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ORIGINAL RESEARCH PAPER

Fed-batch versus batch cultures of *Yarrowia lipolytica* for γ-decalactone production from methyl ricinoleate

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Abstract Constant medium feeding rate and intermittent fed-batch fermentation strategies were investigated aiming to increase the yields of γ-decalactone production by *Yarrowia lipolytica*, using methyl ricinoleate as substrate and ricinoleic acid source. The accumulation of another compound, 3-hydroxy-γ-decalactone, was also analyzed since it derives from the direct precursor of γ-decalactone thereby providing information about the enzymatic activities of the pathway. Both strategies were compared with the traditional batch mode in terms of overall productivity and yield in respect to the substrate. Although the productivity of γ-decalactone was considerably higher in the batch mode (168 mg l^{-1} h^{-1}), substrate conversion to lactone (73 mg γ-decalactone g^{-1}) was greater in the intermittent fed-batch giving 6.8 g γ-decalactone l^{-1}. This last strategy therefore has potential for γ-decalactone production at an industrial level.

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Introduction

Methyl ricinoleate, can be used to produce γ-decalactone, an interesting peach-like aroma compound, with the aerobic yeast *Yarrowia lipolytica*. This yeast degrades fatty acids through peroxisomal β-oxidation (Fig. 1). Several compounds derived from 4-hydroxydecanoic acid, the direct precursor of γ-decalactone, may be detected in the medium, among which 3-hydroxy-γ-decalactone. Despite the accumulation of those compounds decreasing the yields of γ-decalactone, it gives an indication about the activities of the enzymes involved in the pathway, namely acyl-CoA oxidase and 3-hydroxyacyl-CoA dehydrogenase.

Considering that the primary goal of fermentation research is the cost-effective production of the products, it is important to develop a culture method that allows the production of the desired product at high concentrations and with high productivity and yield.

Fed-batch cultures have been widely employed for the production of various bio-products including primary and secondary metabolites, proteins and other biopolymers. Fed-batch operation allows achieving higher cell density than batch mode and is often

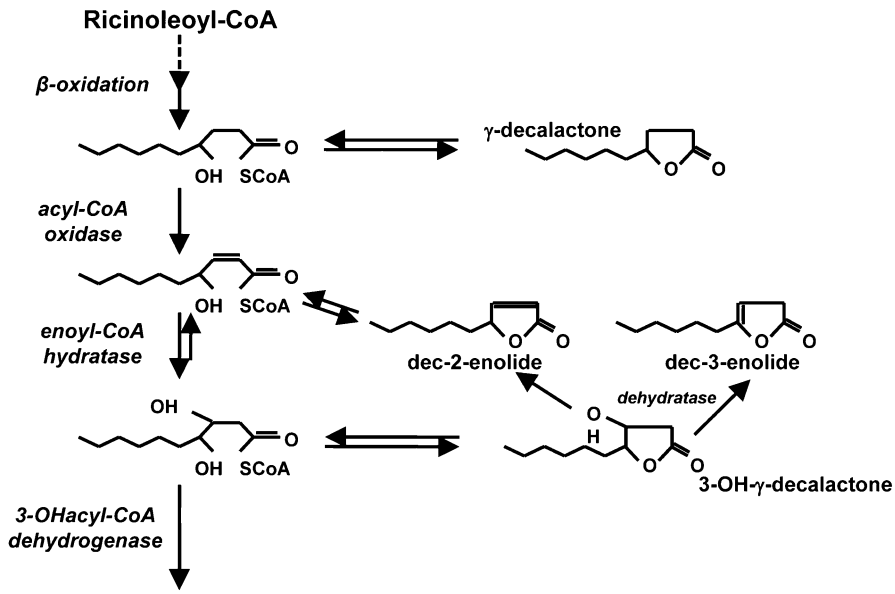

Fig. 1 β-oxidation cycle at the C10 level, during the degradation of ricinoleoyl-CoA (adapted from Bakker et al. 2001)

applied to obtain high yields and productivities of the desired product, by controlling the nutrient feeding (Lee et al. 1999). This feature is especially interesting for our process, considering the potential toxicity of ricinoleic acid to the cells (Lee et al. 1998). With this approach, it is possible to supply more substrate to the cells, simultaneously preventing toxic effects.

