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Improved γ-decalactone production from castor oil by fed-batch cultivation of *Yarrowia lipolytica*

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ABSTRACT

γ-Decalactone is an industrially important flavor compound with a peachy aroma which has been approved by FDA as a food additive. The aim of this study was to compare batch and fed-batch cultivation for production of γ-decalactone using *Yarrowia lipolytica* and castor oil as substrate. Microbial production of γ-decalactone from castor oil using the obligate aerobic yeast *Y. lipolytica* was investigated in a 3 l bioreactor. Batch and fed-batch fermentations were compared for the production of γ-decalactone. Also the effect of enhancing oxygen transfer rate by using higher agitation rates or pure oxygen for aeration was investigated. The highest γ-decalactone concentration (220 mg/l) was obtained in the fed-batch fermentation using pure oxygen which was 3-fold more compared to the batch cultivation. Using pure oxygen instead of atmospheric air in the fed-batch fermentation also resulted in 60% increase in γ-decalactone production.

1. Introduction

Since the dawn of civilization human beings have used flavors and fragrances mostly extracted from plants to improve the quality of life in different ways. Although plants are full of numerous flavors and fragrances, they are subject to several disadvantages including the low concentration, slow growth, and influence of geographical position and climate conditions on the composition and concentration of essential oils (Longo and Sanromán, 2006; Jong and Birmingham, 1993).

Elucidation of the structures of flavor and fragrance compounds enabled their production through chemical synthesis methods (Longo and Sanromán, 2006) which are currently used for production of about 80% of the flavors and fragrances used worldwide (Krings and Berger, 1998). Chemical synthesis of flavors and fragrances is also associated with several problems such as poor yield, side reactions and generation of undesired racemic mixtures and by-products, and environmentally unfriendly production processes (Longo and Sanromán, 2006). More importantly, the flavors produced by chemical synthesis routes are not legally labeled as "natural" (Aguedo et al., 2004) and they are labeled as "nature identical" or "artificial" in case they do not exist in nature (Scharder, 2007). In the last decades, microbial production of flavors as an alternative route to extraction from plants or chemical synthesis has received great deal of attention. Many microorganisms are able to synthesize flavor compounds using simple nutrients. The main driving force for microbial production of flavor compounds is that the flavor compounds produced by microorganisms can be labeled as "natural" (Scharder, 2007; Schrader et al., 2004; Longo and Sanromán, 2006).

Lactones are ubiquitous flavor and aroma constituents of many essential oils and plant volatiles (Başer and Demirci, 2007). The organoleptic properties of lactones contribute to the taste and aroma of many food stuff (Gatfield, 1997). γ-Decalactone, the lactone of 4-hydroxydecanoic acid, is the most widely used flavor lactone exhibiting an oily-peachy aroma. Okui et al. were the first who noticed accumulation of γ-decalactone during the growth of a Candida species on ricinoleic acid. (Okui et al., 1963). After four consecutive oxidation steps of ricinoleic acid through peroxisomal β-oxidation pathway into 4-hydroxy decanoic acid, this compound lactonizes to γ-decalactone. A variety of microorganisms are able to perform de novo biosynthesis of γ-decalactone. Commercial application of microbial systems for large scale production of γ-decalactone led to a sharp drop in the price of this compound from over 10,000 US$ kg^{-1} in the early 1980s to about 300 US$ kg^{-1} in 2004 (Schrader et al., 2004). The obligate aerobic yeast *Yarrowia lipolytica* is one of the best producers of γ-decalactone and titers up to 9.4 g γ-decalactone l^{-1} have been reported using an auxotrophic strain of *Y. lipolytica* (Pagot et al., 1997).

In most of the processes for production of γ-decalactone, ricinoleic acid is transformed into γ-decalactone by yeast strains.

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Ricinoleic acid is a long chain fatty acid that constitutes about 85% of the fatty acids in castor oil (Gomes et al., 2010; Maume and Cheetham, 1991). Therefore it is possible to use castor oil as a cheap and abundant substrate for industrial production of γ-decalactone. Both castor oil (Alchihab et al., 2010; Alchihab et al., 2009; Serrano et al., 1997; Maume and Cheetham, 1991) and castor oil hydrolysates (Maume and Cheetham, 1991; Lee et al., 1995) have been used as precursors for γ-decalactone biosynthesis. A significant difference was not observed in the final γ-decalactone concentrations for castor oil and castor oil hydrolysates. However, using untreated castor oil is preferred as the lag phase is shorter and the process does not need a separate stage for the hydrolysis (Maume and Cheetham, 1991). Although there are several studies on using castor oil as substrate for γ-decalactone biosynthesis, biotransformation of castor oil into γ-decalactone using *Y. lipolytica* has rarely been reported.

