การเหนี่ยวนำยีสต์ *Moniliella* sp. BCC25224 ให้กลายพันธุ์ด้วยรังสียูวีและเอทิลเมทิลซัลโฟเนต (EMS) เพื่อการผลิตเออริทริทอล

Mutagenesis induction of *Moniliella* sp. BCC25224 using combination of UV and ethyl methanesulfonate (EMS) for erythritol production

ชมัยพร สุวรรณเพ็ชร[1*], วิรัตน์ วาณิชย์ศรีรัตนา[1]
Chamiporn Suwanapetch[1*], Wirat Vanichsriratana[1]

Received: 26 March 2022 ; **Revised**: 26 April 2022 ; **Accepted**: 3 May 2022

บทคัดย่อ

เออริทริทอลคือสารให้ความหวานพลังงานต่ำที่ไม่ส่งผลต่อระดับน้ำตาลในเลือดผลิตจากการหมักกลูโคสด้วยยีสต์ *Moniliella* sp. (ยีสต์ผ่านการรับรองให้ใช้ในอาหาร) โดยงานวิจัยนี้มีวัตถุประสงค์เพื่อปรับปรุงและคัดเลือกยีสต์สายพันธุ์ *Moniliella* sp. ให้ผลิตเออริทริทอลสูง ด้วยการเหนี่ยวนำให้เกิดการกลายพันธุ์ในยีสต์ *Moniliella* sp. BCC25224 โดยใช้สารเคมีเอทิลเมทิลซัลโฟเนต (EMS) ร่วมกับฉายแสงรังสียูวีเป็นเวลา 15, 30 และ 60 นาที จากการศึกษาพบว่าสามารถคัดเลือกยีสต์ *Moniliella* sp. BCC25224-M13 ที่สามารถผลิตเออริทริทอล (erythritol production) สูงกว่าสายพันธุ์ดั้งเดิม 1.2 เท่า (44.2 gL^{-1}) ในระดับฟลาสก์ โดยสามารถผลิตเออริทริทอล (erythritol production), ให้ผลได้ของเออริทริทอล (erythritol yield) และมีประสิทธิภาพการผลิตเออริทริทอล (yield coefficients) ในระดับถังหมักขนาด 10 ลิตร เท่ากับ 103.5 กรัมต่อลิตร, 56.9% และ 0.59 กรัมเออริทริทอล/กรัมกลูโคส ตามลำดับ ดังนั้นการศึกษานี้จึงเป็นการพัฒนาและปรับปรุงยีสต์สายพันธุ์ *Moniliella* sp. และได้ *Moniliella* sp. BCC25224-M13 เป็นทางเลือกในการผลิตเออริทริทอล

คำสำคัญ: เออริทริทอล *Moniliella* sp. กระบวนการหมัก การชักนำการกลายพันธุ์

Abstract

Erythritol is a safe, non-caloric and non-insulinemic sweetener present in various foods. It can be produced from glucose as substrate via fermentation with *Moniliella* pollinis (a safe and suitable food-grade osmophilic yeast). This study aims to screen high erythritol producing *Moniliella* sp. strains via mutagenesis. The candidate producer, *Moniliella* sp. BCC25224, was mutated sequentially by UV irradiation and immersion in ethyl methyl sulphonate (EMS) for 15, 30, and 60 min. The mutant named *Moniliella* sp. BCC25224-M13 was selected based on the highest levels of erythritol production at flask scale (44.2 gL^{-1}) which was 1.2-fold improved compared with its wild parent. In a 10-L bioreactor, the erythritol production, erythritol yield and yield coefficients by BCC25224-M13 were 103.5 gL^{-1}, 56.9% and 0.59 gg^{-1}, respectively. This study revealed that *Moniliella* sp. BCC25224 could be used as a parent to produce mutants for superior erythritol production, such as *Moniliella* sp. BCC25224-M13.

Keywords: Erythritol, *Moniliella* sp., Fermentation, Mutagenesis

[1] ภาควิชาเทคโนโลยีชีวภาพ คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์ เขตจตุจักร กรุงเทพฯ 10900
[1] Biotechnology, Faculty of Agro-Industry, Kasetsart University, Chatuchak, Bangkok, 10900, Thailand
[*] Corresponding author: e-mail: fagiwiv@ku.ac.th

Introduction

Erythritol is a unique member of the polyol family which has sweetening organoleptic properties of 70% relative to sucrose (Rzechonek *et al.*, 2018). Erythritol can therefore be used to produce no-sugar added, reduced-sugar, or sugar-free alternatives in food, beverages and pharmaceutical products. The ingestion of erythritol has many benefits which include zero-calories, non-insulinemic, non-cariogenic, non-acidogenic, non-laxative, anti-oxidative properties and increases in malabsorption of fructose (Jacqz-Aigrain *et al.*, 2015, den Hartog *et al.*, 2010, Moon *et al.*, 2010).

