Peptone as a nitrogen source for erythritol production from glycerol by *Yarrowia lipolytica* yeast

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Erythritol is a compound widely distributed in nature. It found application in medicine, cosmetics, chemical and food industry. It has 60–80% sweetness in comparison to sucrose, very low energy value (~0.2 kcal/g), is non-cariogenic and free of gastric side-effects. This sugar alcohol is commercially produced in microbiological processes using glucose and sucrose. Glycerol, which is produced in large amounts by biodiesel industry, can be used as alternative substrate for the production of erythritol by Yarrowia lipolytica yeast.

The aim of the study was to examine the impact of peptone on erythritol production from glycerol by Wratislavia K1 strain of *Y. lipolytica*.

In the 10-days shake-flasks experiment the peptone concentration of 1–12 g/L were examined. Pure glycerol (98% wt/wt) was used as carbon and energy source. The media were supplemented with 2.5% and 5% of NaCl.

The results showed that peptone could be used as nitrogen source in erythritol biosynthesis from glycerol by *Y. lipolytica* yeast. The best results were achieved with 2 g/L of peptone and 5% of NaCl, where yeast produced 18.2 g/L of erythritol, corresponding to 0.23 g/g yield, 0.11 g/(Lh) volumetric productivity and specific production rate of 0.010 g/(gh). In this conditions minimal level of of by-products was formed — arabitol production was not observed while mannitol, citric acids and α-ketoglutaric acid did not exceed 0.4, 4.4 and 2.0 g/L, respectively.

Keywords and phrases: erythritol, glycerol, *Yarrowia lipolytica*, peptone.

Introduction

Erythritol is a four-carbon sugar alcohol that naturally occurs in human diet. It is found in honey, fruits, mushrooms, seaweed, fermented beverages and food. It is applied in chemical industry, cosmetics, medicine and food industry. In food production it is usually added as sucrose replacer but also as flavor enhancer, humectants, stabilizer or sequestrant [1, 2]. Growing interest in this compound is caused by its specific properties — very low caloric value (~0.2 kcal/g) in comparison to sucrose (4 kcal/g) or other polyols (2.4 kcal/g) and it is non-cariogenic. It has moderate sweetness (60–80% in comparison to sucrose) but in contrast to other sugar alcohols, which are currently used as sweeteners, it is free of gastric side-effects in regular consumption and its use in food is not restricted [3–5].

Due to the problems associated with chemical synthesis of eryhtritol, its production is performed by microbiological biosynthesis. Many organisms, such as yeast of the genera: *Candida, Moniliella, Pichia, Torula, Torulopsis, Trichosporon, Trigonopsis, Zygopichia* and also the bacteria species of *Leuconostoc oenos* are known to produce this sugar alcohol. However, because of selectivity of the process, erythritol is industrially produced using *Aureobasidium* on glucose or sucrose media [6–14].

Erythritol, like other sugar alcohols, can be produced by osmophilic yeast-like fungi in response to increased osmotic pressure of the environment [14, 15]. Moreover, more effective biosynthesis have been obtained by species of relatively high tolerance for a high concentrations of salts or sugars [16]. In the recent investigation glycerol, both pure and crude derived from biodiesel production, was reported as the substrate suitable for effective erythritol biosynthesis by *Yarrowia lipolytica* yeast [17--19], the genus known of the ability to grow in the media with even 12% of NaCl [20].

The aim of this study was to examine the impact of peptone on erythritol production from glycerol by Wratislavia K1 strain of *Y. lipolytica*, in media containing NaCl.

Materials and methods

Microorganism

An acetate-negative mutant strain *Yarrowia lipolytica* Wratislavia K1 was from the collection belonging to the Department of Biotechnology and Food Microbiology at Wrocław University of Environmental and Life Sciences, Wrocław, Poland. The yeast strain was maintained on YM slant at 4°C.

Substrate

Pure glycerol (98% wt/wt) derived from POCH Gliwice; Poland was used as carbon and energy source.

Media

The growth medium consisted of: pure glycerol: 50.0 g; yeast extract 3.0 g, malt extract 3.0 g, bactopeptone 5.0 g, distilled water to 1 L. The production medium for the shake-flasks experiment contained: pure glycerol 100 g, $MgSO_4$ x $7H_2O$ 1.0 g, yeast extract 1.0 g, KH_2PO_4 0.2 g, $CaCO_3$ 3.0 g, NaCl 25.0 or 50.0 g, peptone 0–12.0 g, distilled water to 1 L, pH 3.0.

