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ORIGINAL PAPER

Effect of temperature on D-arabitol production from lactose by *Kluyveromyces lactis*

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Abstract D-Arabitol production from lactose by *Kluyveromyces lactis* NBRC 1903 has been studied by following the time courses of concentrations of cell mass, lactose, D-arabitol, ethanol, and glycerol at different temperatures. It was found that temperature is a key factor in D-arabitol production. Within temperatures ranging from 25 to 39°C, the highest D-arabitol concentration of 99.2 mmol l^{-1} was obtained from 555 mmol l^{-1} of lactose after 120 h of batch cultivation at 37°C. The yield of D-arabitol production on cell mass growth increased drastically at temperatures higher than 35°C, and the yield reached 1.07 at 39°C. Increasing the cell mass concentration two-fold after 24 h of culture growth at 37°C, the D-arabitol concentration further increased to 168 mmol l^{-1}. According to the distribution of the metabolic products, metabolic changes related to growth phase were also discussed. The stationary-phase *K. lactis* cells in the batch culture that is started with exposing the precultured inoculum to high osmotic stress, high oxidative stress, and high heat stress are found to be preferable for D-arabitol production.

Keywords D-Arabitol · Lactose · *Kluyveromyces lactis* · Heat stress

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Introduction

Kluyveromyces lactis has been commonly used for the commercial production of β-galactosidase [1]. Lactose utilization by this strain has been known to be accomplished by the induction of both a permease for lactose transport across the cell membrane and an intracellular β-galactosidase [2]. Previous study in our laboratory has shown that *K. lactis* extracellularly produces a high-value rare pentitol D-arabitol directly from lactose [3].

D-Arabitol is a naturally occurring pentitol with a relatively low degree of sweetness and is found in body fluids [4]. Recently, D-arabitol has been used as a raw material, either for the chemical synthesis of enantiopure compounds, immunosuppressive glycolipids, herbicides, and antipathogenic-disease medicines [5–7], or for the biological production of another pentitol, xylitol [8–11]. D-Arabitol is a key compound selected as one of the 12 potent building-block chemicals for biorefinery by the Department of Energy in the United States [12]. Despite a number of studies on the production of D-arabitol from D-glucose utilizing osmophilic yeast *Zygosaccharomyces rouxii* NRRL Y-27624 [13], *Debaryomuces hansenii* [9], *Candida famata* R28 [14], *C. parapsilosis* FERM P-18006 [15], or *Metschnikowia reukaufii* AJ14587 [16], there are remarkably few reports available on the production of D-arabitol from lactose.

The mechanism by which *Kluyveromyces* regulates D-arabitol production remains to be elucidated, however, there are suggestions in the research with *C. albicans* and brewer's yeast. D-arabitol synthesis is regulated by both the high-osmolarity glycerol (HOG) multiple mitogen-activated protein (MAP) kinase pathway, and HOG-independent pathway in *C. albicans* [17]. It has also been reported that heat shock activates the HOG pathway [18].

An inexpensive and large amount of lactose is available as whey, which is an industrial effluent from cheese manufacturing. Approximately 10 m^3 of cheese whey is formed as a by-product when a ton of cheese is produced. Lactose is converted to ethanol or lactic acid in references [30]. Considering that whey is in food-quality industrial effluent, we have been pursuing studies to convert lactose to a higher-value compound D-arabitol. D-Arabitol production from lactose has the potential for industrial applications.

In the present paper, we report results of experiments which were designed to provide information on the effect of heat stress on the production of D-arabitol from lactose by *K. lactis* NBRC 1903. An additional experiment to increase the concentration of *K. lactis* NBRC 1903 under the stress response mechanism to increase D-arabitol concentration was also performed.

Experimental

Chemicals

Complex nutrients like yeast extract, peptone, and yeast nitrogen base w/o amino acids and ammonium sulfate (YNB w/o AA and AS) were obtained from Becton, Dickinson and Co., Franklin Lakes, NJ, USA.

Strain

K. lactis NBRC 1903 was provided by the Biological Resource Center of the National Institute of Technology and Evaluation (Tokyo, Japan). The strain was maintained on a YPD plate (111 mmol l^{-1} D-glucose, 10 g l^{-1} yeast extract, 20 g l^{-1} peptone, and 20 g l^{-1} agar) at 4°C.