Industrial production of erythritol via fermentation utilizes glucose or hydrolyzed wheat or corn starch as substrate for microbial activity. Microbial strains such as Aureobasidium sp. (Guo *et al.*, 2016), *Moniliella* sp. (Lin *et al.*, 2010), Trichosporonoides sp. and Yarrowia lipolytica (Xiaoyan *et al.*, 2017) have been studied for erythritol production (Rzechonek *et al.*, 2018). *Moniliella* sp. and Yarrowia sp. were proven to be safe for use in food processing (Rzechonek *et al.*, 2018). Nevertheless, the search for strains of *Moniliella* sp. and Yarrowia sp. for high erythritol yield and productivity are key for commercial production, and process conditions (Rakicka-Pustułka *et al.*, 2020). In screening and isolating of novel erythritol producing organisms, pollen, honey, beehives, preserved fruits, dry fruit and sugarcane factory's soil have served as sources (Lin *et al.*, 2001).

However, one sure way for screening and isolating superior microbes for a metabolite of interest is by genetic engineering and mutation via UV radiation or chemical mutagenesis by using ethyl methanesulfonate (EMS) (Park *et al.*, 2019, Yan *et al.*, 2021). The widely used technique is mutagenesis followed by selection because of lower cost and sophistication of processes involved. Mutation selection is an efficient and rapid way to obtain desirable traits in crop plants and microbes (Yan *et al.*, 2021) 2021.

UV mutagenesis technique has been applied to isolate yeasts for specific products such as for improved aroma production. Despite obtaining new strains by UV mutagenesis, they are designated as genetically modified organisms (GMO) by the European Union legislation (Article 2 of EU Directive 2001/18/EC). Meanwhile, such organisms do not undergo strict regulation relative to other altering genetic modification techniques in producing GMO because of the long-standing safety record (Dederer & Hamburger, 2022). Random mutagenesis by UV emission revealed that a mutant of Aureobasidium pullulans CGMCC3.0837 (ER 35) produced more erythritol than parent strains by 50.92% (Guo *et al.*, 2016). In other instance, EMS was used to obtain a Candida magnoliae mutant with minimum by-product production (Ghezelbash *et al.*, 2014). The interaction of UV mutagenesis and chemical mutagenesis to obtain high production of erythritol is still an ongoing study.

Moniliella species are regarded as efficient erythritol producers. However, mutagenesis techniques are still applied to screen for superior desired traits among living organism. In this study, a sequential mutagenesis of UV radiation and EMS was applied on *Moniliella* sp. BCC25224 strain to obtain mutants capable of high erythritol production.

Materials and methods

Chemicals

Yeast extract was purchased from DIFCO (Detroit, MI, USA). Corn steep liquor was obtained from Friendship Corn Starch Co., Ltd. (Thailand). Soy bean flour was obtained from Win Change Industries Co., Ltd. (Thailand). Ethyl methyl sulphonate (EMS) was purchased from Sigma-Aldrich. All chemicals used were reagent grade.

Experimental designs

The research design used was completely randomized design (CRD). The strains was treatment and each treatment was performed in triplicate. The analyst data were dry cell weight, glucose consumption, erythritol production and erythritol yield.

Moniliella sp. BCC25224 cultivation and multiplication

Moniliella sp. BCC25224 used in this study was obtained from the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology, Thailand. *Moniliella* sp. BCC25224 was streaked on glucose yeast extract (GYE) agar (300 gL^{-1},

glucose; 10 gL^{-1}, yeast extract, and 20 gL^{-1} of agar) to obtain single colonies. After, cell multiplication of *Moniliella* sp. BCC25224 was carried out in a 100-mL of glucose yeast extract broth in 500-mL flasks in a shaking incubator at 200 rpm at 30°C for 5 days before use (Lin *et al.*, 2010, Burschaper *et al.*, 2002).

Mutagenesis of *Moniliella* sp. BCC25224 via UV and EMS

The mutagenesis protocol was modified from Savergave *et al.* (2011). The culture broth was centrifuged at 4,000 x g for 10 minutes and the cells were washed twice with sterile saline. Five milliliter of cell suspension (1 x 10^6 cell mL^{-1}) was irradiated by Philips 30W G30 T8 lamp at wavelength 254 nm for 3 minutes at a distance of 20 cm. The cell suspension was then treated with 20 µL EMS at a final concentration of 4 µLmL^{-1} and incubated for three different periods (15, 30 and 60 minutes). The EMS was inactivated by adding 0.5 mL of sterile sodium thiosulfate (50 gL^{-1}). The treated cell suspension was washed twice with sterile saline before spread plating on the GYE agar medium. The plated media were incubated at 30°C for 7 days (Guo *et al.*, 2016).

