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Erythritol biosynthesis by *Yarrowia lipolytica* yeast under various culture conditions

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Erythritol is a natural compound of great interest in food production because of its very low caloric value, lack of off-taste and lack of side-effects from the gastric system even when consumed with in excess. In the present study, the effect of agitation rates from 500 to 1100 rev/min on biomass and erythritol production on glycerol media by an acetate-negative mutant of *Yarrowia lipolytica* Wratislavia K1 in batch culture was studied. At a constant aeration rate of 0.36 vvm, the agitation rate between 500 and 900 rev/min was found suitable for efficient erythritol production. In the pure glycerol-containing media the erythritol production of 40.7 g/l, corresponding to a 0.28 g/g yield, was achieved at 800 rev/min. The application of crude glycerol at 800 rev/min and 0.6 vvm elicited an increase in erythritol concentration to 58.2 g/l corresponding to a 0.38 g/g yield and a productivity of 0.78 g/lh.

Key words: *Yarrowia lipolytica*, erythritol, glycerol, agitation rate, aeration rate.

INTRODUCTION

Erythritol is a naturally occurring sugar alcohol with 60-70% of the sweetness of sucrose. It might be applied in the chemical, cosmetic, pharmaceutical and food industries. In food production it is used mainly as sucrose substitute. Its popularity is increasing because of its valuable properties – very low caloric value (0-0.2 kcal/g), lack of off-taste, it is noncariogenic and do not cause gastric side effects (Moon et al., 2010). Erythritol has been commercially produced from glucose, derived from chemical or enzymatic hydrolysis of wheat and corn starch, using *Aureobasidium* sp. and *Pseudozyma tsukubaensis* (Ishizuka et al., 1989). Other erythritol-producing microorganisms include osmophilic yeasts and yeast-like fungi like *Candida magnoliae*, *Moniliella tomentosa*, *Torula* sp., *Trichosporon* sp., and *Trichosporonoides* sp. (Moon et al., 2010). The feasibility of using *Yarrowia lipolytica* yeast for erythritol biosynthesis was reported in a patient of the Mitsubishi Chemical Corporation Chiyoda-ku (Ueda and Yamagishi, 1997). The patient focused on the use of glucose in the concentration of 10 – 60%, however the possibility of erythritol biosynthesis from glycerol was mentioned as well. In the shake flasks culture with 200 g/l of glycerol and *Y. lipolytica* ATCC 8661 strain, the production of erythritol accounted for 43.2 g/l, which corresponded to 0.21 g/g yield. Recently we have reported that *Y. lipolytica* are able to produce high amounts of erythritol, up to 170 g/l, in a fed-batch cultures when use was made of a medium containing crude glycerol (Rymowicz et al., 2009).

Y. lipolytica are not pathogenic yeast that occurs naturally in food – especially dairy products and meat. *Y. lipolytica* has been applied in a large variety of biotechnological processes and the industrial potential of the yeast has been already described (Groenewald et al., 2013).

In this investigation we have conducted a comparative analysis of growth, yield, and dynamics of erythritol production from pure and crude glycerol in batch cultivations at various agitation rates by an acetate-negative mutant

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Wratislavia K1 of *Y. lipolytica*.

MATERIALS AND METHODS

Microorganism

Y. lipolytica Wratislavia K1 used in this study originated from the yeast culture collection of the Department of Biotechnology and Food Microbiology, Wrocław University of Environmental and Life Sciences in Poland (Rywińska et al., 2010).

Culture preparation

The growth culture was prepared as described by Tomaszewska et al. (2012). An inoculum of 0.2 l was introduced into a bioreactor containing 1.8 l of the production medium which consisted of: 150 g of glycerol, 3 g of NH_4Cl, 1 g of $MgSO_4x7H_2O$, 0.2 g of KH_2PO_4, and 1 g of a yeast extract per liter of tap water. Unpurified crude glycerol from methyl ester production (SG BODDINS GmbH; Germany) (containing 86% wt/wt of glycerol, 6.5% wt/wt of NaCl and methanol - below 2 g/l) and pure glycerol were used as carbon and energy sources in the media. To obtain 150 g/l of glycerol in the production medium, 175 g/l of the crude glycerol was added.

