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Optimization of Erythritol Fermentation by High-Throughput Screening Assays

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In this research, the erythritol-producing ability of three *Yarrowia lipolytica* strains was investigated. The focus of our research was to achieve the highest possible erythritol concentration by examining and optimizing the cultivation conditions of erythritol fermentation. The complex utilization of the produced fermentation broth was also sought, e.g. the ergosterol extraction of yeast cells and isolation of a biodetergent from the foam formed during erythritol fermentation. Erythritol is a naturally occurring, widespread sugar alcohol that is gaining popularity, moreover, due to increases in usage, its demand among consumers is rising, which is why the importance of its biological production is becoming all the more critical in the food industry. Erythritol is 60-70% as sweet as sucrose and a low-calorie sweetener. Some microorganisms are capable of producing erythritol from glucose, meanwhile *Yarrowia lipolytica* strains have been reported as glycerol-consuming erythritol producers. These two sources of carbon were compared despite being subjected to further conditions like the initial pH, nitrogen sources and some additives. The largest production of erythritol was achieved by *Y. divulgata* resulting in 44g/L on glycerol compared to only 2g/L on glucose. The best supplementation was found to include ammonium nitrate and sodium citrate resulting in a product yield of 33%.

Keywords: erythritol, fermentation, *Yarrowia,* glycerol, glucose

1. Introduction

Due to the current lifestyle of society, the number of people suffering from diabetes mellitus and obesity is increasing. The desire of consumers to become healthier has created a whole market for sugar-free and zero-calorie foods, an important part of which is the production of sugar alcohols called polyols. Erythritol ($C_4H_{10}O_4$) is a naturally occurring sugar alcohol. Since the synthesis of erythritol is more difficult than those of other polyols, intensive research has been conducted to optimize its production in terms of the erythritol concentration, production rate (productivity) and/or yield [1].

Although erythritol is 60-70% as sweet as table sucrose, it is almost calorie-free (0.2 kcal/g), does not affect the blood sugar level nor cause tooth decay [2], has antioxidant effects, binds free radicals, possesses a glycemic index of 0 and increases the ability to absorb fructose [3]. Many methods are available to produce erythritol, both chemically and biotechnologically. Even though one of the most well-known chemical methods is high-temperature chemical synthesis from dialdehyde starch in the presence of a metal catalyst, namely nickel, which yields equimolar amounts of erythritol and ethylene glycol, this chemical process involves several steps [2,4-5]. However, it is not used in industry due to its very low yield and relatively high costs. Currently, biotechnological methods are far superior to chemical methods. The large-scale production of erythritol uses microbial fermentation processes with pure glucose, sucrose and glucose syrup from chemically and enzymatically hydrolyzed wheat and corn starches [2]. The main microbiological strains in the synthesis of erythritol are osmophilic yeasts, e.g. *Moniliella pollinis*, *Trichosporonoides megachiliensis* and *Y. lipolytica*, as well as many strains of lactic acid bacteria, e.g. *Oenococcus oeni*, *Leuconostoc mesenteroides* and *Lactobacillus sanfranciscensis* [5]. Within the food industry, erythritol is mainly used as a sweetener in finished goods. Sugar-free, reduced sugar and calorie- or sugar-free alternative foodstuffs can be produced. As a sugar substitute, erythritol can be found as a tabletop sweetener as well as a sweetener in drinks, chewing gum, chocolate, candy and baked goods. Polyols are also commonly used in products from the personal care industry such as cosmetics or toiletries. As an additive, it is increasingly included in care products such as toothpastes, mouthwashes, creams, make-up, perfumes or deodorants. Due to its properties, erythritol offers good fluidity and stability as an excipient, making it an ideal candidate for active ingredients in sachets and capsules [6]. *Yarrowia lipolytica* is one of the most