Medium

The preculture medium for *K. lactis* NBRC 1903 contained 27.8 mmol l^{-1} lactose, 3 g l^{-1} yeast extract, 37.9 mmol l^{-1} $(NH_4)_2SO_4$, 14.7 mmol l^{-1} KH_2PO_4, and 4.06 mmol l^{-1} $MgSO_4$ $7H_2O$. The D-arabitol production medium was a synthetic lactose (SL) medium containing 555 mmol lactose, 68.4 mmol glutamine, and 3.4 g YNB w/o AA and AS per liter.

Culture techniques

K. lactis NBRC 1903 was precultured at 30°C in 50 ml of preculture medium in a 500-ml baffled flask, with shaking at 200 rpm on a rotary shaker. Inocula for batch cultures were collected at 12 h from the preculture of *K. lactis* NBRC 1903. The cells were washed once with distilled water and the measured amount of cells was suspended in the medium solution so as to fix the initial dry cell mass concentration at 0.004 g l^{-1}.

The temperature of the batch culture was varied as 25°C (run 1), 28°C (run 2), 30°C (run 3), 35°C (run 4), 37°C (run 5) and 39°C (run 6). The culture of *K. lactis* NBRC 1903 was grown in the 2 ml of SL medium in 20-ml test tubes that were shaken at 200 rpm. An additional experiment was also performed. Cells of *K. lactis* NBRC 1903 were first grown at 37°C for 24 h, and then the cells were harvested by centrifugation. The collected cells were re-suspended in 1 ml of culture supernatant to double the cell mass concentration and the cultivation was restarted (run 7).

Analytical techniques

For the analysis of the concentrations of *Kluyveromyces* cells, residual lactose (c_S), ethanol (c_E), and D-arabitol (c_A), a sample was removed from the culture. The optical density at 600 nm (OD_{600}) was measured using a spectrophotometer to calculate cell mass concentration (X). One unit of absorbance of *K. lactis* NBRC 1903 was equivalent to 0.235 g of dry cell weight per liter. The concentrations of sugars and sugar alcohols in the filtered supernatant were analyzed by high-performance liquid chromatography (HPLC) with an HPLC column (SZ5532, Showa Denko K.K, Tokyo, Japan). Column temperature was set at 60°C. The mobile phase consisted of acetonitrile and water (75:25, v/v). Ethanol concentration was analyzed by gas chromatography with Gasukuropack 54 (GL Science Inc., Tokyo, Japan).

The correlation between temperature (T) and specific growth rate (μ) was evaluated by the following equation [19–21]:

$$\mu = \frac{\beta T \exp\left(-\frac{E_1}{RT}\right)}{1 + \exp\left(\frac{\Delta S_d}{R} - \frac{\Delta H_d}{RT}\right)} \quad (1)$$

where β is a model parameter, E_1 is the activation energy of culture growth, and ΔH_d and ΔS_d are the enthalpy and entropy of deactivation, respectively. The growth rate at any observed time during the batch culture was evaluated by the following equation:

$$\frac{dX}{dt} = \mu\phi X, \quad (2)$$

where ϕ is the growth coefficient [22] and t is the time. The parameter ϕ is 1 in the logarithmic growth phase, between 0 and 1 in the late-logarithmic growth phase and 0 in the stationary phase.

If q_S, q_A, $q_{E,P}$, $q_{E,C}$, $q_{Gly,P}$, and $q_{Gly,C}$ are the specific rates for lactose consumption, D-arabitol production, ethanol production, ethanol consumption, glycerol production, and glycerol consumption, then the rates of consumption

and production of such components can be evaluated by utilizing:

$$\frac{dc_S}{dt} = -q_S X \quad (3)$$

$$\frac{dc_A}{dt} = q_A X \quad (4)$$

$$\frac{dc_E}{dt} = q_{E,P} X \quad \text{for} \quad \frac{dc_E}{dt} > 0 \quad (5)$$

$$\frac{dc_E}{dt} = -q_{E,C} X \quad \text{for} \quad \frac{dc_E}{dt} < 0 \quad (6)$$

$$\frac{dc_{Gly}}{dt} = q_{Gly,P} X \quad \text{for} \quad \frac{dc_{Gly}}{dt} > 0 \quad (7)$$

$$\frac{dc_{Gly}}{dt} = -q_{Gly,C} X \quad \text{for} \quad \frac{dc_{Gly}}{dt} < 0 \quad (8)$$

The maximum values of q_S and q_A during the batch cultivation were obtained, and related to T by the following equations:

$$q_{S,max} = k''_S \exp\left(-\frac{E_2}{RT}\right) \quad (9)$$

$$q_{A,max} = k''_A \exp\left(-\frac{E_3}{RT}\right) \quad (10)$$

where k''_S and k''_A are the frequency factors, and E_2 and E_3 are the activation energy for the lactose consumption reaction and D-arabitol production reaction, respectively.