The cultures were carried out in a 5-l stirred-tank reactor (Biostat B Plus, Sartorius; Germany) with a working volume of 2 g/l at 30°C and the aeration rate fixed at 0.36 or 0.6 vvm. The stirrer speed was set at various rates: 500, 600, 700, 800, 900, 1000, and 1100 rev/min. The dissolved oxygen concentration (DOC) in the fermentation broth was measured using a pO_2 electrode (Oxyferm FDA 325). The pH value was maintained automatically at 3.0 by the addition of a 20% (w/v) NaOH solution. All the cultures were cultivated until the complete consumption of glycerol. Samples were taken 2-3 times per day. All the cultures were carried out in three replications.

Analytical methods

Biomass was determined gravimetrically after drying at 105°C. The supernatant was determined for concentrations of glycerol, erythritol, mannitol, arabitol and citric acid as described earlier (Tomaszewska et al., 2012).

Calculation of fermentation parameters

The yield of erythritol production from glycerol (Y_{ER}), expressed in g/g, was calculated from: Y_{ER} = ER/S, The volumetric erythritol production rate (Q_{ER}), expressed in g/lh, was calculated as: Q_{ER} = ER/t. The specific erythritol production rate (q_{ER}), expressed in g/g h, was calculated from: $q_{ER} = Q_{ER}/X_{max}$. The maximum specific growth rate during exponential phase (μ_{max}) was calculated as: ln(dX)/(dt). In the formulas ER was the erythritol concentration in the culture liquid at the end of the cultivation (g/l); S, total amount of glycerol consumed (g/l); t, duration of the fermentation process (h); X and X_{max}, biomass and maximum biomass concentration (g/l).

Statistical analysis

The mean values and standard deviations were calculated from the data obtained in three different replications of the experiments. The analysis of the variance was performed by one-way ANOVA procedures followed by Duncan tests using Statistica version 9 (StatSoft, Inc.). Statistical differences at $p < 0.05$ were considered to be significant.

RESULTS AND DISSCUSION

The effect of agitation rate in the range of 500 - 1100 rev/min on the growth and erythritol production by *Y. lipolytica* Wratislavia K1 was investigated. The cells were grown in the media containing pure or crude glycerol, while pH was maintained at a constant value of 3.0. Table 1 shows a comparison of total time of erythritol production, biomass concentration and maximum specific growth rate in dependence on the used agitation rate value and kind of glycerol. In Figure 1 as the examples of the time courses of glycerol consumption and biomass production during batch cultivations, the cultures conducted in the media with crude glycerol at 0.36 and 0.6 vvm are presented. Glycerol was completely exhausted within 71 to 79 h (Table 1, Figure 1A). The final concentration of the biomass in the cultures with pure glycerol varied from 19.1 to 20.3 g/l (Table 1). A slightly higher concentration of biomass found in processes with crude glycerol, where reached from 21.3 to 25 g/l. The maximum specific growth rate ranged from 0.149 to 0.22 1/h depending on the culture conditions (Table 1, Figure 1B). For comparison, the value of this parameter reported for *Y. lipolytica* growth on glycerol ranged from 0.06 to 0.41 1/h (Andre et al., 2009; Kamzolova et al., 2008; Papanikolaou et al., 2008; Rywińska et al., 2010).

As shown in Table 2, when pure glycerol was applied the erythritol production was similar at the tested agitation rates, however the highest polyol concentration, 40.7 g/l, was obtained at 800 rev/min. The increased erythritol concentration was correlated with the improved yield observed during cultivation in the medium with crude glycerol (Table 2, Figure 2A). When crude substrate was applied the highest concentration of erythritol, 58.2 g/l, was achieved at 800 rev/min and 0.6 vvm (Table 2).

