Full Length Research Paper

The properties of *Kluyveromyces lactis* for the production of D-arabitol from lactose

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Production of D-arabitol from lactose by *Kluyveromyces lactis* NBRC 1903 was investigated to make a solution for utilization of whey. It turned out that initial concentration of yeast extract, working volume and initial cell mass concentration are important factors in D-arabitol production from lactose by this strain. It was indicated that higher aerobic condition was preferable for D-arabitol production from lactose by *K. lactis*. Highest D-arabitol concentration of 13.5 g L^{-1} was obtained at 96 h cultivation with 0.002 g L^{-1} of initial cell mass concentration, 40 g L^{-1} of initial yeast extract concentration and 2 mL of working volume.

INTRODUCTION

Pentitol D-arabitol ((2R,4R)-1,2,3,4,5–pentanepentol) is one of the 12 building block chemicals from sugars for biorefinery selected by the Department of Energy in the United States (Werpy and Petersen, 2004). Potential products from D-arabitol biorefinery include xylitol (Ohnishi and Suzuki, 1969; Mayer et al., 2002; Suzuki et al., 2002), enantiopure, immuno-suppressive glycolipid, herbicides and anti-pathogenic-disease medicines (Dubey, 2002; Urbansky et al., 2004; Murata et al., 2005). For this purpose biotechnologists have long been involved in technologies to convert D-glucose from natural resource to D-arabitol utilizing osmophilic yeast *Debaryomuces hansenii* (Ohnishi and Suzuki, 1969), *Candida famata* R28 (Ahmed, 2001), *C. parapsilosis* FERM P-18006 (Utsuka et al., 2002), *Zygosaccharomyces rouxii* (Saha et al., 2007) or *Metschnikowia reukaufii* AJ14587 (Nozaki et al., 2003).

Currently, biotechnologists face important research challenges to replace D-glucose of this process by other economically feasible raw materials. Such challenges include the search for a large amount of high quality mono- and polysaccharides in industrial effluents.

Lactose is a disaccharide abundant in the ultrafiltration (UF) permeates of whey, that is, an important aqueous fraction of milk-effluent from cheese and casein manufacturing. Approximately, 10 m^3 of cheese whey is made as a by-product from the process to manufacture one ton of cheese. Cheese whey usually contains 5 - 6% lactose and are characterized by high biological oxygen demand (BOD) indicating their high organic content. Thus, the industrial attempts to convert lactose utilizing lactose fermenting osmophilic yeast *Kluyveromyces* species have been made. Conversion of lactose to ethanol (Browne, 1941; Silveira et al., 2005; Toyoda and Ohtaguchi, 2008) has been studied from the middle of the last century. Production of glycerol (Rapin et al., 1994) and β-galactosidase (Cortes and Trujillo-Roldan, 2005) were also proposed. These conversions are technically possible but have not been economically successful. The results of our recent study showed that *K. lactis* is capable of producing D-arabitol from lactose without generating D-glucose or D-galactose in culture supernatant (Toyoda and Ohtaguchi, 2009). The attainable level of D-arabitol shown in that report was only 4.1 g L^{-1}.

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Nomenclature: c_A, D-arabitol concentration [g L^{-1}]; c_E, ethanol concentration [g L^{-1}]; c_S, lactose concentration [g L^{-1}]; t, Time [h]; V_L, working volume [mL]; X, cell mass concentration [g L^{-1}]; Y, yield coefficient of cell mass on lactose [g g^{-1}]; Y_A, yield coefficient of D-arabitol on lactose [g g^{-1}]; Y_E, yield coefficient of ethanol on lactose [g g^{-1}]; **f**, final; **max**, maximum; **0**, initial.
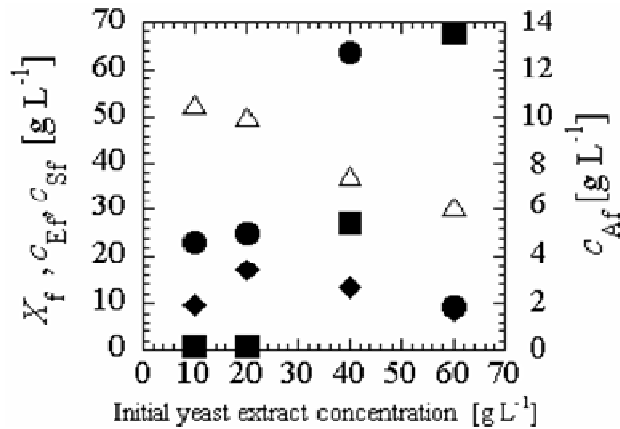
Figure 1. Effect of initial yeast extract concentration on X_f (♦), c_{Sf} (■), c_{Af} (●) and c_{Ef} (△).

