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Screening and Characterization of Flocculent Yeast, *Candida* sp. HY200, for the Production of Xylitol from D-Xylose

KANG, HEUI-YUN, YONG-SUNG KIM, GEUN-JOONG KIM[1], JIN-HO SEO[2], AND YEON-WOO RYU*

Department of Molecular Science and Technology, College of Engineering, Ajou University, Suwon 443-749, Korea
[1]Institute of Biotechnological Industry, Inha University, Incheon 402-751, Korea
[2]Department of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea

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Abstract On the basis of high osmotic tolerance and xylitol production, a novel yeast strain was screened from soils of rice farming. The isolated strain HY200 was systematically characterized by using general approaches of Biolog Microlog™ and 18S rRNA sequence analyses, and consequently was designated as *Candida tropicalis* HY200. Under formulated culture conditions, relatively high xylitol yield (77%) and productivity (2.57 g/l·h) were obtained, in practice, when 200 g/l of xylose was supplemented. In the utilization of nitrogen, inorganic compounds could not serve as nitrogen sources. As a promising phenotype, HY200 steadily flocculated during and/or after growing in the formulated medium. The extent of flocculation was partly affected by nitrogen sources. However, regardless of the kinds of carbon source fed, the flocculent cells were always observed at the end of the exponential growth phase. These observations strongly suggest that the strain HY200 could effectively be used as a potential candidate for the production of xylitol from xylose, especially in repeated batch mode, because of its flocculation ability and tolerance to high substrate concentrations.

Key words: *Candida* sp., flocculant yeast, repeated batch, osmotic tolerance, xylitol

Xylitol, a five-carbon sugar alcohol, is found in various fruits and some vegetables [30]. Because of its high sweetening power and no insulin requirement for its digestion, xylitol has recently become attractive as an alternative sweetener for the treatment of diabetics and glucose-6-phophate dehydrogenase-deficient diseases [21, 32]. It is also well known as an anticariogenic sugar alcohol, because the causing agents, *Streptococcus mutans* and *sobrinus*, are able to metabolize it as a carbon source [21]. Additionally, xylitol extends the shelf life of food by inhibiting saprophytes such as *Clostridium butyrican* and *Salmonella typhii* [4]. Therefore, xylitol is incorporated as an ingredient in various consumer products, such as chewing gum, soft drink, ice cream, and toothpaste.

Xylitol has routinely been produced by chemical reduction of xylose, mainly derived from hemicellulose hydrolysates of birch wood or other xylose-rich resources. The hemicellulosic fractions of these raw materials, however, contain various sugars, and their polymers result in inevitable problems such as the high cost of separation steps and disposal of wastewater [9]. Microbial production of xylitol, therefore, became a more attractive process, since its downstream processing is simple and its fermentation process provides high cell density, thus resulting in high xylitol yields. In these contexts, some microorganisms have been screened and evaluated as xylitol producers, and most of them have been found to belong to the genus *Candida* (*C. boidinii*, *C. guilliermondii*, *C. parapsilosis*, *C. pelliculosa*, *C. shehatae*, and *C. tropicalis*) and related strains, *Debaryomyces hansenii*, and *Pichia stipitis* [1, 3, 12, 14, 15, 18, 19, 28]. Xylitol is the main product of xylose reduction in these yeasts, although the factors that regulate the production and excretion of xylitol have not yet been clearly identified. Among them, *C. tropicalis*, *P. stipitis*, and recombinant *Saccharomyces cerevisiae* have mainly been employed in a batch or fed-batch culture process for xylitol production [13, 17, 27] and approximately 80% of high yields obtained. The key steps for the batch process are turnaround time for washing, autoclaving of fermenter, and then regrowing the cells. The loss of a portion of cells during a fed-batch process is unavoidable, thereby resulting in a fluctuation period of readjustment time in operation. To overcome these problems or improve the yield of xylitol, the use of flocculent cells or immobilization strategy have been considered as an alternative [11, 24, 31] for easy separation of cells from culture broth, which could

*Corresponding author
Phone: 82-31-219-2449; Fax: 82-31-216-8777;
E-mail: ywryu@ajou.ac.kr

also minimize the risks of contamination and washout of cells.