Results and discussion

The time courses of culture of *K. lactis* NBRC 1903 were observed. The value of X_f decreased with an increase in T, if subscript f represents final state. The X_f value at 37°C was only 0.37 times that at 30°C. The cells scarcely grew and consumed lactose at 39°C, while 4.76 mmol l^{-1} of D-arabitol was produced at 96 h (data not shown). Figure 1 shows the time courses of culture variables in cultures at 25, 30, and 37°C (runs 1, 3, and 5). Conversion of lactose after 96 h was 97.4% at 30°C. Temperature shift from preculture (30°C) to main culture resulted in an increase in c_{Sf}. Conversion of lactose after 96 h was 67.7% at 37°C while that after 96 h was 75.7% at 25°C. Production of D-arabitol, glycerol, and ethanol started simultaneously at 37°C, while D-arabitol production was delayed at 25 and 30°C. The highest c_{Af} of 99.3 mmol l^{-1} was achieved at 37°C. Ethanol production continued during cultivation at temperatures other than at 37°C. Produced glycerol was consumed at a latter stage of the cultivation. The observation of glycerol consumption in this study is borne out by a previous work, which showed that the aerobic assimilation of accumulated glycerol in culture by *K. lactis* NBRC 1903 cells was due to the activity of glycerol 3-phosphate shuttle [23].

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 D-arabitol on cell mass Y_{AX} ($=c_{Af}/(X_f - X_0)$) were calculated. Subscript 0 shows the initial states of batch culture. Table 1 shows the effect of T on c_{Af}, Y_{AX}, and Y_A. The increase in T resulted in a decrease of Y_A when T increased from 25 to 30°C and an increase of Y_A when T increased from 30 to 37°C. An extremely high Y_A of 1.07 was obtained at 39°C. The lowest c_{Af} was observed at 30°C. While relatively higher c_{Af} was obtained at 25°C, Y_{AX} at 25°C was only 17.1% of that at 37°C. While there was no significant difference in Y_{AX} at T between 298 K and 30°C, it increased drastically at T higher than 35°C. The precursor of

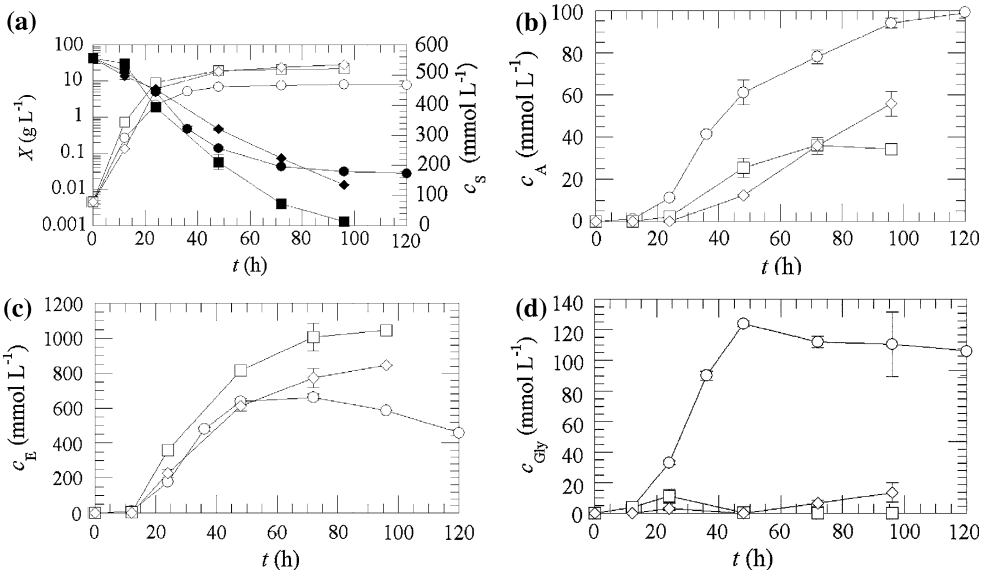

Fig. 1 Time courses of culture parameters at different T. Keys: (*filled diamond*, *open diamond*), 25°C; (*filled square*, *open square*), 30°C; (*filled circle*, *open circle*), 37°C. Concentrations of **a** cell mass; X (*open*), lactose; c_S (*closed*), **b** D-arabitol; c_A, **c** ethanol; c_E and **d** glycerol; c_{Gly}