Oxygen availability has been shown to be a limiting factor for the production of γ-decalactone (Gomes et al., 2007; Aguedo et al., 2005). Hence, applying appropriate strategies to improve oxygen availability in the medium could be of great importance in microbial production of γ-decalactone. Batch cultivation has been widely used for microbial production of γ-decalactone. Fed-batch cultivation is advantageous over batch cultivation as it allows working at higher cell densities and lower residual substrate concentrations thereby reducing the inhibitory effect of substrate. However, oxygen transfer is more complicated in the fed-batch cultivation.

The aim of this study was to compare batch and fed-batch cultivation for production of γ-decalactone using *Y. lipolytica* and castor oil as substrate. Moreover, the effect of increased oxygenation on the performance of cultivation was investigated.

2. Materials and methods

2.1. Microorganism and culture conditions

Y. lipolytica (DSM 3286) was grown on liquid YPD medium at 29 °C and then maintained at 4 °C on YPD-agar medium. The biotransformation medium was composed of 6 g/l peptone, 3 g/l yeast extract, and 25 g/l castor oil.

2.2. Batch cultivations

Batch and fed-batch cultivations were carried out in a 3 l bioreactor (MS-F1, Major Science) with a working volume of 2 l. The bioreactor was equipped with two Rushton impellers and four baffles to ensure proper mixing. The pH was controlled between 5.8 and 6.2 by automatic addition of 2 M NaOH and 2 M HCl. The temperature was kept constant at 29 °C. The air flow rate was 1 vvm and the inlet air was sterilized by filtration. Carbon dioxide and oxygen concentrations were continuously monitored in the off-gas by a gas analyzer (FerMac 368, Electrolab, UK). Batch fermentations were carried out at two different conditions. In both conditions aeration rate was kept constant at 1 vmm. In the first case, however, agitation was also performed at constant rate (300 rpm) and the dissolved oxygen (DO) varied depending on oxygen consumption rate. In the second case, DO was set above 30% of air saturation by automatic increase of agitation rate when needed. Batch cultures were inoculated with 5 (v/v)% of liquid precultures prepared in shake flasks.

2.3. Fed-batch cultivations

Fed-batch cultivations were initially run similar to a batch culture. Feeding was started 18 h after inoculation with a rate of 5 ml castor oil per hour. For fed-batch cultivations, aeration was done both with air and pure oxygen. The DO levels for aeration with air and pure oxygen were set above 30% and 50% of air saturation, respectively, and variable agitation rate was used for controlling DO level.

2.4. Cell dry weight determination

Biomass concentration was measured by cell dry weight (CDW) determination of culture medium. A certain amount of sample was filtered under vacuum using 0.45 μm pore-size nitrocellulose filters (Sartorius AG, Germany). The filtered biomass was dried at 60 °C until the weight became constant.

2.5. Lactone extraction and quantification

Two milliliters of sample was taken from the bioreactor and after removing the biomass by centrifugation, 50 mg/l of γ-valerolactone was added to the supernatant as internal standard (Alchihab et al., 2009). Afterwards, lactone extraction was performed by a liquid–liquid extraction using diethyl ether as organic phase (Aguedo et al., 2004; Groguenin et al., 2004). The ether layer was recovered and the analysis was performed with GC (HP6890, Agilent Technologies) coupled to an FID detector on an HP-5 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). Helium at a flow rate of 3 ml/min was used as carrier gas. A split/splitless injector was used in split mode (split ratio, 1:30). The injector and FID detector temperatures were set to 200 and 250 °C, respectively. The oven temperature was programmed to increase from 60 to 195 °C at a rate of 20 °C/min and then at a rate of 10 °C/min to 270 °C. The oven temperature was then kept constant at this temperature for 5 min.

3. Results and discussion

3.1. Batch cultivations

Biomass, γ-decalactone, DO, agitation rate, and CO_2 concentration in the off-gas were monitored as a function of time for batch cultivations (Figs. 1 and 2). After a lag phase period, cells entered the exponential phase of growth which continued for about 24 h. Accumulation of biomass caused increased oxygen consumption rates and therefore a drastic decrease observed for DO. For the second case where DO level was set above 30% of air saturation, the higher agitation compensated for the drop in DO though in the end of exponential phase,.when the growth phase was high, it was not possible to keep the DO above the set point solely by varying agitation rate (Fig. 2). Growth of biomass was concomitant with accumulation of γ-decalactone in the medium which reached maximum almost at the end of exponential growth phase. The maximum γ-decalactone concentration reached 65 and 70 mg/l for the first and second conditions, respectively. This indicates that controlling DO did not stimulate γ-decalactone production. The DO reached below the set point nearly at the end of exponential phase when the γ-decalactone concentration was already at maximum. It is evident that higher agitation rate at this point does not help much. However, we speculate that at higher castor oil concentrations and therefore higher biomass concentrations for which the oxygen demand is higher, increased agitation rates could be helpful.

The lactone concentration drastically reduces when cells enter the stationary phase of growth. This is because yeast cells are able to reconsume the produced lactones (Pagot et al., 1998; Wache et al., 1998; Wache et al., 2000; Wache et al., 2001; Guo et al., 2011). The first step in the ß-oxidation pathway catalyzed by