In order to increase the xylitol production through the process of repeated batch fermentation and simplify the downstream steps, we attempted to isolate a novel strain that had the ability of flocculation and tolerance to high xylose concentration. By using the isolated strain, repeated batch operation with a high cell mass, and easy means of cell retention and reuse, and high xylitol production and yield were achieved.

MATERIALS AND METHODS

Enzymes and Chemicals

D-Xylose and xylitol were purchased from Sigma (St. Louis, MO, U.S.A.). Restriction enzymes, *Taq* DNA polymerase, pGEM-T vector, and genomic DNA isolation kit were purchased from Promega (Madison, U.S.A.). All other reagents and chemicals used were of analytical grade.

Screening and Enrichment Media

Various soil samples were obtained from rice farming fields located in Hwasong and Suwon, Korea. Aliquots of soil samples were vigorously suspended overnight in a saline buffer or enriching media to release most of adhering cells. Suspended cells were harvested by centrifugation or directly seeded into enrichment medium (pH 6.0) containing 300 g/l xylose, 10 g/l yeast extract, and 10 g/l bacto peptone. The enriched cells were spread on solid agar plates (100 g/l xylose, 10 g/l yeast extract, 10 g/l bacto peptone, and 20 g/l agar) for further analysis. After incubation at 30°C for 24 h, morphologically distinct cells dominantly present on the plates were finally evaluated for xylitol production by using the enrichment medium at high xylose concentrations (300 g/l).

Strain Identification

From successive screening steps, a superior strain HY200 was isolated. The microorganism was first identified as one of the *Candida tropicalis* species by using the Biolog Microstation automated microbial identification system (Biolog Micrologᵀᴹ release 3.5, Hayward, CA, U.S.A.). This strain was also analyzed with the information of 18S rRNA sequencing. A set of universal primers (forward; 5'-GGA TCC TAT CTG GTT GAT CCT-3' and reverse; 5'-GTC GAC TAA TGA TCC TTC CGC-3') was used to amplify the respective 18S rRNA gene (1,785 bp) from the isolate. PCR-amplification was performed in a Thermal Cycler (Techne, England), and the reaction condition was as follows: an initial denaturation step at 96°C for 3 min, followed by 35 cycles of denaturation at 96°C for 30 sec, annealing at 56°C for 40 sec, and extension at 72°C for 1 min, with a final extension step at 72°C for 3 min. The amplified DNA fragment was loaded on 0.8% (w/v) agarose gel and then eluted. The resulting DNA fragment was subcloned into a TA cloning vector, pGEM-T, for sequence analysis.

Culture Conditions

The isolated strain *Candida tropicalis* HY200 was routinely grown and periodically transferred at 30°C to the medium containing 100 g/l xylose, 10 g/l yeast extract, 10 g/l bacto peptone, and 20 g/l agar. Seed cultures were grown at 30°C and 200 rpm in 500-ml Erlenmeyer flasks containing 100-ml of the xylose medium containing 300 g/l xylose, 10 g/l yeast extract, and 10 g/l bacto peptone. To formulate the medium for xylitol production, the main culture was carried out in 2.5-l jar fermenter (KoBioTech, Korea) with 1-l working volume. Under these conditions, cell cultures were optimized in terms of agitation, aeration, nutrient sources, temperature, and initial substrate concentration. Cell growths of all cultures were conducted at 30°C, and foams were controlled by the addition of an antifoam agent (antifoam 289, Sigma).

Analyses

Cell growth was monitored at 600 nm, using spectrophotometery (UV-1201, Shimadzu, Japan) with harvested cells after centrifugation (4,000 rpm, 5 min). Dry cell weight was also determined according to the general procedure. The culture broths were analyzed by using high performance liquid chromatography (Waters, U.S.A.). As for the analyses of xylose and xylitol, a carbohydrate analysis column (Waters, U.S.A.) equilibrated with acetonitrile and distilled water (80:20) was operated at the constant flow rate of 2 ml/min, and the eluent was recorded by using a RI detector (Waters) at room temperature.