Mutated colonies that survived were picked up and cultivated in GYE broth medium for 2 days at 30°C and 200 rpm for the erythritol production test. The inoculum (20 gL^{-1}) was transferred into 100-mL GYE broth and incubated at 30°C, at 200 rpm mechanical shaker for 5 days. The fermented broth was centrifuged at 4,000 x g for 10 min at 4°C, the supernatant was then filtered through 0.45 µm nylon filter before erythritol analysis via HPLC. The strain with the highest erythritol production and highest glucose consumption were compared with the parent strain and chosen (Ghezelbash *et al.*, 2014, Lin *et al.*, 2010) for further investigation in a 10 L jar fermenter.

Production of erythritol in fermenters

Moniliella sp. BCC25224 and selected mutant strains were grown in a 10 L B. E. Marubishi fermenter containing 5 L broth (200 gL^{-1} glucose and 13 gL^{-1} soybean flour, pH 5.3) with agitation speed of 350 rpm and aeration rate of 0.7 vvm at 30°C. Broth samples were harvested and analyzed every 11 or 12 hours for 9 days to calculate the dry cell weight, erythritol concentration, glucose consumption, erythritol yield, yield coefficients and productivity.

Physico-chemical analysis of fermented broth

High performance liquid chromatography analytical methods (HPLC)

The filtrate supernatants were analyzed for glucose and erythritol by HPLC under isocratic conditions a refractive index detector (Waters 2414) at 30°C, using an amino column APS-2 Hypersil column. The column was kept at 30°C with a mobile phase constituting of water and acetonitrile (25:75 v/v) at a flow rate of 1 mL min^{-1}.

Dry cell weight analytical methods

Culture broth were centrifuged at 5,000 x g for 15 minutes. The cultured cells were wash twice with sterile distilled water and dried to constant weight in a hot air oven at 105°C (Ghezelbash *et al.*, 2014). The dry cell weight (DCW) was calculated using the following equations.

Statistical analysis

All data were analysed for ANOVA using SPSS program. The results are means ± S.D. for triplicate experiments and significantly different at 99% ($p<0.01$)

Results and discussion

Mutagenesis

The synergistic effect of UV irradiation and EMS on *Moniliella* sp. BCC25224 in screening for superior erythritol production was investigated. The sequential treatment of *Moniliella* sp. BCC25224 by UV, and immersion in EMS for 15, 30, and 60 min resulted in 3%, 1% and 0% survival of *Moniliella* sp. BCC25224, respectively. Surviving colonies were inoculated in GYE medium and then further analyzed by HPLC. Screening of mutants via culturing in broth for erythritol production and glucose utilization resulted in the selection of three mutants that were *Moniliella* sp. BCC25224-M13, BCC25224-M14 and BCC25224-M15 that produced more erythritol than parent cells (Table 1). A total of 138 survivor mutant colonies were analyzed of which 97.8% produced erythritol at lower amount than did the parent strain (3.8 - 9.3 gL^{-1}).

Mutant strains increased in cell mass because of an increase in glucose consumption (Table 1) hence greater metabolic activity increased cell multiplication led to higher erythritol production. Statistically, *Moniliella* sp.

BCC25224-M13 varied significantly ($p < 0.01$) relative to the parent culture. The mutant BCC25224-M14 and BCC25224-M15 produced less erythritol lower than BCC25224-M13 with low glucose consumption (Table 1). Nevertheless, BCC25224-M14 and BCC25224-M15 had similar erythritol yields which were higher than that of BCC25224-M13. The relatively low erythritol yield of BCC25224-M13 was attributed to the high cell mass production during fermentation.

Candida magnolia mutant were generated by ultraviolet and EMS chemical mutagenesis (Ghezelbash *et al.*, 2014). The mutant named 12-2 gave a 2.4-fold and 2.2 fold increased in erythritol production (20.32 gL^{-1}) and yield (10.99%) from 200 gL^{-1} glucose compared to the parent strain. The glucose consumption of parent and mutant was 84.87% and 92.41%, respectively. PCR product of the mutant revealed a minor change in the sequence of genes involved in a production pathway which can lead to a significant increase in protein translation.

Aureobasidium sp SN-124A successfully mutated with UV irradiation and NTG treatment (Ishizuka *et al.*, 1989). The mutant produced erythritol with 47.6% yield on a medium containing 22.5% glucose compared to 41.8% yield obtained from parent strain. The erythritol concentration produced reached 164.8 gL^{-1} with the mutant while the parent was 110 gL^{-1}.