Culture conditions

The growth cultivation was conducted in 0.3-L flasks containing 0.05 L of growth medium on a rotary shaker (CERTOMAT IS; Sartorius Stedim Biotech GmbH) at 29.5°C and 140 rpm for 72 h. The production media were inoculated with 1 mL of the culture from growth medium. The shake-flasks experiment run for 10 days in 0.3-L flasks containing 0.03 L of production medium under the same conditions as described above. The cultures were performed in three replications. The samples for the analysis were taken at the end of the experiment.

Analytical methods

The samples were centrifuged for 10 min at 4°C and 5500 rpm. The biomass was determined gravimetrically after drying in a dryer at 105°C. In the supernatants the concentrations of glycerol, erythritol, mannitol, arabitol, ketoglutaric and citric acids were analyzed by the HPLC method using the Carbohydrate H^+ Column (Thermo Scientific) coupled to a UV (λ = 210 nm) and RI detector (Shodex). The column was eluted with 25 mM trifluoroacetic acid (TFA) at 65°C and at flow rate of 0.6 mL/min.

Results and discussion

In the shake-flasks experiment peptone, in the concentration from 0 to 12 g/L, was examined as a nitrogen source for erythritol biosynthesis by *Yarrowia lipolytica* Wratislavia K1. It was demonstrated that NaCl addition to the media enhanced erythritol production by *Yarrowia lipolytica* when grown on glycerol media [21]. Hence, peptone was applied in the glycerol media supplemented with 2.5% and 5% of NaCl.

The pH of the media after 10 days of the experiment ranged from 2.9–3.3 and 3.3–4.0 in media with 2.5% and 5% of the salt, respectively. It should be mentioned

Table 1. The effect of peptone on biomass, polyols and organic acids production by *Y. lipolytica* Wratislavia K1 in glycerol containing media supplemented with 2,5% and 5% of NaCl.

Peptone [g/L]	X [g/L]	ERY [g/L]	MAN [g/L]	ARA [g/L]	CA [g/L]	KA [g/L]
2.5% of NaCl						
0	4.3	13.0	1.0	0.0	9.9	0.2
2	8.8	14.0	1.1	0.1	4.3	1.2
4	9.0	10.2	0.8	0.0	1.6	2.4
6	9.1	8.9	0.6	0.2	1.1	2.8
8	9.0	11.7	0.6	0.4	0.8	3.2
10	9.1	11.3	0.6	0.1	0.6	3.7
12	9.6	12.1	0.6	0.2	0.5	3.9
5% of NaCl						
0	4.5	11.5	1.1	0.1	11.0	0.0
2	10.5	18.2	0.4	0.0	4.4	2.0
4	10.3	12.0	0.4	0.0	1.5	2.4
6	10.4	9.5	0.2	0.2	1.0	1.9
8	11.0	8.3	0.2	0.0	0.9	1.4
10	9.9	9.1	0.2	0.0	0.0	1.4
12	9.2	7.9	0.0	0.0	0.0	1.3

X — biomass, ERY — erythritol, MAN — mannitol, ARA — arabitol, CA — citric acid, KA — α-ketoglutaric acid.

Table 2. Fermentation parameters of erythritol production by *Y. lipolytica* Wratislavia K1 in dependance on pepton and NaCl concentration

Peptone [g/L]	Y [g/g]	Q [g/(Lh)] **2,5% of NaCl**	q [g/(gh)]	Y [g/g]	Q [g/(Lh)] **5% of NaCl**	q **[g/(gh)]**
0	0.20	0.08	0.018	0.20	0.07	0.015
2	0.20	0.08	0.010	0.23	0.11	0.010
4	0.16	0.06	0.007	0.18	0.07	0.007
6	0.15	0.05	0.006	0.15	0.06	0.005
8	0.18	0.07	0.008	0.14	0.05	0.005
10	0.17	0.07	0.007	0.16	0.05	0.005
12	0.17	0.07	0.007	0.13	0.05	0.005

Y — erythritol production yield (g of produced erythritol / g of glycerol consumed), Q — erythritol volumetric productivity, q — specific production rate of erythritol.

that in the cultures with *Y. lipolytica* pH values above pH 5.5 of the glycerol media favors citric acids production, while lower pH values (pH 3.0) enhance erythritol formation [18]. Therefore, in this study initial pH of the production media was set up at pH 3.0. As presented in Table 1, irrespective of NaCl, biomass concentration was the lowest in cultures without peptone presence in the media (~4.4 g/L). In other cultures biomass ranged from 8.8 to 11.1 g/L and only slightly higher concentrations were observed when 5% of NaCl was applied. The biomass level was comparable to results obtained in other study (6.1–10.9 g/L) during biosynthesis of erythritol on glycerol using different strains of *Y. lipolytica* [19].