Table 1 Relations between T and concentration of D-arabitol c_{Af} (*filled circle*), yield of D-arabitol on lactose Y_A (*open square*), yield of D-arabitol on cell mass Y_{AX} (*filled triangle*)

T (°C)	c_{Af} (mmol l^{-1})	Y_A (−)	Y_{AX} (mmol g^{-1})
25	55.9	0.133	2.00
28	42.9	0.0950	1.93
30	34.4	0.0635	1.55
35	54.0	0.128	8.06
37	94.1	0.251	11.6
39	4.76	1.07	26.0

D-arabitol from D-glucose is either D-ribulose or D-xylulose [24–26], which is a key intermediate of the pentose phosphate pathway (PPP). Cell mass and D-arabitol are the products from PPP and Y_{AX} reveals the ratio of the fluxes at D-ribulose-5-phosphate that is the branch point of carbon metabolism in PPP. This result indicates that metabolic flux was directed to D-arabitol production rather than culture growth at higher temperature. Our observation leads to an assumption that *K. lactis* NBRC 1903 responded to high temperature by extracellularly accumulating higher amount of D-arabitol. This is supported by the previous observation utilizing *C. albicans* [17].

Figure 2 shows the time courses of specific rates at 25, 30, and 37°C (runs 1, 3, and 5). The values of $q_{S,max}$, $q_{E,Pmax}$, and $q_{Gly,P,max}$ were observed around the end of the logarithmic growth phase at each run. It is of special interest that $q_{A,max}$ at 25 and 30°C were observed at 24 h, while that at 37°C was observed at 12 h. It has been reported that *K. lactis* NBRC 1903 mainly produced D-arabitol during the late-logarithmic growth phase and the stationary phase at 30°C when osmotic stress caused by lactose and oxidative stress caused by high aeration rate were loaded at the beginning of the culture [27, 28]. D-Arabitol production was elevated under either osmotic stress, oxidative stress, or heat stress at the beginning of batch growth. The stress caused by the shift in different physical parameters resulted in the different timing when D-arabitol was mainly produced and the different distributions of metabolic products. Compared to the effect of osmotic stress and oxidative stress, strong repression of culture growth due to heat stress is also characteristic. Glycerol production was enhanced under heat stress and oxidative stress, though not under osmotic stress. These results are supported by a previous work in which stress response could be divided into common environmental response and environment specific response [29].