Several bioprocesses involving *Y. lipolytica* use the fed-batch operation mode successfully (Fickers et al. 2009; Kim et al. 1999; Kyong and Shin 2000; Nicaud et al. 2002; Rymowicz et al. 2009; Turki et al. 2010). However, there are very few works using fed-batch fermentation for γ-decalactone production (Kapfer et al. 1989; Kumin and Munch 1998; Lee et al. 1995) and, to the best of our knowledge, no reports have been published to date on its application for γ-decalactone production from methyl ricinoleate by *Y. lipolytica*. Thus, the main aim of this study was to explore the fed-batch operation strategy to increase the yields of γ-decalactone production.

Materials and methods

Microorganism, media and culture conditions

Yarrowia lipolytica W29 (ATCC20460) was cultured and grown as described by Gomes et al. (2011), unless otherwise stated. The biotransformation medium components were then added as a solution to start the biotransformation stage. The source of ricinoleic acid used was methyl ricinoleate (MR). The composition of the biotransformation medium was: 6.7 g YNB (Yeast Nitrogen Base) with amino acids l^{-1}, 2.5 g NH_4Cl l^{-1}, 30 g MR l^{-1} and 3 g Tween 80 l^{-1}.

All chemicals were purchased from Sigma-Aldrich, except for methyl ricinoleate that was kindly supplied by Biotor Industries Limited (Mumbai, India).

Operating conditions

Batch and fed-batch cultures were conducted in the bioreactor at the optimal conditions for γ-decalactone production (pH 6.2 and a dissolved O_2 concentration of 44%) (Gomes et al. 2011).

Two different strategies of fed-batch culture were investigated: constant and intermittent feeding rate. In the first case, growth occurred in 1 l glucose medium to which the biotransformation medium components were added. After the maximum peak of γ-decalactone production was reached, 1.5 l biotransformation medium was fed to the bioreactor at 1 ml min^{-1} (initial dilution rate = 0.06 h^{-1}). In the second approach, the growth occurred in 1.7 l YPD medium, to which the biotransformation medium components were added. When no more MR was detected in the medium, 30 g MR l^{-1} were added to the bioreactor. This addition occurred in two cycles.

Sampling and analytical methods

Samples were collected at intervals for analysis of cell concentration and viability and for lactones and MR quantification. Cell concentration was estimated using a Neubauer-improved counting chamber (Mather and Roberts 1998). For viability, the Methylene Blue method was used (Bonora and Mares 1982).

Lactones and MR were extracted from 2 ml samples with 2 ml diethyl ether. The organic phase was analyzed by GC as described by Gomes et al. (2010).

Results and discussion

Yarrowia lipolytica can use γ-decalactone as a carbon source when the substrate is completely consumed (Pagot et al. 1998) resulting in its complete disappearance from the medium after some hours of batch operation. To avoid this, as well as to prevent the inhibitory effects of the substrate (ricinoleic acid) on the cells, two different fed-batch operation strategies were investigated. In a first approach, the biotransformation medium was fed continuously to the bioreactor when γ-decalactone concentration began to decline; secondly, an intermittent fed-batch strategy was attempted in which MR, after its exhaustion, was added in pulses to the bioreactor.

Growth was low during the biotransformation in batch and fed-batch modes of operation. Cell numbers in the medium slightly increased through time and generally varied between 10^9 cells ml^{-1} and 2.5 × 10^9 cells ml^{-1}. The viability of the cells remained practically constant at 100% during the whole experiments.

The low cellular growth in lipidic substrates was also reported by Turki et al. (2010), being responsible for the cellular mass concentration decrease in fed-batch cultivation, compared to the high-cell mass concentration attainable when glucose is used (Kim et al. 1999).

The production of two lactones, γ-decalactone and 3-hydroxy-γ-decalactone, was monitored under different culture strategies. The accumulation of the latter is a result of a deviation in the metabolic pathway responsible for the formation of γ-decalactone, which thus gives indications on the role of the enzymes involved.

The accumulation of both lactones in the biotransformation medium on batch and fed-batch modes is depicted in Fig. 2.