In all the cultures yeast were able to produce erythritol, however its amount depended on peptone and salt concentrations in the media and ranged between 7.9 and 18.2 g/L (Table 1). In this study in media with 5% addition of NaCl generally more erythritol was produced than in media with 2.5% of the salt, which confirmed that high salt concentration had positive effect on erythritol formation by Wratislavia K1 strain. The best results were achieved when 2 g/L of peptone and 5% of NaCl were applied. In this conditions yeast were able to produce 18.2 g/L of erythritol, corresponding to 0.23 g/g yield, 0.11 g/(Lh) volumetric productivity and specific production rate of 0.010 g/(gh) (Table 1 and 2). Although lower amounts of erythritol were produced in the media without peptone, higher values of specific production rate were achieved (0.015–0.018 g/(gh)), which is explained by very low biomass level observed in these cultures.

When NH_4Cl (2 g/L) was used in the glycerol-media as the nitrogen source for strain A-10 of *Y. lipolytica*, yeast produced 33.6 g/L of erythritol with the 0.45 g/g yield [19]. Glycerol was mentioned as the substrate for erythrtiol production in a patent of the Mitsubishi Chemical Corporation Chiyoda-ku [22]. In the shake--flask culture with 200 g/L of glycerol *Y. lipolytica* ATCC8661 produced 43.2 g/L of erythritol corresponding to yield of 0.21 g/g, thus the yield was comparable to the results presented in this study. In comparison to presented work lower concentration of erythritol (11.3 g/L) was achieved when glycerol was used as a carbon source for biosynthesis of erythritol with *Pseudozyma tsukubaensis* [23]. However, the same yeast were able to produce 134 g/L of erythritol when glucose media were applied. In the study performed by Hajny et al. [7] on erythritol production from glucose by *Torula* sp. application of 5 g/L of yeast extract resulted in erythritol production yield 0.39 g/g. Similar value of this parameter (0.36 g/g) was observed using 12 g/L of corn steep liquor, however production yield decreased to 0.21 g/g when concentration of the nitrogen source in the media reached 25 g/L. The impact of yeast extract and corn steep liquor on erythritol biosynthesis was investigated also with *Moniliella* sp. [24]. On 30% glucose-media erythritol was produced with the yield of 0.32 and 0.27 g/g when 10 g/L of yeast extract and 60 g/L of corn steep liquor were used, respectively.

Apart from erythritol during biosynthesis by-product such as organic acids and other polyols are produced. In the culture broth sugar alcohols like mannitol, arabitol and rybitol could be found when using yeast of the genera e.g. *Debaryomyces, Hansenula, Pichia, Zaygosaccharomyces* [25]. In this study production of unwanted polyols was minimal. Arabitol was produced only in some cultures in amounts of about 0.1–0.4 g/L (Table 1). The level of mannitol was insignificantly higher and its concentrations did not exceed 1.1 g/L. Low production of mannitol may be explained by the addition of NaCl to the production media (see Materials and methods) which enhanced erythritol but simultaneously inhibit mannitol formation by *Y. lipolytica* [21].

Citric acids were produced in the amounts of 0.5--11.0 g/L (Table 1). It was clearly seen that its amount decreased with increasing concentration of peptone in the medium. The results were consistent with the

literature, because it is well known that citric acid production appeared in nitrogen-limited media [26]. The other organic acid present in the culture broth was α-ketoglutaric which concentration ranged from 0.2 to 3.9 g/L.

Conclusions

The results confirmed that glycerol could be applied as suitable substrate for erythritol biosynthesis by *Y. lipolytica* Wratislavia K1. Due to the present situation in the market — enhanced biofuels production, therefore low costs of easy-available glycerol — application of this substrate as a carbon source in the biosynthesis of erythritol by *Y. lipolytica* yeast may be of great interest. Based on the presented results it may be concluded that peptone could be used as a nitrogen source for erythritol production from glycerol by *Y. lipolytica* yeast. The best results were achieved with 2 g/L of peptone and 5% of NaCl, where 18.2 g/L of erythritol was produced, corresponding to 0.23 g/g yield, 0.11 g/(Lh) volumetric productivity and specific production rate of 0.010 g/(gh). However, further studies are necessary to eliminate the by-products, such as citric and α-keto-glutaric acids, and enhance the erythritol production.

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References

[1] Bernt, W., et al. "Erythritol: a review of biological and toxicological studies". *Reg. Toxicol. Pharmacol.* 24 (1996): 191–197.

[2] Goossens, J., and H. Röper. "Erythritol: a new sweetener". *Food Sci. Technol. Today* 8 (1994): 144–149.