RESULTS AND DISCUSSION

Screening and Identification of Novel Flocculent Yeast

Since the expected strains should tolerate high xylose concentration and flocculate after or during fermentation procedures, the successive screening steps of solid and solution cultures were repeated to confirm the strain selected. From various soils of rice farming fields, 10 samples were found to have a distinct cell growth in enrichment media supplemented with 300 g/l of xylose. These strains were seeded into the same media supplemented with 100 g/l of xylose and then analyzed for their flocculation ability. Depending on the flocculation observed in several flasks, culture broths were appropriately diluted and spread on the same solid plates. As a result, about 38 colonies were primarily screened, and they were further examined for their colony forming ability on solid plates at high xylose concentration (300 g/l). The strains, having a high growth rate and colony size, were compared again for their

potential of xylitol-producing activity and flocculation in solution cultures under various conditions. Finally, one strain, named HY200, which had the highest yield, was selected as a possible candidate for further analyses. The maximum cell and xylitol concentrations obtained were 10.8 g/l and 73.3 g/l, respectively, when the isolate was cultivated in the enrichment medium supplemented with 100 g/l of xylose, 10 g/l yeast extract, and 10 g/l bacto peptone (data not shown). The selected strain was routinely cultivated at a relatively low xylose concentration (100 g/l) due to relatively higher cell growth and less lag time.

The selected strain HY200 was systematically identified based on the phenotypic and genotypic characters for strain classification. This strain was immotile, cream-colored, with a slightly mycelial border, and was an aerobic microorganism. It showed both catalase- and oxidase-positive reactions. HY200 utilized glucose, fructose, maltose, mannitol, xylose as well as inositol and raffinose as carbon sources. The optimal growth temperature range was 30–34°C and it could not grow at 40°C. Comparisons of ribosomal RNA (rRNA) gene sequences offer a powerful means for estimating phylogenetic relationships. The identification of strain is available for either the large (26S) or the small (18S) rDNA subunits [16]. The 18S rRNA sequence analysis revealed a quite high homology (>99%) to the typical species of *Candida*, especially to the species of *C. tropicalis*. The level of 18S rRNA identity, therefore, together with physiological and taxonomic properties, strongly suggested that the strain HY200 is a strain of *C. tropicalis* and was thus named taxonomically as *C. tropicalis* HY200.

Although the screened strain was identified as a species of *C. tropicalis*, some properties were distinctly different from the typical *C. tropicalis* ATCC20336 that is generally employed in industrial use for practical application. The strain HY200 consumed xylose more rapidly than ATCC20336 and was tolerable to high xylose concentrations of above 300 g/l. Thus, the cell growth of HY200 was not severely inhibited at this concentration. As a promising feature, the strain HY200 distinctly flocculated when the cultures were not agitated or shaken, thereby readily settling down (Fig. 1).

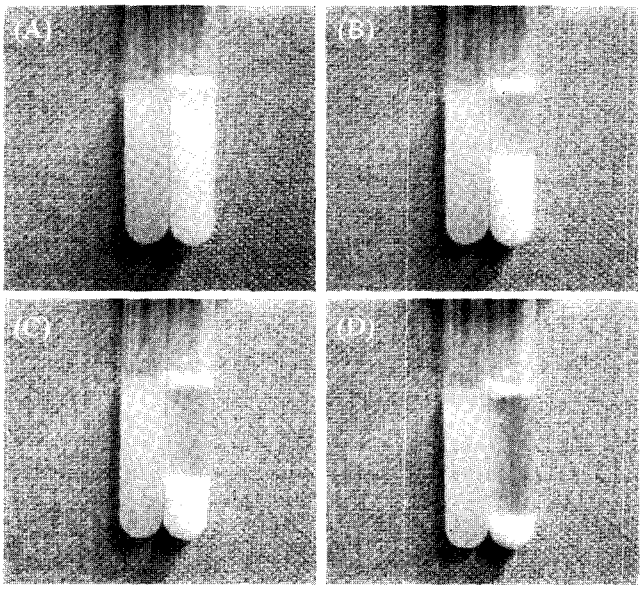

Fig. 1. Comparison of HY200 (right) with a related strain (left) for its flocculating ability.
The figures represent (A) 0 min, (B) 1 min, (C) 2 min, and (D) 3 min after stopping agitation.