The DOC profile versus time and erythritol production from crude glycerol are shown in Figure 2. At the beginning of all the processes, the DOC was about 100% and decreased rapidly during the first 30 h to the value ranging from 36 to 62%, depending on the agitation rate. According to the study of Wentworth and Cooper (1996), when the nitrogen source was completely exhausted the organism's respiration rate decreased, resulting in an increasing concentration of dissolved oxygen. A constant level of DOC was observed in the stationary phase of cell growth. However, as it can be seen in Figure 2A, time courses of erythritol production were similar when the tested agitation rate was from 600 to 1000 rev/min. *Y. lipolytica* Wratislavia K1 was able to produce from 42.0 to 58.2 g/l of erythritol in a wide range of DOC (25 to 55%) (Figure 2B). In the case of citric acid biosynthesis, conducted at pH 5.5, the air saturation seemed to be the main determining factor, rather than agitation or aeration rate (Rywińska et al., 2012). The efficient conversion of

Table 1. Comparison of the total cultivation time, maximal biomass concentration and maximum specific growth rate (μ_{max}) during erythritol biosynthesis by *Y. lipolytica* Wratislavia K1 strain at various culture conditions

Agitation rate (rev/min)	Time (h)	Biomass* (g/l)	μ_{max}* (1/h)
Pure glycerol			
500[1]	77.5	19.8[ab]	0.149[f]
600[1]	73.5	18.8[a]	0.169[ac]
700[1]	74.5	19.1[a]	0.170[ac]
800[1]	78.0	19.3[ab]	0.177[ab]
900[1]	77.0	19.4[ab]	0.18[ab]
1000[1]	79.0	20.2[ab]	0.22[e]
1100[1]	75.5	20.3[ab]	0.217[e]
Crude glycerol			
600[1]	71	24.0[c]	0.152[cf]
800[1]	75.5	25.0[c]	0.191[bd]
800[2]	74.5	24.2[c]	0.21[de]
1000[1]	71.0	21.3[b]	0.197[bd]

*Letters along the same column indicate the results of the Duncans test (P<0.05); values with shared alphabet letters along the same column are not significantly different. [1]0.36 vvm; [2]0.6 vvm.

Table 2. Summary of agitation rate effects on erythritol production from glycerol by *Y. lipolytica* Wratislavia K1.

Agitation rate (rev/min)	Concentration (g/l)				Y_{ER} (g/g)	Q_{ER} (g/lh)	q_{ER} (g/gh)
	Erythritol*	Mannitol*	Arabitol	Citricacid			
Pure glycerol							
500[1]	39.5[bc]	11.7[ab]	7.9	1.2	0.26	0.51	0.026
600[1]	36.8[a]	9.6[ac]	6.0	2.1	0.25	0.50	0.027
700[1]	38.0[ac]	14.8[be]	5.6	1.8	0.26	0.51	0.027
800[1]	40.7[bd]	15.1[e]	2.9	2.6	0.28	0.52	0.027
900[1]	40.1[b]	12.0[abe]	3.1	0.4	0.27	0.52	0.027
1000[1]	37.0[a]	11.6[ab]	4.8	1.6	0.24	0.47	0.023
1100[1]	33.1[e]	11.8[ab]	3.2	2.1	0.21	0.44	0.022
Crude glycerol							
600[1]	42.0[d]	8.1[c]	2.1	2.7	0.27	0.59	0.025
800[1]	55.0[g]	4.3[d]	1.9	3.1	0.37	0.73	0.029
800[2]	58.2[h]	7.1[cd]	2.2	3.2	0.38	0.78	0.032
1000[1]	51.0[f]	7.4[cd]	2.3	1.9	0.34	0.72	0.034

*Letters along the same column indicate the results of the Duncans test (P<0.05); values with shared alphabet letters along the same column are not significantly different. [1]0.36 vvm; [2]0.6 vvm

glycerol to citric acid occurred when the DOC was maintained within the range of 50 - 80%, which was feasible at an agitation rate of 800 or 900 rpm and aeration rate between 0.24 to 0.6 vvm.