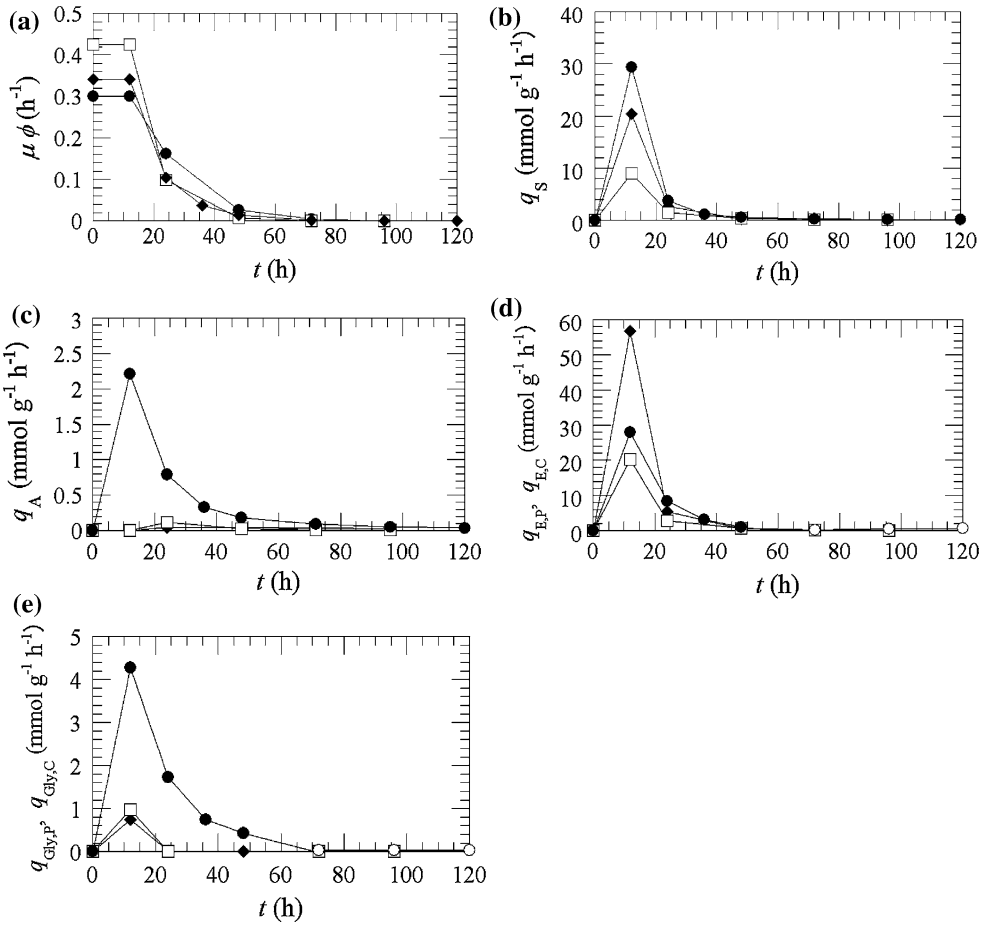

Fig. 2 Time courses of specific rates during cultivation at 25°C (*filled diamond*), 30°C (*open square*), and 37°C (*filled circle*, *open circle*). Specific rates of **a** culture growth; $\mu\phi$ (*filled diamond*, *open square*, *filled circle*), **b** lactose consumption; q_S (*filled diamond*, *open square*, *filled circle*), **c** D-arabitol production; q_A (*filled diamond*, *open square*, *filled circle*), **d** ethanol production; $q_{E,P}$ (*filled diamond*, *open square*, *filled circle*), ethanol consumption; $q_{E,C}$ (*open circle*), **e** glycerol production; $q_{Gly,P}$ (*filled diamond*, *open square*, *filled circle*), glycerol consumption; $q_{Gly,C}$ (*open circle*)

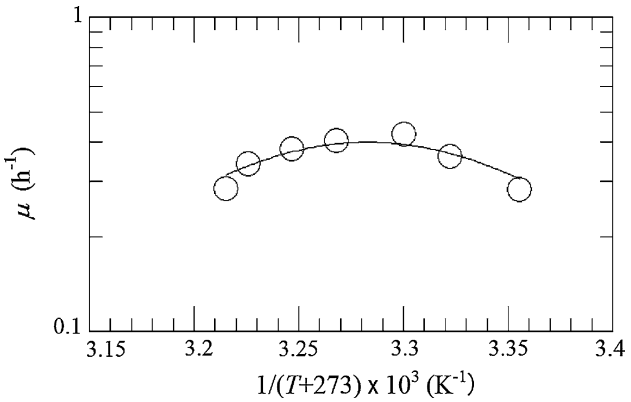

Fig. 3 Arrhenius plot of specific growth rate μ

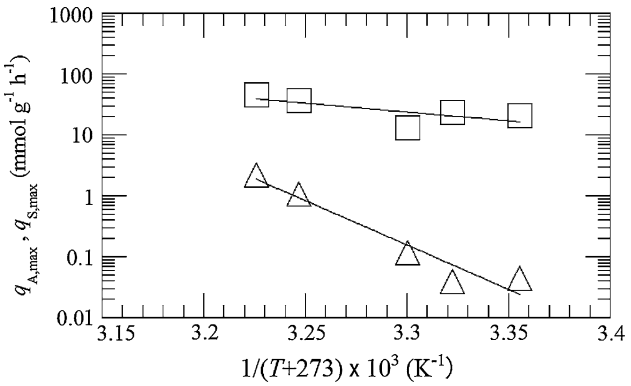

Fig. 4 Arrhenius plots of maximum specific rates of lactose consumption; $q_{S,max}$(*open square*) and of D-arabitol production; $q_{A,max}$(*open triangle*)

Figure 3 shows the Arrhenius plot for μ. The highest μ of 0.425 h^{-1} was achieved at 30°C. The values of μ at 25 and 37°C were 0.282 and 0.341 h^{-1}, respectively. The solid curve was estimated from Eq. (2) utilizing the parameter values: $E_1 = 85.2$ kJ mol^{-1}, $\Delta H = 176$ kJ mol^{-1}, $\Delta S = 0.579$ kJ mol^{-1} K^{-1} and $\beta = 1.07 \times 10^{12}$ h^{-1} K^{-1}. Arrhenius behavior is seen at T between 25 and 30°C.