Chemical mutagenesis of *Moniliella* sp. 440 by N-methyl-N´-nitro-N-nitrosoguanidine (NTG) resulted in N61188-12 mutant which produced two times more erythritol (237.8 gL^{-1}) than parent strain in fermentation medium comprise of 400 gL^{-1} glucose and 10 gL^{-1} yeast extract (Lin *et al.*, 2010). Although the mutant strain consumed the glucose completely for efficient erythritol production with 55.6% yield, the cell density (A_{660}) was lower than parent strain (26.1 and 56.8). The high erythritol production and yield was attributed to the low by-product production e.g. glycerol and ribitol. Glucose concentration produces osmotic stress on biomass and erythritol formation (Jeya *et al.*, 2009).

Although, the improvement in erythritol production of the *Moniliella* sp. BCC25224 in this study was achieved by mutagenesis, the production performance of the mutant was lower than the other erythritol-producing strains. The low production efficiency probably resulted from the small number of cells. Anyway, *Moniliella* sp. BCC25224-M13 was further examined for cell growth and erythritol formation in batch fermentation and compared with the parent strain to confirm the mutagenesis achievement.

Table 1 Erythritol production of wild type strain and derived mutant at flask scale (Volume of flask) after 5 days of fermentation

Strain	Dry cell weight (gL^{-1})	Glucose consumption (%)	Erythritol production (gL^{-1})	Erythritol yield (%)
Moniliella sp. BCC25224 (Parent)	13.2 ± 1.0^{b}	74.0 ± 7.8^{b}	35.1 ± 3.5^{b}	24.7 ± 3.7^{b}
Moniliella sp. BCC25224-M13	18.0 ± 2.0^{a}	98.7 ± 2.3^{a}	44.2 ± 0.1^{a}	25.6 ± 0.1^{b}
Moniliella sp. BCC25224-M14	17.6 ± 1.3^{a}	77.7 ± 0.3^{b}	39.1 ± 0.1^{b}	28.7 ± 1.2^{a}
Moniliella sp. BCC25224-M15	16.7 ± 0.6^{a}	78.2 ± 0.9^{b}	38.8 ± 0.1^{b}	28.4 ± 0.7^{a}

Mean values with different letters in columns indicates a significant difference ($p<0.01$)
Results are means ± S.D. for triplicate experiments.

Up-scale production of erythritol in 10 L fermenter

A batch fermentation was carried out for both *Moniliella* sp. BCC25224 and *Moniliella* sp. BCC25224-M13 mutant strain in a 10-L jar fermenter to observe the cell growth, glucose utilization, erythritol production pattern, and especially for erythritol production efficiency. The cell growth, glucose consumption and erythritol production rate should indicate the ideal conditions for the further fed-batch operation. The parent *Moniliella* sp. BCC25224 and *Moniliella* sp. BCC25224-M13 produced erythritol during cell growth (Figure 1 and 2) and fermentation which indicated that erythritol is a primary metabolite (Sanchez & Demain, 2008). Erythritol is also synthesised during the exponential phase of growth and synthesis is an integral part of the normal growth process. The cells showed continuous growth throughout the cultivation period (216 hours) but during the stationary phase growth could not be clearly determined.

In the first 48 hours of the fermentation, dry cell weight value was interfered with by the soy bean flour. *Moniliella* sp. BCC25224 showed a higher growth rate than the mutant strain after 143 hours (0.15 $gL^{-1}h^{-1}$ and 0.07 $gL^{-1}h^{-1}$). Erythritol formation of the parent and mutant began at 23 hours with different production rates. Glucose was completely consumed by parent and mutant strain after fermentation for 216 and 204 hours respectively, which reflected the lower dry cell weight of the mutant. The pH of fermentation broth of both strains decreased from 5.4 to 4.0 after 48 hours and continued to decrease with the lowest pH value of 3.5 at 95 hours. The rate of growth and of substrate consumed reflected the cell's ability to respond to the environment (Figure 1 and 2). High glucose concentration caused inhibition of cell growth and synthesis of erythritol. Our results agreed with Kobayashi *et al.* (2013) who reported erythritol production in response to oxidative stress and altered redox balance by *Moniliella* megachiliensis.

For the parent strain, the erythritol production, erythritol yield, yield coefficients and volumetric production rate were 86.6 gL^{-1}, 46.7%, 0.47 gg^{-1} with 0.40 $gL^{-1}h^{-1}$, respectively. While *Moniliella* sp. BCC25224-M13 produced 103.5 gL^{-1} erythritol; 56.9%, erythritol yield; 0.59 gg^{-1}, yield coefficients, and 0.47$gL^{-1}h^{-1}$ volumetric production rate. The effect of UV and EMS immersion was maintained in *Moniliella* sp. BCC25224-M13 as a 1.2-fold increase in erythritol production and yield was observed (Table 2).