Media Formulation for Xylitol Production

Since the xylitol production by *Candida* species was known to depend on the utilization of xylose and/or xylitol as carbon sources, much effort had previously been expended to reduce the xylitol utilization *in vivo* by blocking or modulating the further metabolism by the enzyme xylitol dehydrogenase. In addition, selection of an alternative carbon source for cell growth has also been considered to diminish the xylitol utilization, but still support the cell growth and cofactor regeneration. In spite of various experiments conducted, the levels of xylose utilization and xylitol productivity have not significantly been improved [7, 8, 10, 23]. This was mainly due to the fact that the cell growth and xylitol production were severely affected at high xylose concentrations in a batch or fed-batch culture. Therefore, the tolerance of cells to high xylose concentrations or use of alternative carbon sources for cell growth was considered as possible strategies for further progress.

In order to maximize the cell productivity for xylitol production in semicontinuous fed-batch culture, it was first necessary to formulate the effective medium on high cell mass, since xylitol was also produced by xylose reductase as a nongrowth-associated metabolism in grown cell, thereby requiring a high cell mass. Basically, xylose was chosen as the carbon source, because it is generally known to be the most effective carbon source for the induction of xylose reductase and inevitably xylitol production. However, various carbon sources were also tested to select suitable carbon sources for high cell density, because they were occasionally co-fed to reduce the xylose assimilation for energy and carbon source, and to support the whole cell growth when a mutation, such as defective xylitol dehydrogenase [13], was introduced. The strain HY200 was tested for its assimilation activities for 18 representative carbon sources. HY200 could metabolize fructose, glucose, maltose, and mannitol well, which revealed relatively high cell concentrations. However, the strain HY200 showed no growth on gluconic acid and lactose as carbon sources. In contrast to the results reported by some researchers [7, 8, 20, 23, 29] with other *C. tropicalis* strains, HY200 could use inositol, raffinose, and rhamnose as carbon sources. As a notable property, HY200 could not easily utilize xylitol as a sole carbon source, thus strongly

Table 1. Effect of nitrogen sources on the xylitol production of HY200.

Nitrogen sources	Concentration[a] (g/l)	DCW (g/l)	Yield (g-xylitol/g-xylose)
Yeast extract	10.0	5.2	0.74
Bacto peptone	5.7	3.8	0.42
Liquid starch waste	87.4	11.4	0.70
Corn steep powder	13.7	9.7	0.72
Soytone	10.9	3.5	0.69
Tryptone	6.9	6.8	0.67

[a]Concentration of each source was determined, based on the real content of nitrogen of yeast extract.

suggesting this strain as a superior candidate for xylitol production from xylose.

To select suitable nitrogen sources for cell growth and xylitol production, some representative nitrogen sources were also tested (Table 1). As a commercially valuable nitrogen source, liquid starch waste showed the highest cell concentration (11.5 g/l), whereas xylitol yield reached the maximum when yeast extract was used as a nitrogen source. Soytone gave a relatively low cell concentration, but resulted in no flocculation. Tryptone revealed two-fold higher cell concentration than that with peptone and also showed distinct flocculation. These observations strongly suggest that the characters of HY200 such as hyperproductivity and flocculation, could be well maintained if yeast extract or tryptone was used as a nitrogen source. The combination of yeast extract (10 g/l) and tryptone (7 g/l) also supported these characters. All the inorganic nitrogen sources tested were inappropriate for cell growth and xylitol production. These results are in agreement with the observations of Sirisansanneeyakul *et al.* [26].

The next experiment was carried out to establish the effect of initial xylose concentration on the cell growth and xylitol production (Table 2). It is generally known that high xylose concentrations lead to decreased yield of ethanol and increased yield of xylitol [2, 25, 27]. Nolleau *et al.* [22] reported that increasing initial xylose concentration reduced cell growth by substrate inhibition and/or osmotic pressure, however, Sirisansanneeyakul *et al.* [26] reported that increasing xylose concentration resulted in increasing rates of xylose uptake and xylitol formation at similar specific growth rate.

