Journal of Cleaner Production 139 (2016) 905–913

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Technology of efficient continuous erythritol production from glycerol

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ARTICLE INFO

Article history:
Received 17 April 2016
Received in revised form
17 August 2016
Accepted 24 August 2016
Available online 26 August 2016

Keywords:
Chemostat
Yarrowia lipolytica
Erythritol
Glycerol
Desalination

ABSTRACT

Erythritol production in chemostat culture from pure and crude glycerol with different nitrogen sources by *Yarrowia lipolytica* Wratislavia K1 was investigated. Moreover, a process of erythritol purification based on ion exchange is proposed. The highest erythritol production (103.4 g L^{-1} with a volumetric erythritol production rate of 1.12 g L^{-1} h^{-1} and a yield of 0.52 g g^{-1}) was obtained by applying an inorganic nitrogen source (4.6 g L^{-1} of ammonium sulfate). Very promising results were also obtained when pure glycerol was replaced with crude glycerol as a carbon source. During this culture yeast produced 81.9 g L^{-1} of erythritol, which corresponded to a 0.9 g L^{-1} h^{-1} volumetric erythritol production rate and a yield of erythritol production of 0.4 g g^{-1}. The fermentation broth was desalinated and decolorized by ion exclusion, ion exchange and sorption on activated carbon. The final solution contained only erythritol. The presented technology follows the vision of a circular economy by turning waste into products and using renewable materials as a feedstock. This study supports the potential of the industrially relevant wild type *Yarrowia lipolytica* strain for intensified and efficient erythritol production. The proposed technology is ecofriendly, requires a small number of purification steps and generates low amount of wastes. The final solution is rich in erythritol and can be applied directly as a sweet low-calorie food additive.

1. Introduction

Continuous systems for biotechnological processes undoubtedly have many advantages, among which the most important include the possibility to set the physiological state of the cells by selecting the flow rate of feeding medium and composition of the culture medium, physical and chemical homogeneity of the culture, automation of the process, higher process efficiency, maximization of apparatus utilization, and even spread of the workload (Saldanha et al., 2004). However, a drawback of these methods is the difficulty in ensuring aseptic conditions of the process, the possibility of degeneration or mutation of strains, as well as formation of populations with worse production capacity (Hoskisson and Hobbs, 2005). Chemostat cultures applying yeast are successfully used in biotechnological processes, e.g. in the production of biodiesel (Papanikolaou and Aggelis, 2002), bulk chemicals (Temudo et al., 2008), and xylitol (Granström et al., 2001). A well-known process is production of citric acid in chemostat culture by *Y. lipolytica* using glycerol as a carbon source (Rywińska et al., 2013).

Glycerol is widely recognized as a cheap raw material obtained as a byproduct from biodiesel production. In addition, glycerol is also formed during bio-ethanol production (Fangxia et al., 2012). Glycerol can be applied in many processes, including those in the pharmaceutical, cosmetic and food industries. Due to a pronounced glut of glycerol on the market, it is advantageous to find further areas for its application. One of them is the possibility of changing it into a zero-caloric sweetener – erythritol (Park et al., 2016). As shown in our previous studies, *Y. lipolytica* yeast has a great potential for biotechnology and can be successfully used in the biosynthesis of erythritol from glycerol (Rymowicz et al., 2009). Past experiments focused on batch, fed-batch and repeated batch cultures (Tomaszewska et al., 2014). So far, the possibilities of using this yeast in a continuous culture in production of erythritol have been unexplored. Even if during cultivation the high concentration of a desirable product is received, the product has to be discolored and purified of some components of the culture broth or byproducts of biosynthesis.

The cost of removing byproducts from the culture broth often

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http://dx.doi.org/10.1016/j.jclepro.2016.08.126
0959-6526/

makes the process expensive. Thus, not only high yield and selectivity of bioconversion are important, but also an efficient method of product recovery. Ion exchange resins are often used in the processing of fermentation broths, e.g., in the separation of lactic acid (González et al., 2006). Also well known is purification of molasses (beet and cane), which is acidic and brown colored, caused by the products of alkali decomposition, melanoidin pigments and products of sucrose caramelization (Satyawali and Balakrishnan, 2008). In addition to biological methods, molasses can be purified by the use of activated carbon (Krzywonos and Szymańska, 2011) or other sorbents, e.g. halloysite (Lee et al., 2004). Apart from sorbents, there are also a number of commercially available ion exchange resins, which allow simultaneous discoloration and desalination, and are used in the food, chemical, petrochemical and pharmaceutical industries (http://lanxess.com/). However, there are no reports on the purification of fermentation broth rich in erythritol.

Recently, one of the most important challenges for modern technologies is to fulfill the main principles of cleaner production, energy saving and sustainable development. Important issues are also to enhance the process integration, process intensification and environmentally friendlier production in comparison to conventional methods (Nemet et al., 2016). Cleaner production can be achieved either by improvement of existing processes or by developing completely new technologies. Excellent examples of cleaner processes are high efficiency and waste reduced biofuel production (Mahmood et al., 2016) as well as CO_2 removal from post-combustion coal-fired flue gases by membrane absorption technology (Molina and Bouallou, 2016).

The ability of the *Y. lipolytica* yeast to produce well-dispersed, unicellular populations offers the possibility of enhancing erythritol biosynthesis from glycerol by the Wratislavia K1 strain in the classical single-step continuous chemostat culture. The effect of inorganic and organic nitrogen sources on the parameters and stability of the long-term system of erythritol production was examined in this study. Moreover, a novel process for erythritol purification based on ion exchange is proposed.