In our earlier investigation, we have demonstrated that NaCl addition to the medium significantly enhanced erythritol biosynthesis (Tomaszewska et al., 2012). It is worth noting that crude glycerol applied in the presented experiment originally contained NaCl which was probably the cause of the improvement of erythritol production.

The achieved results are difficult to discuss as the biosynthesis of sugar alcohols from glycerol by *Y. lipolytica* yeast is not very common and erythritol has generally been produced from glucose, sucrose or starch (Moon et al., 2010). Recently it have been mentioned that it is feasible to produce erythritol from glycerol but the reported yield of erythritol production was low (0.038 g/g) in comparison to the application of glucose (0.44 g/g) (Jeya et al., 2009). In our work, the yield of erythritol production was the highest (0.37 g/g) in the experiment

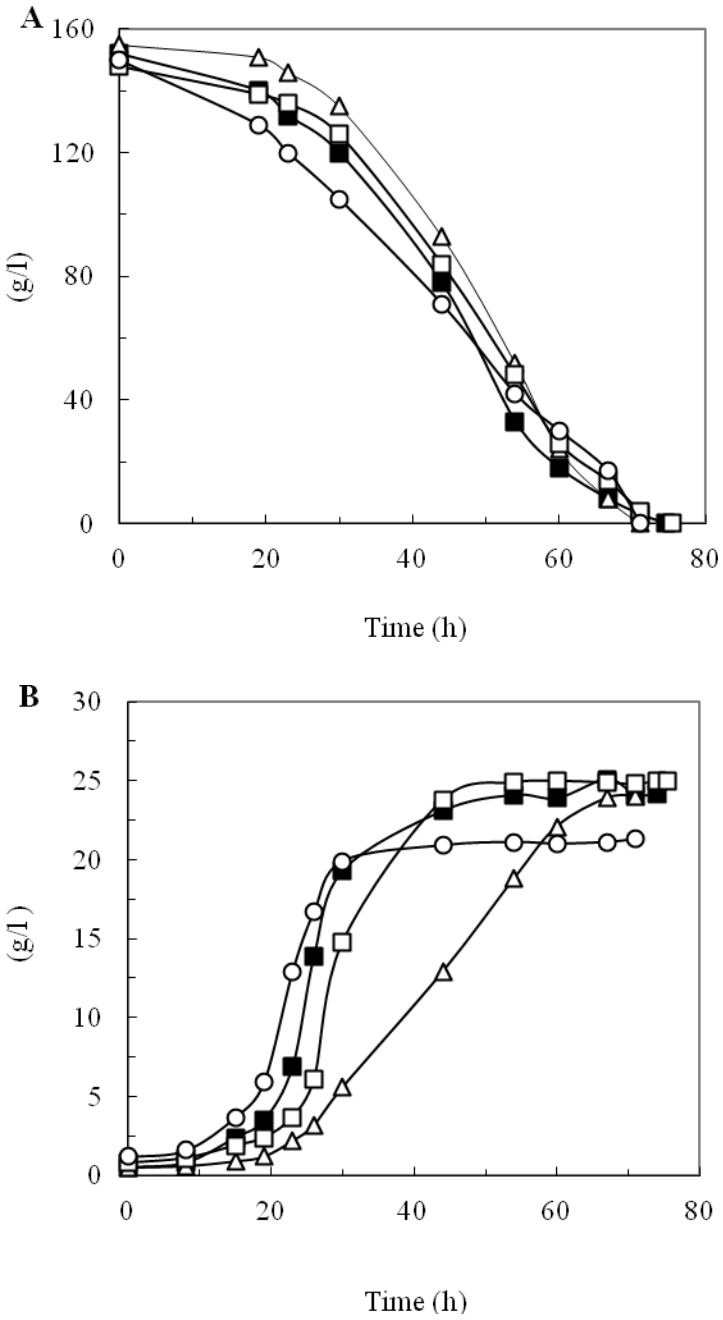

Figure 1. Glycerol consumption (A) and biomass production (B) by *Y. lipolytica* Wratislavia K1 at various agitation rates at an aeration rate of 0.36 vvm: 600 rev/min (Δ), 800 rev/min (□),1000 rev/min (○), and 800 rev/min at an aeration rate of 0.6 vvm (■). Substrate: crude glycerol.