Lactose utilization of *K. lactis* has been known to be accomplished by induction of both a permease for lactose transport across the cell membrane and an intracellular β-galactosidase (Sheetz and Dickson, 1981).

In an exploratory experiment, it was found that there was a marked increase in D-arabitol production in *K. lactis* by reducing inoculum size. The possibility exists that proper choice of culture conditions elevates D-arabitol production. The conversion of lactose to D-arabitol by *K. lactis* appears to be very attractive. The present research is aimed at the elucidation of effect of initial yeast extract concentration, working volume and initial cell mass concentration upon production of D-arabitol utilizing *K. lactis* NBRC 1903.

MATERIALS AND METHODS

Organisms

K. lactis NBRC 1903 was purchased from the NITE Biological Resource Center (NBRC). The strain was maintained on yeast peptone dextrose (YPD) plate (20 g L^{-1} of glucose, 10 g L^{-1} of yeast extract, 20 g L^{-1} of peptone and 20 g L^{-1} of agar) at 277 K.

Medium

The composition of the preculture medium was 9.5 g L^{-1} of lactose, 3 g L^{-1} of yeast extract, 5 g L^{-1} of $(NH_4)_2SO_4$, 2 g L^{-1} of KH_2PO_4 and 1 g L^{-1} of $MgSO_4$ $7H_2O$. The medium containing 190 g L^{-1} lactose and 40 g L^{-1} yeast extract was used for D-arabitol production as the basal medium.

Culture techniques

Inocula for batch culture were collected from 12 h-preculture in 500-mL baffled flask with 50 mL of preculture medium at 200 rpm and 303 K. Three series of batch experiments were conducted in 20-mL test tube at 303 K. Standard initial cell mass concentration and working volume were set at 0.004 g L^{-1} and 2 mL, respectively.

Shaking rate was fixed at 200 rpm. In the first series, initial yeast extract concentration was changed ranging from 10 to 60 g L^{-1}. Test tubes containing 2 mL culture were shaken at 200 rpm for 72 h. In the second series, aerobic condition was varied by shaking the 20-mL test tubes containing basal media at different working volumes (1 - 5 mL). Culture was conducted for 96 h. In the third series, initial concentration of *Kluyveromyces* was varied from 0.002 to 2 g L^{-1}.

Analytical techniques

For the analysis of the concentrations of *Kluyveromyces*, residual lactose, ethanol and D-arabitol, the sample solution was removed from the culture. The optical density at 600 nm (OD_{600}) was measured using a spectrophotometer (UV-120-02, Shimadzu Corp.) to calculate cell mass concentration (X). One unit of absorbance of *K. lactis* (NBRC 1903) was equivalent to 0.225 g of dry cell weight per liter. After centrifugation, the supernatant was filtered and the concentrations of lactose (c_S), D-arabitol (c_A) and ethanol (c_E) in filtered supernatant were analyzed by high performance liquid chromatography (HPLC) (LC-10AD, Shimadzu Corp.) and refractive index detector (RID-6A, Shimadzu Corp.). Temperature of HPLC column (SZ5532, Showa Denko K.K) was set at 333 K. The carrier was acetonitrile /H_2O (75: 25) and the flow rate was 1.0 mL min^{-1}.

RESULTS AND DISCUSSION

Effects of initial yeast extract concentration

A series of experiments in which the initial yeast extract concentration was varied is designated as Run1. This run was designed to investigate the effect of the concentration of yeast extract on D-arabitol production. Figure 1 shows the culture variables values at the end of the batch culture, X_f, c_{Af}, c_{Sf} and c_{Ef}, in Run 1. Subscript f represents final state of batch culture. The highest c_{Af} value of 12.8 g L^{-1} was obtained at the initial yeast extract concentration of 40 g L^{-1}; whereas the highest X_f value of 17.2 g L^{-1} was obtained at the initial yeast extract concentration of 20 g L^{-1}. The value of c_{Ef} decreased with increase in the initial yeast extract concentration. Change in initial yeast extract is proportional to the change of initial concentration of nitrogen source. D-Arabitol is produced from the pentose phosphate pathway (PPP). Such effects of yeast extract upon synthesis of D-arabitol are supported by the previous work which showed that the activity of glucose-6-phosphate dehydrogenase, the first enzyme of the PPP, during cultivation was affected by the ratio of the amount of D-glucose to the amount of nitrogen source (Thomas et al., 1996).