In the present study, a sweetener production technology based on waste feedstock using genetically unmodified, potent erythritol producer is proposed. The main advantages of this process, over existing technologies, include intensification of the titer, productivity and yield of the final product in comparison to simple batch or fed-batch cultures. Moreover, high concentration of feedstock waste can be incorporated in biosynthesis step without any initial purification. Due to the low pH of the culture, biosynthesis of erythritol can be performed in non-aseptic conditions, which leads to lower energy consumption and reduce the costs of the entire process (the technological process does not require sterilization). The presented technology is ecofriendly, requires a small number of purification steps and generates low amount of wastes. The final solution is rich in erythritol and can be applied directly as a sweet low-calorie food additive. The process is based on closed recirculating water system. All wastewater containing some carbon sources can be used for biomass production. Thus, *Y. lipolytica* biomass, as byproduct of this technology, can be used as animal feed. The biomass of *Y. lipolytica* produced from crude glycerol is approved by European Feed Manufacturers Federation to trade within the European Union (Rywińska et al., 2013).

2. Materials and methods

2.1. Microorganisms

The Wratislavia K1strain of *Y. lipolytica* used in this study belongs to the yeast culture collection of the Department of Biotechnology and Food Microbiology, Wroclaw University of Environmental and Life Sciences.

2.2. Substrate

Pure glycerol 960 g kg^{-1} (POCH, Gliwice, Poland) and crude glycerol from the biodiesel industry 830 g kg^{-1} (Wratislavia-Bio, Wroclaw, Poland) containing 4.3% NaCl, methanol 0.5%, nitrogen content 0.014%, ash 6.34%, and water 5.85% were used as a source of carbon and energy.

2.3. Media and culture conditions

Growth medium for inoculum preparation consisted of glycerol (50 g), yeast extract (3 g), malt extract (3 g), and peptone (5 g) in 1 L of distilled water. Growth of cells was carried out in 0.3 L flasks containing 0.05 L of growth medium on a rotary shaker (CERTOMAT IS, Sartorius, Germany) at 30 °C and 2.3 Hz for 72 h. In order to inoculate the media for bioreactor experiments 0.2 L of the growth culture was used.

The experiment proceeded according to the scheme as shown in Fig. 1. All bioreactor culturing took place in a 5-L stirred tank reactor (BIOSTAT B-PLUS, Sartorius, Germany) at 30 °C, and the working volume was maintained at 1.5 L. Aeration and stirring rates were set at 0.6 min^{-1} and 13.3 Hz, respectively.

The process of erythritol production was started as a batch culture in medium consisting of (in g L^{-1}): glycerol – 100, $(NH_4)_2SO_4$ – 2.3, KH_2PO_4 – 0.22, $MgSO_4$ x $7H_2O$ – 1.0, YE – 1.0, NaCl – 26.5, in tap water. After 24 h of batch culture the continuous biosynthesis with chemostat cultures began at the dilution rate of 0.01 h^{-1}. The feeding medium with the inorganic $(NH_4)_2SO_4$ and organic (yeast extract) nitrogen source consisted of (in g L^{-1}): pure glycerol – 200, KH_2PO_4 – 0.22, $MgSO_4$ x $7H_2O$ – 1.0, NaCl – 26.5 in tap water. The concentration of $(NH_4)_2SO_4$ was 2.3 g L^{-1} or 4.6 g $L^{-1}$and of yeast extract was 9.75 g L^{-1}. The nitrogen content in yeast extract was about 10% and the concentration of yeast extract was calculated in order to obtain 0.49 g L^{-1} of nitrogen (as in 4.6 g L^{-1} of $(NH_4)_2SO_4$). Yeast extract as a thiamine source in the concentration of 1.0 g L^{-1} was applied in the case of medium with ammonium sulfate.

The feeding medium for continuous culture with crude glycerol consisted of (g L^{-1}): glycerol – 200, $(NH_4)_2SO_4$ – 4.6, KH_2PO_4 – 0.22, $MgSO_4$ x $7H_2O$ – 1.0, YE – 1.0 in tap water. To obtain 26.5 g L^{-1} of NaCl in the feeding medium, 21.3 g L^{-1} NaCl was added.

The pH was maintained automatically at 3 by the addition of 20% NaOH solution. The bioreactor with appropriate medium was sterilized in the autoclave at 121 °C for 20 min. All cultures were conducted in two biological replicates and standard deviations were calculated.

2.4. Analytical methods

Ten milliliters of culture broth was centrifuged (5 min, 5000 rpm) and then the biomass was washed with distilled water and filtered on 0.45 μm pore-size membranes. The biomass was determined gravimetrically after drying at 105 °C. Biomass was expressed in grams of cell dry weight per liter (g L^{-1}). Protein concentration in the biomass during the cultivation processes was analyzed using the Kjeldahl method (1883). Concentrations of glycerol, erythritol mannitol, citric acid and α-ketoglutaric acid were measured in culture supernatants by high-performance liquid chromatography (Dionex-Thermo Fisher Scientific, UK) using a Carbohydrate H + Column (Thermo Scientific, Waltham, MA) coupled to a UV (k = 210 nm) and refractive index detector (Shodex, Ogimachi, Japan). The column was eluted with 25 mM