The productivity of *Moniliella* sp. BCC25224-M13 were compared with that of other microorganisms which have been reported previously to produced erythritol (Table 2). *Moniliella* sp. BCC25224-M13 showed similar higher values in terms of erythritol yield and yield coefficients when compared to most other strains cultivated in traditional batch culture. However, erythritol production and productivity is lowest which was similar to the results at flask scale.

Focusing on simple batch culture (Table 2), Pseudozyma tsukubaensis gave the highest erythritol production, erythritol yield, yield coefficients and productivity. In addition, Jeya *et al.*, (2009) reported the dry cell weight after fermentation for 36 hours was 25.2 gL^{-1} which was higher than with *Moniliella* sp. BCC25224-M13 (13.9 gL^{-1} at 216 hours). Thus, increasing the cell formation during fermentation should result in higher erythritol production.

In the case of fed-batch fermentation, the erythritol productivity was higher than batch fermentation because the batch culture at high glucose level inhibited the maximum volumetric productivity (Rzechonek *et al.*, 2018, Jeya *et al.*, 2009). Maintaining a glucose concentration during fermentation can increase erythritol production and dry cell weight (Jeya *et al.*, 2009). Also, *Moniliella* sp. BCC25224-M13 should maintain the glucose content at 100 gL^{-1} during the exponential phase of growth (Figure 2).

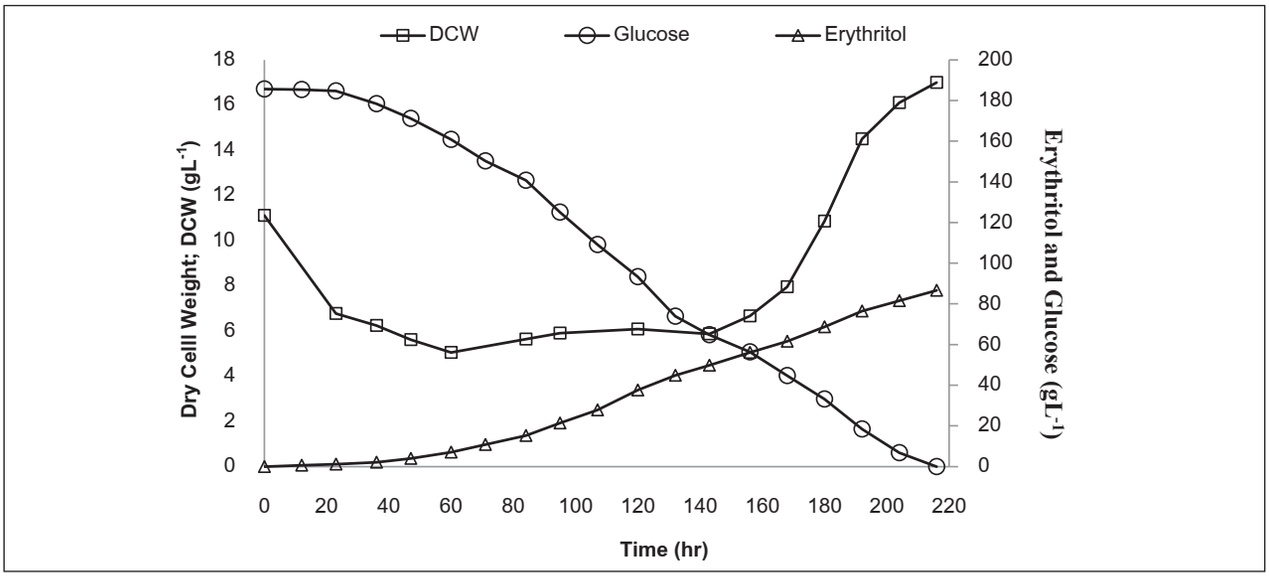

Figure 1 Biomass and erythritol production of *Moniliella* sp. BCC25224 parent strain in 10 L jar fermenter (200 gL^{-1} glucose with 13 gL^{-1} soybean flour)