In the fed-batch cultures, the concentration of both lactones increased after the feeding of additional medium (in the experiments at constant medium feeding rate) and after each MR addition (in the intermittent fed-batch strategy).

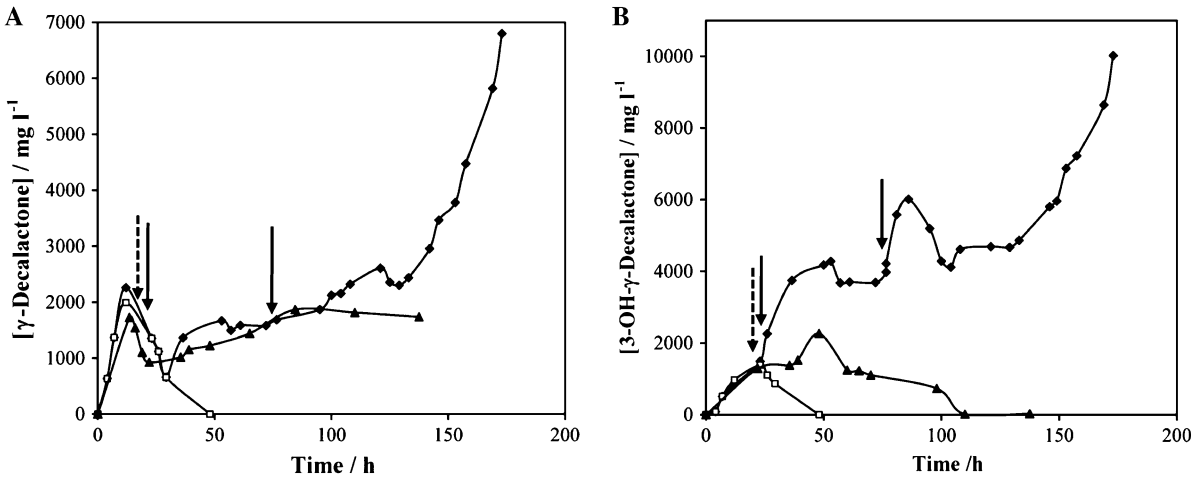

Fig. 2 Production of γ-decalactone (**a**) and 3-hydroxy-γ-decalactone (**b**) by *Y. lipolytica* cultures in batch (*open square*), fed-batch at a constant medium feeding rate of 1 ml min^{-1} (*filled triangle*) and intermittently (*filled diamond*). The *dashed arrow* indicates the time at which the biotransformation medium started to be fed to the bioreactor in the fed-batch experiments at constant feeding rate and the solid arrows indicate MR addition to the medium in the intermittent fed-batch strategy

In the first fed-batch strategy (using a constant feeding rate of 1 ml min^{-1}), a maximum of 1,867 mg γ-decalactone l^{-1} was obtained after 85 h and was slightly less than 1,993 mg l^{-1} achieved in the batch culture after 12 h of biotransformation. In batch culture, after reaching its peak, the aroma concentration decreased progressively until complete disappearance from the medium: this behavior was not observed with this fed-batch strategy. After the feeding of fresh medium, the aroma concentration increased and was maintained in the biotransformation medium during the whole experiment. This reinforces the hypothesis of the consumption of lactone by the yeasts only in the absence of oil.

Regarding the accumulation of 3-hydroxy-γ-decalactone, a maximum of 2,265 mg l^{-1} was obtained after 48 h in the fed-batch culture compared to 1,388 mg l^{-1} achieved in the batch trial at 23 h. In both cases, after reaching the peak of production, the concentration of this lactone decreased until complete disappearance from the medium. As far as we know, this has not been previously reported.

γ-Decalactone started to accumulate at a relatively constant concentration when 3-hydroxy-γ-decalactone concentration began to decrease. Thus, it is possible that, at this point, the β-oxidation pathway is controlled by acyl-CoA oxidase, which implies a higher accumulation of γ-decalactone in detriment of 3-hydroxy-γ-decalactone.