with crude glycerol (Table 2). Similar value of this parameter was obtained by Ryu et al. (2000) in the culture with *Candida magnoliae* and glucose as a carbon source. The highest reported yield of erythritol production was obtained in the culture with *Moniliella* sp. 440 N61188-12 and reached 0.63 g/g (Lin et al., 2010). As calculated on the basis of the reaction 1 and 2, the theoretical yield for erythritol biosynthesis from glucose is 67.7%, whilst from glycerol it is only slightly lower, 66.3%:

Reaction 1: $C_6H_{12}O_6 + 3/2O_2 \rightarrow C_4H_{10}O_4 + 2CO_2 + H_2O$;
Reaction 2: $2C_3H_8O_3 + 5/2O_2 \rightarrow C_4H_{10}O_4 + 2CO_2 + 3H_2O$

The volumetric production rate of erythritol reached 0.78 g/lh when crude glycerol was applied (Table 2). The obtained erythritol productivity was significantly higher than

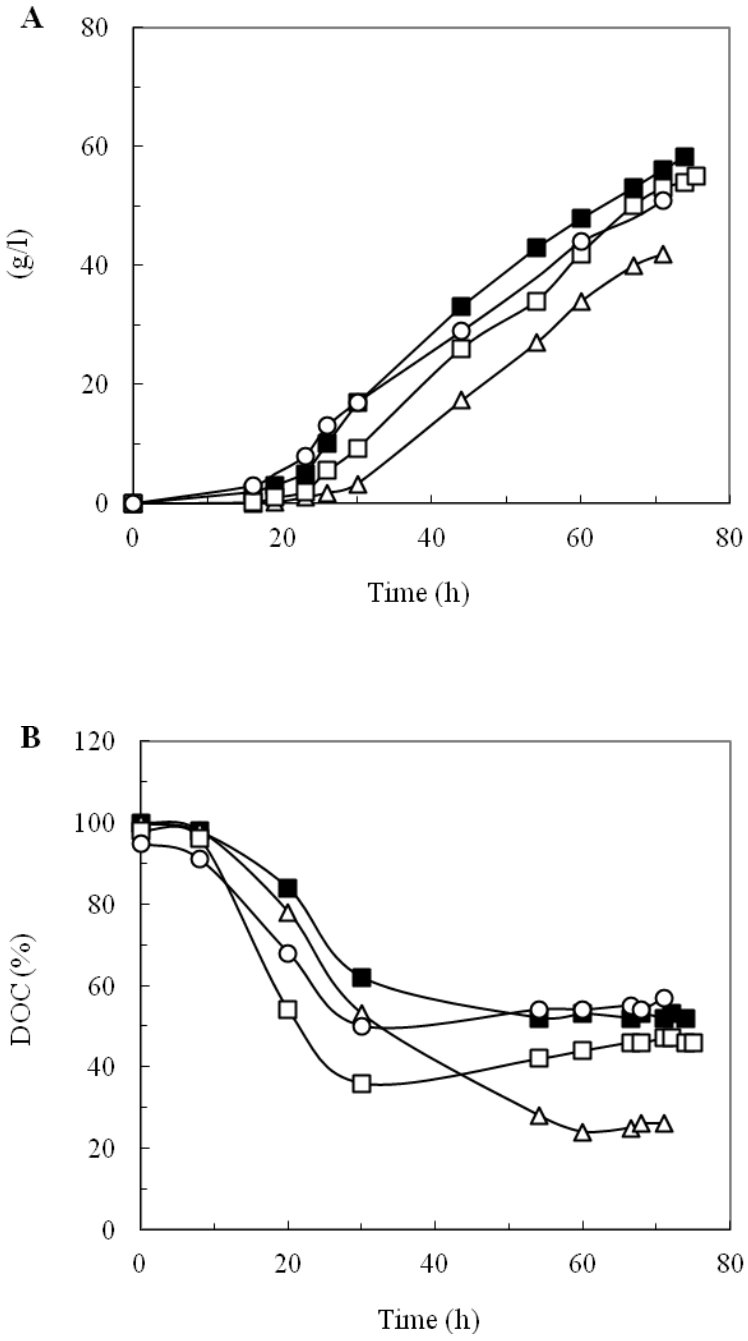

Figure 2. Erythritol production (A) and dissolved oxygen concentration (B) during batch processes with *Y. lipolytica* Wratislavia K1 strain at various agitation rates at an aeration rate of 0.36 vvm: 600 rev/min (Δ), 800 rev/min (□),1000 rev/min (○), and 800 rev/min at an aeration rate of 0.6 vvm (■). Substrate: crude glycerol.

the values achieved by Kim et al. (1997) and comparable to the results reported by Lin et al. (2001) for various strains of yeast belonging to the *Moniliella* genus. Depending on glucose concentration, which ranged from 14 to 30%, the erythritol productivity by *Trichosporon* sp. yeast reached 1.08 - 1.23 g/lh (Park et al., 1998). Higher values of this parameter were obtained in batch cultures with *Pseudozyma tsukubaensis* KN75 (1.65 g/lh) by Jeya et al. (2009) and with *Moniliella* sp. (1.98 g/lh) by Lin et al. (2010).

In the present study, the applied culture medium was characterized by a high concentration of NH_4Cl (3 g/l), therefore high biomass level and relatively low values of specific production rate were obtained. It is important to

note that the composition of the medium was adjusted to the biosynthesis of citric acid as reported in our previous investigation focused on citric acid production (Rymowicz et al., 2008; Rywińska et al., 2010), therefore it requires optimization for erythritol biosynthesis. Our recent investigation demonstrated that at lower concentration of NH_4Cl (2 g/l) similar amounts of erythritol were produced but in longer time (Tomaszewska et al., 2012).