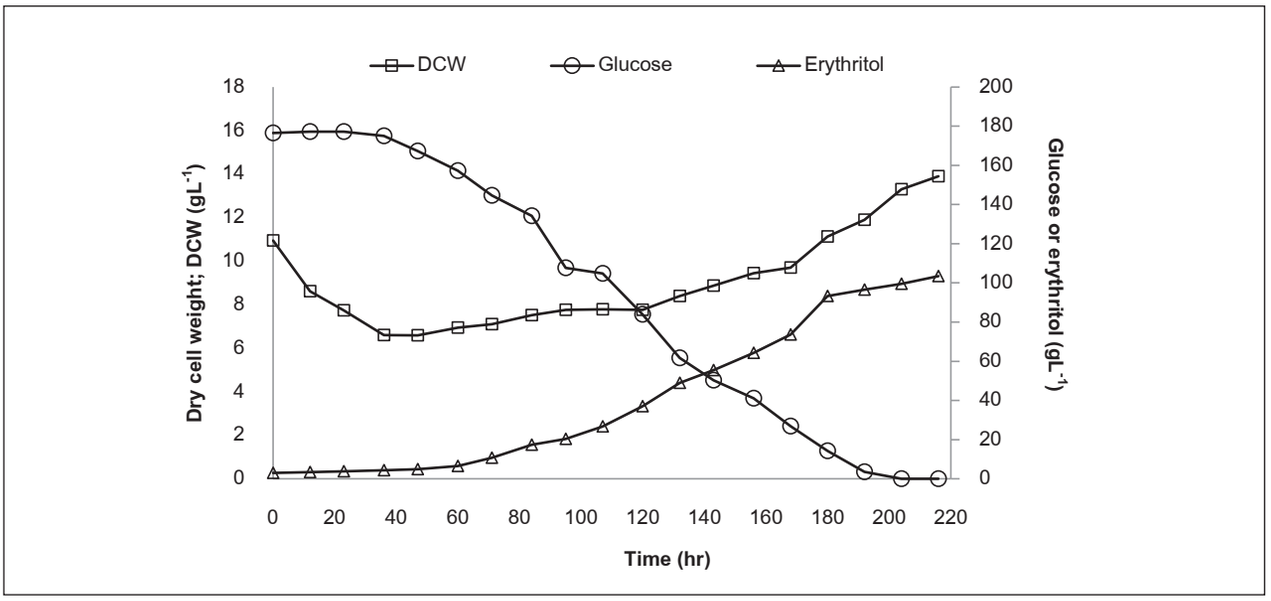

Figure 2 Biomass and erythritol production of *Moniliella* sp. BCC25224-M13 mutant strain in 10 L jar fermenter (200 gL^{-1} glucose with 13 gL^{-1} soybean flour)

Table 2 Erythritol productivity comparison of producing strains in various reactor operation types using glucose as substrate

Strain	Operation type	Erythritol production (gL^{-1})	Erythritol yield (%)	Yield coefficients (gg^{-1})	Productivity ($gL^{-1}h^{-1}$)	Reference
Aureobasidium sp. (mutant SN-124A)	Batch	170.0	37.7	N.A.*	1.82	Rzechonek *et al.* (2018)
Candida magnoliae	Fed-batch	200.0	43.0	N.A.*	1.2	Rzechonek *et al.* (2018)
Candida magnoliae	Two-stage Fed-batch	187.0	41.0	N.A.*	2.8	Rzechonek *et al.* (2018)
Candida magnolia JH110 (mutant)	Fed-batch	200.0	43.0	N.A.*	1.2	Rzechonek *et al.* (2018)
Torula sp.	Fed-batch	192.0	48.0	N.A.*	2.26	Rzechonek *et al.* (2018)
Trichosporon sp.	Batch, Stirred tank	138.0	46.0	0.49	1.23	Park *et al.* (1998) ; Jeya *et al.* (2009)
Trichosporon sp.	Fed-batch, Stirred tank	316.8[a]	44.0	0.51	1.33	Park *et al.* (1998)
Moniliella megachilliensis sp. 440 mutant (mutant N53199-12)	Batch, Stirred tank	152.4	43.6	N.A.*	0.65	Rzechonek *et al.* (2018)
Moniliella 618A-01	Fed-batch, Stirred tank	100.0	39.3	N.A.*	0.19[a]	Hirata *et al.* (1999)
Moniliella 278-3 (or *Moniliella* sp. 166-2)	Batch, Stirred tank	111.0	37.0	0.37[a]	0.77[a]	Lin *et al.* (2001)
Moniliella sp. N61188-12 (mutant)	Batch, Stirred tank	142.0	40.5[a]	N.A.	1.18[a]	Lin *et al.* (2010)
Moniliella sp. N61188-12 (mutant)	Fed-batch, Stirred tank	189.4	48.0	N.A.*	0.80	Lin *et al.* (2010)

Table 2 Erythritol productivity comparison of producing strains in various reactor operation types using glucose as substrate (cont.)