The cell growth was examined in a fermentor containing 10 g/l of yeast extract, 7 g/l of tryptone, and different initial xylose concentrations ranging from 100 to 300 g/l. Unexpectedly, the cell growth and resulting cell mass were moderately influenced by the initial xylose concentration. Higher cell concentration and productivity were obtained with an increasing xylose concentration up to 200 g/l, whereas only a minor difference in the carbon yield relative to xylitol production was observed with increasing xylose concentration. A further increase in the xylose concentration (up to 300 g/l) resulted in a drastic increase in the culture time and decrease in the cell concentration and productivity. Culture time is indicated by the complete consumption period of xylose in batch cultivation. These results are in good agreement with those reported by Gong *et al.* [7] and Ikeuchi *et al.* [10], who obtained the best yields at the initial xylose concentration of 200 g/l.

Optimization of Culture Conditions

Some researchers earlier described the effect of aeration on cell growth, xylose consumption, and xylitol production, and reported that excessive aeration resulted in a high cell mass but significantly reduced xylitol production. It is also generally known that a lower level of oxygen could limit cell growth and result in a significantly decreased xylitol production [5, 6, 25, 29], although some reported that xylitol production was maximized at microaerobic condition. In our case, a decrease of xylitol production was found at microaerobic condition (0.2% of DO level).

To optimize culture conditions, a 2.5-l jar fermenter with 1-l working volume was used to produce xylitol under various culture conditions. The rate of aeration in this fermenter, containing 100 g/l xylose, 10 g/l yeast extract, and 7 g/l tryptone, varied from 0.5- 1.5 vvm (Table 3). At an aeration rate of 1.5 vvm, the cell concentration reached the maximum of about 14.8 g/l, whereas the conversion yield of xylose to xylitol was relatively lower than those of other conditions. The specific productivity and conversion yield of xylitol were 1.15 g/l·h and 0.72 g xylitol/g xylose, respectively, at 0.5 vvm. Although these values appeared to be significant for further consideration, delayed cell growth and culture time severely limited the efficient process. Based on these observations, the optimal aeration rate was determined to be about 1.0 vvm.

Table 2. Effect of initial xylose concentration on the xylitol production of HY200.

Concentration (g/l)	Culture time (h)	DCW (g/l)	Yield (g xylitol/g xylose)	Productivity (g/l·h)
100	42	9.3	0.69	1.74
150	48	11.2	0.73	2.17
200	60	11.7	0.74	2.52
250	90	11.5	0.76	2.23
300	120	11.2	0.72	1.68

Cell cultures were carried out at 1 vvm and 300 rpm in the fermentation medium containing 10 g/l of yeast extract and 7 g/l of tryptone.

Table 3. Effect of aeration on the xylitol production of HY200.

Aeration (vvm)	Time (h)	DCW (g/l)	Yield (g xylitol/g xylose)	Productivity (g/l·h)
0.5	54	3.6	0.72	1.15
1.0	42	9.3	0.69	1.74
1.5	30	14.8	0.62	2.12

Cell cultures were carried out at the constant agitation speed of 300 rpm in the medium containing 100 g/l of xylose, 10 g/l of yeast extract, and 7 g/l of tryptone.

In addition, whether or not agitation could improve the production of cell mass and xylitol was also investigated. The experimental condition was the same as the above-mentioned, and the aeration was kept constant at 1.0 vvm. As shown in Table 4, the final cell mass and xylitol productivity were increased proportionally to the increasing agitation speed. However, the culture time and xylitol yield were sharply decreased with increasing agitation speed. This was probably due to oxygen-mediated NADH consumption that lowers the level of NADH/NAD^+, thus accelerating the further metabolism of xylitol to xylulose. The maximum xylitol yield was observed at agitation speed of 330 rpm. Specifically, in this condition, the flocculation phenotype was well revealed, and the grown cells easily settled down.