Citric acid is a by-product occurring during the polyols biosynthesis and it is worth noting that in this study its concentration was very low (Table 2). It might be explained by application of a low pH value, which inhibits the citric acid production but stimulates the biosynthesis of sugar alcohols, as it was described earlier by Rymowicz et al. (2009). Our results indicate that in these conditions the acetate-negative mutant Wratislavia K1 of *Y. lipolytica* produced erythritol and mannitol as the predominant polyols although arabitol was also detected. Mannitol concentration depended on glycerol used. As it can be seen in Table 2, pure glycerol enabled mannitol production at a level of 9.6 to 15.1 g/l. A lower concentration of mannitol was achieved when crude glycerol was applied, which was associated with the presence of salt in this substrate (Tomaszewska et al., 2012). Literature provides sparse data on the production of mannitol by *Y. lipolytica* (Andre et al., 2009; Chatzifragkou et al., 2011). Mannitol, in the concentration of 5.1 and 6.0 g/l, was the principal extra-cellular metabolite produced in the shake-flask experiments, in nitrogen-limited culture conditions and at pH 4.5, by the strain LFMB 19 and LFMB 20, respectively (Andre et al., 2009). According to the study of Chatzifragkou et al. (2011), *Y. lipolytica* LFMB 19 strain in the nitrogen-limited media with a increased glycerol concentration produced more mannitol, up to 19.4 g/l.

Based on the present and previous results (Rymowicz et al., 2009; Tomaszewska et al. 2012), it may be concluded that *Y. lipolytica* represents a high potential for the industrial production of erythritol. Low pH, simple cultivation medium and possibility of using crude glycerol are the main advantages of this process. The erythritol biosynthesis by Wratislavia K1 strain was accompanied by relatively low amount of by-products, such as other sugar alcohols and organic acids. Moreover, this investigation showed that high amounts of erythritol might be produced in a wide spectrum of agitation rate and dissolved oxygen concentration.

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