Strain	Operation type	Erythritol production (gL^{-1})	Erythritol yield (%)	Yield coefficients (gg^{-1})	Productivity ($gL^{-1}h^{-1}$)	Reference
Moniliella tomentosa var pollinis	Batch, Stirred tank	90.0	36.1	0.35	0.59[a]	Burschapers *et al.* (2002a)
Moniliella tomentosa var pollinis	Fed-batch, Stirred tank	170.0	38.8	N.A.*	1.62[a]	Burschapers *et al.* (2002a)
Moniliella tomentosa var pollinis	Batch, Airlift tower loop	105.0	62.0	0.40	N.A.*	Burschapers *et al.* (2002b)
Moniliella tomentosa var pollinis	Fed-batch, Airlift tower loop	175.0	54.0	0.39	N.A.*	Burschapers *et al.* (2002b)
Moniliella Mutant HAT 101	Batch, Airlift tower loop	150.0	65.0	0.57	N.A.*	Burschapers *et al.* (2002b)
Moniliella Mutant HAT 101	Fed-batch, Airlift tower loop	175.0	70.0	0.58	N.A.*	Burschapers *et al.* (2002b)
Pseudozyma tsukubaensis	Batch, Stirred tank	243	61.0	N.A.*	1.65	Jeya *et al.* (2009)
Pseudozyma tsukubaensis KN75	Fed-batch, Stirred tank	245	61.0	N.A.*	2.86	Jeya *et al.* (2009)
Moniliella sp. BCC25224	Batch, Stirred tank	86.6	46.7	0.47	0.40	this study
Moniliella sp. BCC25224-M13 (mutant)	Batch, Stirred tank	103.5	56.9	0.59	0.47	this study

Moniliella, Trichosporonoides and Aureobasidium sp. were thought to be synonymous

[a] calculation estimation

* not available

Conclusions

In this study, *Moniliella* sp. BCC25224 was subjected to UV and EMS mutagenesis. The mutant *Moniliella* sp. BCC25224-M13 produced a higher erythritol concentration and erythritol yield during fermentation with glucose as substrate than in *Moniliella* sp. BCC25224. Sequential random mutagenesis through UV irradiation and EMS treatments were achieved and obtained a potential candidate mutant for erythritol production. Attempts at further erythritol improvement should study process operation to optimize biomass and erythritol production. Erythritol production and productivity should increase influence of osmotic pressure or the glucose concentration regulation.

References

Burschapers, J., Schustolla, D., Schugerl, K., Roper, H. & Troostembergh, JC.de. (2002). Engineering aspects of the production of sugar alcohols with the osmophilic yeast *Moniliella*tomentosa var pollinis. Part I. Batch and fed-batch operation in stirred tank. *Process Biochemistry, 38*, 497-506. DOI:10.1016/S0032-9592 (02) 00178-4

Burschapers, J., Schustolla, D., Schugerl, K., Roper, H. & Troostembergh, JC.de. (2002). Engineering aspects of the production of sugar alcohols with the osmophilic yeast *Moniliella* tomentosa var pollinis Part 2. *Batch and fed-batch operation in bubble column and airlift, Process Biochemistry, 38*, 559-570. DOI:10.1016/S0032-9592 (02) 00179-6

Dederer, H-G. & Hamburger, D. (2022). Are genome-edited micro-organisms covered by Directive 2009/41/EC?-implications of the CJEU's judgment in the case C-528/16 for the contained use of genome-edited micro-organisms. *Journal of Law and the Biosciences, 31*, 9. doi: 10.1093/jlb/lsab033. eCollection Jan-Jun 2022.

den Hartog, G.J.M., Booths, A.W., Adam-Perrot, A., Brouns, F., Verkooijen, I.W.C.M., Weseler, A.R., Haenen, G.R.M.M. & Bast, A. (2010). Erythritol is a sweet antioxidant. *Nutrition, 26*, 449-58. doi: 10.1016/j.nut.2009.05.004. Epub 2009 Jul 24.

Hirata, Y., Igarashi, K., Ezaki, S. & Atomi, H. (1999). High-level production of erythritol by strain 618A-01 isolated from pollen. *Journal of Bioscience and Bioengineering, 87*, 630-635.

Ghezelbash, G.R., Nahvi, I. & Malekpour, A. (2014). Erythritol production with minimum By-product using Candida magnoliae mutant. *Applied Biochemistry and Microbiology, 50*, 292-296. DOI:10.1134/S000368381403003X

Guo, J., Li, J., Chen, Y., Guo, X. & Xiao, D. (2016). Improving Erythritol Production of Aureobasidium pullulans from Xylose by Mutagenesis and Medium Optimization. *Applied Biochemistry and Biotechnology, 180*, 717-727. doi: 10.1007/s12010-016-2127-3.

Ishizuka, H., Wako, K., Kasumi, T. & Sasaki, T. (1989). Breeding of a mutant of Aureobasidium sp. with high erythritol production. *Journal of Fermentation and Bioengineering, 68*, 10.1016/0922-338x (89) 90003-2.