As for the final set of experiments, the possibility of cultivation temperature to improve xylitol production was tested, and the result showed that when the cultivation temperature was kept at 30°C, the maximum xylitol yield and productivity obtained were about 0.72 g xylitol/g xylose and 1.72 g/l·h, respectively. Cultivation temperatures lower than 27°C resulted in a drastic decrease in cell growth and also caused a severe morphological change to the pseudomycelium. At cultivation temperatures higher than 32°C, the rate of specific cell growth was lowered and xylitol productivity was also decreased.

The initial pH of medium was about 6.0 and steadily decreased to about 4.0. Variable initial pH exhibited a negligible effect on the xylitol production. The maximum cell growth, however, was obtained at an initial pH of 4.0.

Under optimized conditions, the cell growth and xylitol production of HY200 were not significantly inhibited, as

Table 4. Effect of agitation speed on the xylitol production of HY200.

Agitation (rpm)	Time (h)	DCW (g/l)	Yield (g xylitol/g xylose)	Productivity (g/l·h)
250	84	8.5	0.68	0.65
300	42	9.3	0.69	1.74
330	36	10.8	0.72	2.04
350	30	21.7	0.59	2.24
400	24	27.2	0.43	2.38
500	20	33.3	0.29	2.04

Cell cultures were carried out in the fermentation medium containing 100 g/l of xylose, 10 g/l of yeast extract, and 7 g/l of tryptone at 1 vvm.

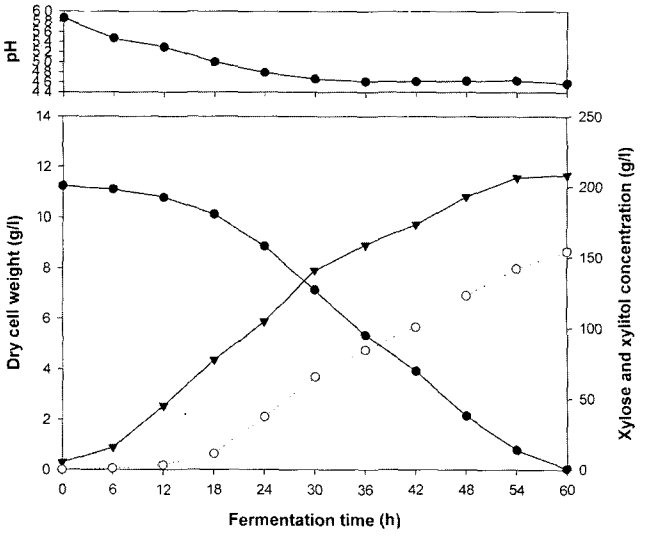

Fig. 2. Profiles of batch fermentation for xylitol production of *C. tropicalis* HY200 in the medium containing 200 g/l of xylose, 10 g/l of yeast extract, and 7 g/l of tryptone.
Fermentation was carried out at 1.0 vvm, 330 rpm, and 30°C. ▼: dry cell mass; ●: xylose; ○: xylitol.

expected (Fig. 2). Xylitol production and xylose consumption rates were linearly increased and decreased, respectively, indicating that higher xylitol concentration could be obtained when the initial xylose concentration was increased. In addition, the apparent lag phase of cell growth was not observed at this high concentration of xylose. As shown in Fig. 2, when 200 g/l xylose was fed, relatively high xylitol yield (0.77 g xylitol/g xylose) and productivity (2.57 g/l·h) were obtained.

In summary, we reported herein a novel flocculation yeast *C. tropicalis* HY200 that provides high level production of xylitol at high xylose concentration, thus indicating a possibility of application for practical use. The HY200 strain was found to belong to a species of *Candida tropicalis*; however, some phenotypes, such as flocculation, and utilization and tolerance of carbon sources, are distinctly different. Therefore, this is the first report on a novel species of *C. tropicalis* with promising properties. The members of this subspecies share almost all other phenotypes related to the entire metabolism and related activity. Thus, the strain HY200 is expected to play an important role in further research on identification of related enzymes responsible for flocculation *in vivo*. Together with the complete genome sequences available from related strains, detailed genetic studies are expected to provide some clues for this information.

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