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widely studied species of yeast [7]. *Y. lipolytica* exhibits strong proteolytic and lipolytic activity [8-9]. One of its most important products is lipase, an enzyme widely used in various areas of industry. *Y. lipolytica* can produce succinic acid [15] as well as erythritol and mannitol using glycerol as a substrate both in the presence and absence of sodium chloride [10]. Erythritol (170 g/l) was produced at pH 3.0 with the acetate-negative strain *Y. lipolytica* Wratislavia K1 by fed-batch fermentation [11]. In terms of its industrial use, it is most widespread in the food industry, moreover, is known for both its positive and negative effects [8]. To increase erythritol production, the effects of various additives, including sodium citrate and mannitol, were investigated in order to down-regulate the formation of by-products during erythritol fermentation. Furthermore, the supplementation of metal ions provides cofactors for key enyzmes [10], various nitrates [13] and polyethylene glycol (PEG) as an osmoticum [12].

2. Experimental

The planned experiment in terms of erythritol production was performed by three *Yarrowia lipolytica* strains from the National Collection of Agricultural and Industrial Microorganisms (NCAIM, Hungary), namely NCAIM *Yarrowia lipolytica* 00597, NCAIM *Yarrowia lipolytica* 00594 and NCAIM *Yarrowia divulgata* 1485. During the fermentations, a 24-well deep-well microtiter plate (*Fig.1*) was applied with a sandwich cover by enzyscreen.com (The Netherlands) for each strain separately since many small-scale experiments can be conducted at the same time. Due to the high osmotic pressure and low pH during erythritol production, cells grow slowly, so a thorough study of many parallel fermentations needs to be performed. Using the microtiter plate, 24 experiments in different or even the same media in parallel fermentations were performed simultaneously to provide a comparison.

The ability of the strains to produce erythritol and the conditions required for erythritol fermentation were examined in order to increase the efficiency of production and achieve the highest possible erythritol concentration. The effects of the initial pH and C:N ratio were investigated during the fermentation. Given that previous studies [12,14] have shown that proper osmolarity has a significant effect on erythritol production, the effect of changing it was also investigated to determine the most appropriate range of osmolarity for erythritol production with these strains. The main consideration was the comparison of two substrates, that is, glycerol and glucose, in terms of erythritol production. After determining the most efficient fermentation conditions, our intention was to scale up the process. In the case of significant erythritol production, extraction of the product from the fermentation medium was sought. Our aim was to use the residual cells, e.g. *Yarrowia lipolytica* lysates, to further investigate cosmetic purposes after ultrasonic treatment.

Figure 1. Deep-well microplate

All the inoculum medium was contained, that is, glycerol (50 g/l), yeast extract (3 g/l), malt extract (3 g/l) and peptone (5 g/l). The erythritol fermentation medium was also contained, namely glycerol (150 g/l), NH_4Cl (3 g/l), $MgSO_4*7H_2O$ (1 g/l), KH_2PO_4 (0.2 g/l) and yeast extract (1 g/l) [11]. In order to increase erythritol production, the effects of the following additives was also tested. 20 g/l of both mannitol and sodium citrate were added during the fermentation. Metal ion supplementation resulted in the following concentrations of salts in the medium: $CuSO_4*5H_2O$ (2.5 mg/l), $FeSO_4*7H_2O$ (10 mg/l), $MnSO_4*7H_2O$ (25 mg/l) and $ZnSO_4*7H_2O$ (20 mg/l). 100 g/l of the supplement PEG (Polyethylene Glycol) was added, moreover, 4.6 g/l of nitrate supplementation was applied. Furthermore, co-fermentation was investigated in combination with the two carbon sources, namely glucose and glycerol.

The strains were inoculated in 100 ml of inoculum medium in 250 ml flasks and incubated on a rotary shaker (New Brunswick Innova 40) at 250 rpm for 3 days at 25°C. All wells of the microplate contained 5 ml of fermentation medium and were incubated on a rotary shaker at 250 rpm for at least 20 days at 25°C. The pH was measured by a Mettler Toledo FiveEasy pH meter and controlled at 3.0 with NaOH (6N).