The Arrhenius plots for $q_{S,max}$ and $q_{A,max}$. are also shown in Fig. 4. The solid curves were estimated from Eqs. (3) and (4) using the parameter values: $E_2 = 55.8$ kJ mol^{-1}, $E_3 = 279$ kJ mol^{-1}, $k''_S = 9.95 \times 10^{10}$ h^{-1} and $k''_A = 1.85 \times 10^{47}$ h^{-1}. The activation energy for D-arabitol production was found to be exceedingly high compared to that of culture growth and that of lactose consumption. Different from the plot of μ vs. T, the plots of $q_{A,max}$ and $q_{S,max}$ vs. T show Arrhenius behavior in wider T range from 25 to 37°C.

The instantaneous yield of D-arabitol on lactose (q_A/q_S) and that of cell mass on lactose ($\mu\phi/q_S$) were also calculated. Figure 5 exemplifies the relation between q_A/q_S and $\mu\phi/q_S$, which are replotted utilizing the data of run 5.

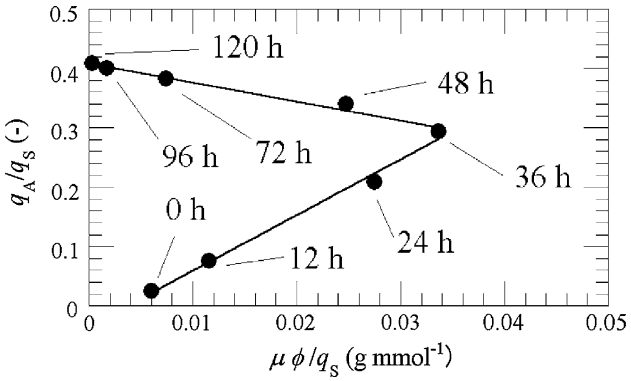

Fig. 5 Relation of q_A/q_S and $\mu\phi/q_S$

Table 2 Kinetic parameters in Eq. (11) at different T

T (°C)	25	28	30	35	37
(a) Logarithmic growth phase and late-logarithmic growth phase					
k_1 (−)	−0.0239	−0.00406	−0.144	−0.0214	−0.0343
k_2 (mmol g^{-1})	1.57	0.359	0.373	3.09	9.38
(b) Stationary phase					
k_1 (−)	0.505	0.285	0.0692	0.197	0.408
k_2 (mmol g^{-1})	−3.52	−3.14	0.0286	−1.71	−3.21

Plotted data shows that both q_A/q_S and $\mu\phi/q_S$ increased with time during the logarithmic growth phase and the late-logarithmic growth phase. A decrease in $\mu\phi/q_S$ and an increase in q_A/q_S were seen after 36 h. The data in this period shows that the metabolic flux entering PPP from lactose assimilation was directed for the production of D-arabitol rather than for the production of cell mass. The value of q_A/q_S reached the maximum value of 0.409 at 120 h, while the value of Y_A was 0.251. The plotted data until 36 h and after 36 h fit into straight lines in succession, from which the following empirical correlation can be assumed as previously described [27]:

$$q_A = k_1 q_S + k_2 \mu\phi \tag{11}$$

where k_1 and k_2 are the model parameters of Eq. (11). Linear relations between q_A/q_S and $\mu\phi/q_S$ were also observed in the time course data of runs 1–4 (data not shown). The parameters k_1 and k_2 are found to be under the influence of growth phases. Table 2 lists the values for k_1 and k_2 in terms of T and growth phases. Hence these findings appear to be characteristic of the D-arabitol synthesis from lactose by *K. lactis* NBRC 1903.

The results of batch culture experiments suggest that elevation of the concentration of *K. lactis* NBRC 1903 has promise for increasing the D-arabitol concentration by increasing the D-arabitol production rate dc_A/dt ($=q_A X$).