Jacqz-Aigrain, E., Kassai, B., Cornu, C., Cazaubiel, J. M., Housez, B., Cazaubiel, M., Prevel, J-M., Bell, M., Boileau, A. & Decock, P. (2015). Gastrointestinal tolerance of erythritol-containing beverage in young children: a double-blind, randomised controlled trial. *European Journal of Clinical Nutrition, 69*, 746-51. DOI:10.1038/ejcn.2015.4

Jeya, M., Lee, K-M., Tiwari, M. K., Kim, J-S., Gunasekaran, P., Kim, S-Y., Kim, I-W. & Lee, J-K. (2009). Isolation of a novel high erythritol-producing Pseudozyma tsukubaensis and scale-up of erythritol fermentation to industrial level. *Applied Microbiology and Biotechnology, 83*, 225-231. doi: 10.1007/s00253-009-1871-5.

Kobayashi, Y., Yoshida, J., Iwata, H., Koyama, Y., Kato, J., Ogihara, J. & Kasumi, T. (2013). Gene expression and function involved in polyol biosynthesis of Trichosporonoides megachiliensis under hyper-osmotic stress. *Journal of Bioscience and Bioengineering, 115*, 645-50. doi: 10.1016/j.jbiosc.2012.12.004. Epub 2013 Jan 5.

Lin, S-J., Wen, C-Y., Wang, P-M., Huang, J-C, Wei, C-L., Chang, J-W. & Chu, W-S. (2010). High-level production of erythritol by mutants of osmophilic *Moniliella* sp. *Process Biochemistry, 45*, 973-979, https://doi.org/10.1016/j.procbio.2010.03.003

Lin, S-J., Wen, C-Y., Liau, J-C. & Chu, W-S. (2001). Screening and production of erythritol by newly isolated osmophilic yeast-like fungi. *Process Biochemistry, 36*, 1249-1258. https://doi.org/10.1016/S0032-9592 (01) 00169-8

Moon, H.J., Jeya, M., Kim, I-M. & Lee, J-K. (2010). Biotechnological production of erythritol and its applications. *Applied Microbiology and Biotechnology, 86*, 1017-1025. DOI: 10.1007/s00253-010-2496-4

Park, Y-K., Korpys, P., Kubiak, M., Celinska, E., Soudier, P., Trebulle, P., Larroude, M., Rossignol, T. & Nicaud, J-M. (2019). Engineering the architecture of erythritol-inducible promoters for regulated and enhanced gene expression in Yarrowia lipolytica. *FEMS Yeast Research, 1*, 19. doi: 10.1093/femsyr/foy105.

Park, J., Seo, B., Kim, J. & Park, Y. (1998). Production of erythritol in fed-batch cultures of Trichosporon sp. *Journal of Fermentation and Bioengineering, 86*, 577-580. https://doi.org/10.1016/S0922-338X (99) 80010-5.

Rakicka-Pustułka, M., Mirończuk, A.M., Celińska, E., Białas, W. & Rymowicz, W. (2020). Scale-up of the erythritol production technology - Process simulation and techno-economic analysis. *Journal of Cleaner Production, 257*, 120533. https://doi.org/10.1016/j.jclepro.2020.120533

Rzechonek, D.A., Dobrowolski, A., Rymowicz, W. & Mironczuk, A.M. (2018). Recent advances in biological production of erythritol. *Critical Reviews in Biotechnology, 38*, 620-633. https://doi.org/10.1080/07388551.2017.1380598

Savergave, L., Gadre, R.V., Vaidya, B.K. & Narayanan, K. (2011). Strain improvement and statistical media optimization for enhanced erythritol production with minimal by-products from Candida magnoliae mutant R23. *Biochemical Engineering Journal, 55*, 92-100. DOI:10.1016/j.bej.2011.03.009

Sanchez, S. & Demain, A.L. (2008). Metabolic regulation and overproduction of primary metabolites. *Microbial Biotechnology, 1*, 283-319. doi: 10.1111/j.1751-7915.2007.00015.x

Xiaoyan, L., Xinjun, Y., Jinshun, Lv., Jiaxing, Jun, X., Zhen, W., Tong, Z. & Yuanfang, D. (2017). A cost-effective process for the coproduction of erythritol and lipase with Yarrowia lipolytica M53 from waste cooking oil. *Food and Bioproducts Processing, 103*, 86-94. https://doi.org/10.1016/j.fbp.2017.03.002

Yan, W., Deng, X.W., Yang, C. & Tang, X. (2021). The Genome-Wide EMS Mutagenesis Bias Correlates With Sequence Context and Chromatin Structure in Rice. *Frontiers in Plant Science, 12*, 579675. https://doi.org/10.3389/fpls.2021.579675