The cell density was measured optically at 600 nm by a Camspec M501 Single Beam UV/Vis spectrophotometer every other day. Fermentation products were determined by a Waters Breeze isocratic HPLC system equipped with a Bio-Rad Aminex HPX-87H column at 65°C. An RI detector was applied at 40°C and the eluent was 5mM H_2SO_4 in ultrapure water (Simplicity, Millipore). To determine the osmotic pressure of the fermentation, 60 µl of fermentation medium was analyzed by measuring the freezing-point depression using an osmometer (Gonotec Osmomat 3000).

3. Results and Discussion

3.1. Yarrowia lipolytica strain 594

Y. lipolytica strain 594 was produced using the least amount of erythritol compared to the other two tested strains. The highest concentration (10.95 g/l) was achieved on the 14th day of the fermentation in the basic fermentation media containing 100 g/l glycerol with 50 g/l glucose supplementation, i.e. co-fermentation. The highest product yield of 14.81% was also recorded using the same experimental setup, where glycerol in the media was completely consumed but not the glucose. In the case of *Y. lipolytica* strain 594, it can be stated that erythritol was not produced when only glucose was used as a substrate together with any of the other supplements. The highest erythritol concentration of 8.65 g/l was achieved when glycerol was used as the substrate supplemented with PEG, which is also rather low in comparison to other reports. Glucose as a substrate was not useful with regard to erythritol production using *Y. lipolytica* strain 594.

3.2. Yarrowia lipolytica strain 597

Y. lipolytica strain 597 was able to produce more erythritol than *Y. lipolytica* strain 594. The highest concentration (14.04 g/l) was produced in glycerol containing a medium supplemented with $NaNO_3$, which was achieved on the 20th day of fermentation and corresponded to a yield of 30.31%. The average osmolarity with a slight decrement was 2243 mOsmol/kg, which is twice that of glucose. The maximum osmolarity of the medium containing glucose was approximately 1200 mOsmol/kg, which decreased in correlation with the reduction in glucose concentration. This may explain why erythritol was produced in larger quantities in the medium containing glycerol. Glycerol was only completely consumed by the end of the fermentation when the media were combined, containing 100 g/l of glucose and 50 g/l of glycerol. On the 14th day, erythritol production had reached its maximum (11.13 g/l), after which its amount began to decrease. Although the osmolarity was 1043 mOsmol/kg on the 14th day, it was initially 1346 mOsmol/kg. In the case of glucose, the highest concentration of erythritol was 2.04 g/l, which was achieved in a medium supplemented with ammonium sulfate and corresponded to a yield of 4.54%. Using this setup, even though the average osmolarity was 1165 mOsmol/kg, which is very low for erythritol production, 4.24 g/l of mannitol was produced. Therefore, when glucose was used as the substrate supplemented with ammonium sulfate, mannitol was produced by *Y. lipolytica* strain 597 instead of erythritol.

3.3 Yarrowia divulgata strain 1485

Once again, two different substrates, namely glycerol and glucose, were compared. Among the tested additives (*Fig.2A*), the largest increase in erythritol concentration was experienced in the case of sodium citrate compared to the control samples. The highest concentration of