The use of the cells that are triggered to express the heat stress metabolism at the beginning of the batch culture and then switched to the stationary phase metabolism are more appropriate for D-arabitol production.

However, this method is accompanied by an increase in the oxygen consumption rate. As previously suggested [28], an increase in the ethanol production rate due to oxygen deficiency should be avoided by increasing the oxygen supply. Open keys in Fig. 6 show the results of the additional experiments (run 7) in which the concentration of *K. lactis* NBRC 1903 was doubled at 24 h and the oxygen supply was increased by reducing the liquid volume from 2 to 1 ml. Solid keys in this figure show the results of run 5. The amount of consumed lactose of run 7 increased 20.3%. The value of c_{Af} in run 7 was 168 mmol l^{-1}, which is 1.78 times higher than that in run 5. The time course of ethanol concentration shows that the production of ethanol increases steeply after doubling X at 24 h (runs 5 and 7), however, it was suppressed under the higher supply of oxygen (run 7).

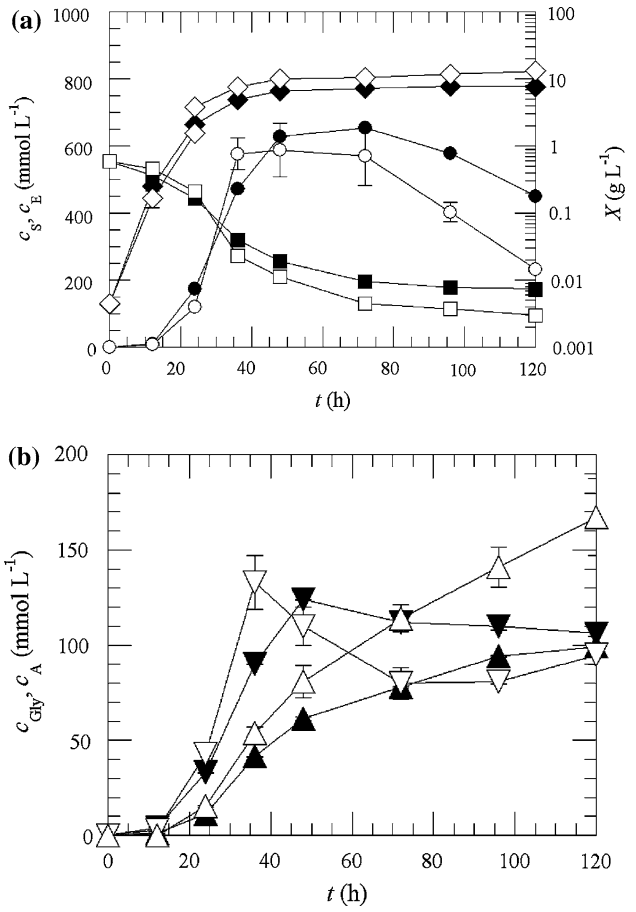

Fig. 6 The time courses of culture parameters in run 5 (*closed*) and in run 7 (*open*). Concentrations of cell mass; X(*filled diamond*, *open diamond*), lactose; c_S (*open square*, *filled square*), ethanol; c_E (*open circle*, *filled circle*), glycerol; c_{Gly} (*open downward triangle*, *filled downward triangle*), D-arabitol; c_A (*open triangle*, *filled triangle*)

The time course of glycerol concentration shows that glycerol production was also accelerated by increasing cell mass concentration although attainable level of glycerol in run 7 was comparable to that in run 5. From these observations, it would appear that final D-arabitol concentration is elevated by shifting up the D-arabitol production rate, which is achieved by increasing the cell mass concentration and by supplying a sufficient amount of oxygen.

In regard to the culture temperature, the yield of D-arabitol production on cell mass growth and that of cell mass growth on lactose consumption showed a trade-off relation. It was found that the culture at 37°C was the for D-arabitol production. It was also confirmed that increasing the cell mass concentration was effective for further increasing the D-arabitol concentration. Since the aim of this research is industrial applications, the results in this study could be of use for industrial purposes. While, the optimum timing of elevation of cell mass and elevated cell mass concentration are not defined. The time course of transcriptional analysis is also required for comprehensive understanding of the relation between the role of metabolic flux and temperature stress response of *K. lactis* NBRC 1903 in the next step of this research.

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