[3] de Cock, P., and C.-L. Bechert. "Erythritol. Functionality in noncaloric functional beverages". *Pure Appl. Chem.* 74 (2002): 1281–1289.

[4] Röper, H., and J. Goossens. "Erythritol, a new raw material for food and non-food applications". *Starch* 45(11), 1993: 400–405.

[5] Makinen, K., et al. "Comparison of erythritol and xylitol saliva stimulants in the control of dental plaque and mutants *Streptococci*". *Caries Res.* 35 (2001): 129–135.

[6] Aoki, M., G. Pastore, and Y. Park. "Microbial transformation of sucrose and glucose to erythritol". *Biotechnol. Lett.* 15 (1993): 383–388.

[7] Hajny, G., J. Smith, and J. Garver. "Erythritol production by a yeast-like fungus". *Appl. Microbiol.* 12 (1964): 240–246.

[8] Hiele, G., et al. "Metabolism of erythritol in humans: comparison with glucose and lactitol". *Nr. J. Nutr.* 69 (1993): 169–176.

[9] Kim, K.A., et al. "Optimization of culture conditions for erythritol production by *Torula* sp". *J. Microbiol. Biotechnol.* 10 (2000): 69–74.

[10] Onishi, H. "Studies on osmophilic yeasts. Isolation of a new obligate halophilic yeast and some consideration on halophilism". *Bull. Agric. Chem. Soc. Jpn.* 24 (1960): 226–230.

[11] Park, J.B., et al. "Production of erythritol in fed-batch cultures of *Trichosporon* sp". *J. Ferment. Bioeng.* 86 (1998): 577–580.

[12] Pfeifer, V., et al. " Two stage process for dialdehyde starch using electrolytic regeneration of periodic acid". *Ind. Eng. Chem. Res.* 52 (1960): 201–206.

[13] Sawada, K., et al. "Key role for transketolase activity in erythritol production by *Trichosporonoides megachiliensis* SN-G42". *J. Biosci. Bioeng.* 108 (2009): 385–390.

[14] Veiga-da-Cunha, M., H. Santos, and E. van Schaftingen. "Pathway and regulation of erythritol formation in *Leuconostoc oenos*". *J. Bacteriol.* 175 (1993): 39–41.

[15] Kayingo, G., S.G. Kilian, and B. Prior. "Conservation and release of osmolytes by yeasts during hypo-osmotic stress". *Arch. Microbiol.* 177 (2001): 29–35.

[16] Lucca, M.E., J.F.T. Spencer, and L.I.C. de Figueroa. "Glycerol and arabitol production by an intergeneric hybrid, PB2, obtained by protoplast fusion between *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*". *Appl. Microbiol. Biotechnol.* 59 (2002): 472–476.

[17] Rymowicz, W., A. Rywińska, and W. Gładkowski. „Simultaneous production of citric acid and erythritol from crude glicerol by *Yarrowia lipolytica*". *Chem. Pap.* 60 (2008): 391–394.

[18] Rymowicz, W., A. Rywińska, and M. Marcinkiewicz. "High-yield production of erythritol from raw glycerol in fed-batch cultures of *Yarrowia lipolytica*". *Biotechnol. Lett.* 31 (2009): 377–380.

[19] Tomaszewska, L., et al. „Skrining szczepów drożdży *Yarrowia lipolytica* do produkcji erytrytolu z glicerolu". *Acta Sci. Pol.* 10 (2011): 15–28 [in Polish].

[20] Andreishcheva, E.N., et al. "Adaptation to salt stress in a salt-tolerant strain of the yeast *Yarrowia lipolytica*". *Biochemistry* (Moscow) 64 (1999): 1061–1067.

[21] Tomaszewska L., and A. Rywińska. "Utilization of glycerol into polyols by *Yarrowia lipolytica* yeasts". *Curr. Opin. Biotechnol.* 22 (2011): 94.

[22] Ueda, M., and K. Yamagishi. "Method for producing erythritol". *European Patent Application EP0770683* (1997).

[23] Jeya, M., et al. „Isolation of a novel high erythritol--producing *Pseudozyma tsukubaensis* and scale-up of erythritol fermentation to industrial level". *Appl Microbiol. Biotechnol.* 83 (2009): 225–231.

[24] Lin, S.J., et al. "Screening and production of erythritol by newly isolated osmophilic yeast-like fungi". *Proc. Biochem.* 36 (2001): 1249–1258.

[25] Moon, H.J., et al. "Biotechnological production of erythritol and its applications". *Appl. Microbiol. Biotechnol.* 86 (2010): 1017–1025.

[26] Papanikolaou, S., et al. "*Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol". *J. Appl. Microbiol.* 92 (2002): 737–744.