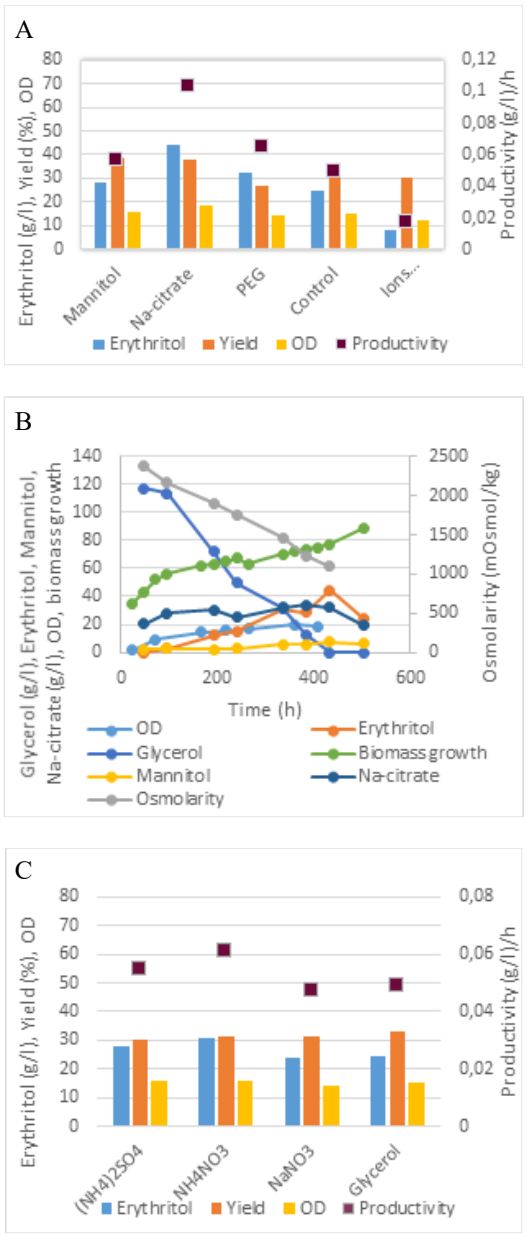

Figure 2. Results of *Y. divulgata* strain 1485 - A) effect of supplements, B) time course of best run, C) effect of N-sources

erythritol (44.38 g/l) was produced by *Y. divulgata* on the 18th day of the fermentation using glycerol as a substrate supplemented with sodium citrate (*Fig.2B*). The glycerol was completely used up in the medium. The highest productivity of 0.1 (g/l)/h and highest yield of 37.86% were achieved with this supplementation. The osmolarity of the medium decreased in correlation with the exhaustion of glycerol from 2371 mOsmol/kg initially to 1099 mOsmol/kg by the end of the fermentation.

In the case of PEG and metal supplementation, 32.44 and 8.31g/L of erythritol had been produced by the end of the fermentation, respectively. Among the tested nitrogen sources (*Fig.2C*), ammonium nitrate yielded the highest erythritol concentration of 30.75 g/l, corresponding to a yield of 31.11% based on glycerol. Compared to the control media, neither of the nitrogen

sources could significantly increase the erythritol production. By the end of the fermentation supplemented with ammonium nitrate, 5.13 g/l of mannitol had been produced. Regarding the fermentations supplemented with ammonium sulfate and sodium nitrate, 27.60 and 23.87 g/l of erythritol had been produced by the end of the fermentation, respectively. In the case of the substrate glucose, the highest production of erythritol (12.95 g/l) was achieved in the medium supplemented with sodium citrate.

4. Discussion

In the present work, 3 strains of *Yarrowia* species were examined on two carbon sources, namely glycerol and glucose, in terms of erythritol fermentation. Glycerol proved to be more usable. Among the 3 *Yarrowia* strains tested, *Y. divulgata* was the most productive, achieving 44.38 g/l of erythritol in the media supplemented with sodium citrate during the microplate fermentation. In the case of *Y. lipolytica* strain 594, the highest erythritol concentration of 10.95 g/l was achieved without supplementation but in a medium containing 100 g/l of glycerol supplemented with 50 g/l of glucose on the 14th day of the fermentation. Finally, in the case of *Y. lipolytica* strain 597, the highest concentration of erythritol was 14.04 g/l in the medium supplemented with sodium nitrate. The tested supplementations could increase the erythritol concentration from 24.70 to 44.38 g/L, yield from 33.15 to 37.86% and productivity from 0.049 to 0.102.

Among the reported *Yarrowia* results, although the aforementioned erythritol concentrations achieved are not particularly high, *Y. divulgata* may reach the recently used strains after optimization. In addition to the use of erythritol alone, the complex utilization of the broth for the application of ergosterol and cosmetics may be of greater interest and feasibility.

In the future, given the high number of variables influencing erythritol productivity, a neural network will be used to optimize erythritol fermentation for *Y. divulgata* strain 1485, which proved to be the best. Additionally, isolation and determination of the ergosterol content of the cells as well as the produced biodetergent will also be investigated

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