would be of greater significance in the xylitol world market.

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