Effect of working volume

A series of experiments in which working volume (V_L) was varied is designated as Run 2. Figure 2 shows the values of X_f, c_{Af}, c_{Sf}, c_{Ef} in Run 2. Yeast extract concentration of this run was fixed at 40 g L^{-1} that was the optimal condition shown in Figure 1. Shaking rate, medium composition and inoculum size were fixed in all experiments for Run 2; hence mole of oxygen that was
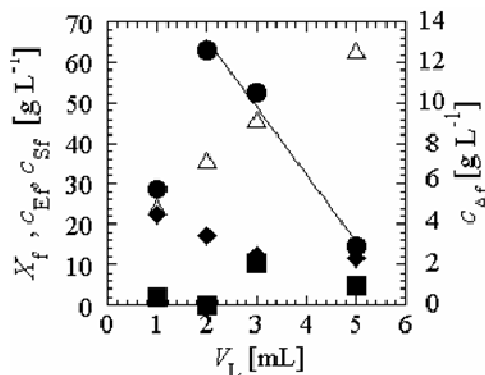
Figure 2. Effect of working volume on X_f (♦), c_{Sf} (■), c_{Af} (●) and c_{Ef} (Δ).
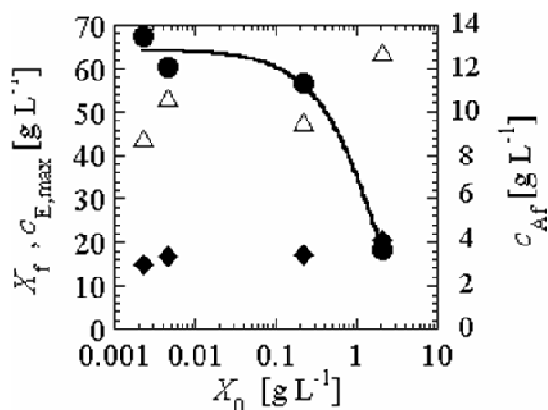
Figure 3. Effect of initial cell mass concentration on X_f (♦), c_{Af} (●) and $c_{E,max}$ (Δ).

Figure 4. Time course of culture parameters in the runs. Initial cell mass concentrations were (a) 0.002 g L^{-1} and (b) 2 g L^{-1}. ♦ = X; ■ = c_S; ● = c_A; Δ = c_E.

reported that ethanol production by *K. marxianus* was strongly affected by aerobic condition (Silveira et al., 2005). Reduction of ethanol production is important for D-arabitol production and adequate supply of oxygen is found to be a key factor for D-arabitol production.

Effect of initial cell mass concentration

A series of experiments in which initial cell mass concentration (X_0) was varied is designated as Run 3. Figure 3 shows the values of X_f, c_{Af}, $c_{E,max}$ in Run 3. Subscript max represent maximum. Decrease in X_0 resulted in a remarkable increase in c_{Af}, slight decrease in X_f and decrease in $c_{E,max}$. A curve in this graph gives the following empirical relation:

$$c_{Af} = 12.8 \exp(-0.560 \, X_0) \tag{2}$$

The highest c_{Af} value of 13.5 g L^{-1} was obtained from the batch culture with X_0 value of 0.002 g L^{-1}. Initial specific respiration rate of *K. lactis* is inversely proportional to X_0; hence Equation (2) appears to indicate the high degree of correlation between c_{Af} and the initial specific respiration rate. The highest $c_{E, max}$ value of 63.7 g·L^{-1} was obtained in the run with the X_0 value of 2 g L^{-1}.

D-Arabitol production in batch cultures

Figure 4 shows the time courses of D-arabitol production

transferred from gas phase to liquid phase per unit time appeared to be fixed. Under this condition, V_L is inversely proportional to initial volumetric oxygen transfer coefficient $k_L a$. Except for the experiment with V_L of 1 mL, final D-arabitol concentration c_{Af} increased with decrease in V_L. Linear line in this graph gives the following empirical relation:

$$c_{Af} = 19.7 - 3.32 \, V_L \tag{1}$$

The highest c_{Af} value of 12.6 g L^{-1} was obtained from batch culture with the V_L value of 2 mL. Initial specific respiration rate of *K. lactis* is proportional to $k_L a$ and hence Equation (1) appears to indicate the high degree of correlation between c_{Af} and the initial specific respiration rate. More than 90% of lactose was consumed in all experiments in Run 2. Decrease in working volume resulted in an increase in X_f value and a decrease in the value of c_{Ef}. Higher cell mass were produced at higher aerobic condition. *Kluyveromyces* is respirofermentative yeast in which respiration and ethanol production occur simultaneously (Gonzalez-Siso et al., 2000). It has been
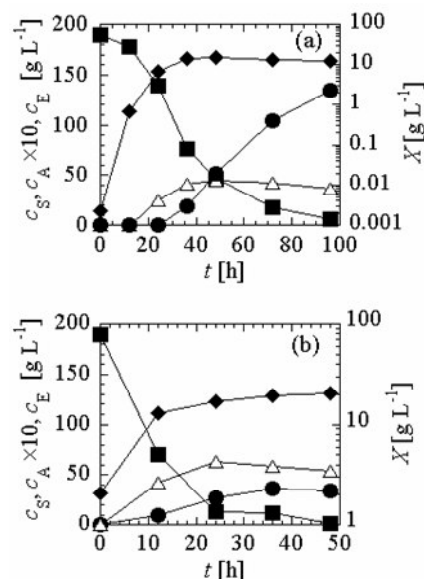
Table 1. Kinetic parameters in cultivation with different X_0.

Fractional yield	Run 4	Run 5
X_0 [g L^{-1}]	0.002	2
Y [g g^{-1}]	0.0694	0.0974
Y_A [g g^{-1}]	0.0741	0.0179
Y_E [g g^{-1}]	0.240	0.338

at X_0 value of 0.002 g L^{-1} (Run 4) and 2 g L^{-1} (Run 5). Although almost all lactose was consumed in both cultures, time required for the complete conversion of lactose in Run 5 was about a half of that in Run 4. D-Arabitol production was still active in the late-logarithmic growth phase and the stationary phase in both runs. The value of c_{Af} in the Run 4 was 4.01 times higher than that in Run 5. Ethanol production was observed right after the beginning of batch culture in Run 5 and the highest ethanol concentration of 63.7 g L^{-1} was observed at 24 h. In Run 4 the highest c_E value of 44.0 g L^{-1} was observed at 48 h. Taking into account that 1 mol of lactose is, theoretically, converted to 4 mol ethanol and 4 mol CO_2, it can be assumed that lactose assimilated for ethanol production in Run 5 is 125 g L^{-1}, while that in Run 4 is 86.1 g L^{-1}.

The overall fractional yield of D-arabitol on lactose Y_A [= $c_{Af}/(c_{S0} - c_{Sf})$], the overall fractional yield of cell mass on lactose Y [= $(X_f - X_0)/(c_{S0} - c_{Sf})$] and the overall fractional yield of ethanol on lactose Y_E [= $c_{E,max}/(c_{S0} - c_{Sf})$] were calculated for Run 4 and Run 5 (Table 1). It turned out that production of D-arabitol and ethanol were competitive in terms of lactose conversion. The relation between production of ethanol and D-arabitol affected by aerobic condition was also observed in a previous work utilizing *Hansenula polymorpha* (Escalante et al., 1990). Increase in X_0 resulted in an increase of total consumption rate of oxygen in culture. Shifting-up the specific respiration rate of *K. lactis* cells from preculture to main batch culture appears to activate the enzymes in the PPP and hence direct the metabolic flow from the production of ethanol to the production of biomass and D-arabitol. It was supported by previous report in which higher oxygen tension promoted D-arabitol production and oxygen uptake and reduced ethanol production when *Saccharomyces rouxii* was utilized for conversion of D-glucose (Spencer and Shu, 1957). Change in the respiration rate is found to be utmost important to convert lactose to D-arabitol. Elevation of initial k_La or reducing X_0 were preferable for increasing D-arabitol yield with higher selectivity.

Conclusion

We have designed this research to provide a solution for utilization of whey. Biotransformation of lactose to D-arabitol by *K. lacits* NBRC 1903 was investigated. It turned out that adequate oxygen supply is important in D-arabtiol production. Highest D-arabitol concentration of 13.5 g L^{-1} was achieved with 40 g L^{-1} of initial yeast extract concentration, 2 mL of working volume and 0.002 g L^{-1} of initial cell mass concentration, which is 3.3 times higher than that of our previous study. Further research is needed to get better understanding of D-arabitol production from lactose by *K. lactis*.

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