Since this fed-batch approach led to the production of approximately the same amount of γ-decalactone produced in batch but taking more time, the overall productivity of the process was inferior to that obtained in batch. For this reason, and in the expectation of achieving higher γ-decalactone concentrations and productivities, an intermittent fed-batch strategy, based on the intermittent addition of MR was developed. Figure 2 shows that the length of each step of MR addition became longer: in the first step (corresponding to the batch mode), 24 h were needed for the total disappearance of MR from the medium (data not shown); in the second step, 50 h; and in the last step, it took 100 h to completely degrade the substrate.

The fact that cells need more time to convert ricinoleic acid into lactones is most likely due to the toxic effect resulting from the exposure of cells to such high lactone concentrations, affecting their performance in the degradation of the substrate. The toxicity that lactones exhibit on microorganisms is potentially a major problem for reaching higher yields (Feron et al. 1996; Feron et al. 1997). Nevertheless, the concentration of both lactones increased along the whole experiment and high amounts of γ-decalactone and 3-hydroxy-γ-decalactone were achieved with this strategy: 6,798 mg l^{-1} and 10,018 mg l^{-1}, respectively, after 173 h of biotransformation. The γ-decalactone concentration achieved was slightly higher than the values obtained by Alchihab et al. (2010) that used batch cultures of *Rhodotorula aurantiaca* with Macronet resins to recover the γ-decalactone produced, thus reducing the product toxicity effects. Similar behavior describing the product increase until the end of the experiment herein reported was described in other works involving fed-batch cultures (Kim et al. 1999; Kyong and Shin 2000; Nicaud et al. 2002; Rymowicz et al. 2009).

An increase on γ-decalactone production in fed-batch compared to batch cultures was also reported by Lee et al. (1995). These authors attempted two fed-batch strategies to produce γ-decalactone by *Sporobolomyces odorus* from castor oil hydrolysate as source of ricinoleic acid. Initially, the substrate was added intermittently to the bioreactor, to maintain the ricinoleic acid level at approx. 0.06% in the medium. With this strategy, an improvement on γ-decalactone production was achieved: 164 mg l^{-1} compared to 60 mg l^{-1} obtained in batch culture. In accordance with our results, that research group obtained even better results when they used a three-step feeding of castor oil: a maximum of 208 mg γ-decalactone l^{-1} was obtained over 168 h.

The use of fed-batch cultures also allowed Kumin and Munch (1998) to claim, in a patent, a yield of 10.5 g γ-decalactone l^{-1} after 60 h culture with Mucor circillenoides and ethyl decanoate as substrate, which was continuously added at a nutrient broth feeding rate of 2–3 g l^{-1} h^{-1}.

The productivities (P) and the overall lactone yields relative to the substrate ($Y_{L/S}$) for each mode of operation were calculated according to Eqs. 1 and 2.

$$P = \frac{L_f V_f - L_0 V_0 + \sum_i L_{si} V_{si}}{(V_f - V_0) t_f} \quad (1)$$

$$Y_{L/S} = \frac{L_f V_f - L_0 V_0 + \sum_i L_{si} V_{si}}{S_0 V_0 - S_f V_f + S_f \int_0^t F(t) dt} \quad (2)$$

where L, is the concentration of lactone; V, the volume of medium; t, the time; S_F, the concentration of

Table 1 Productivities (P) and yields in respect to the substrate ($Y_{L/S}$) achieved in each of the biotransformation strategies investigated (batch, fed-batch with constant medium feeding rate and intermittent fed-batch) for both lactones

Operation strategies	γ-Decalactone		3-OH-γ-decalactone	
	P (mg l^{-1} h^{-1})	$Y_{L/S}$ (mg g^{-1})	P (mg l^{-1} h^{-1})	$Y_{L/S}$ (mg g^{-1})
Batch	168	66	61	46
Fed-batch at constant feeding rate	40	38	81	46
Intermittent fed-batch	43	73	64	105

substrate in the feeding medium; F, the medium feeding rate; and the subscripts f, 0 and si correspond to the final, initial and samples conditions, respectively.

The calculated values of productivities and yields were adjusted, taking into account the loss of lactone that occurred due to sampling. For this purpose, the sum of the amount of lactone removed from the reactor in the samples was introduced in the numerator of the equation.

As a summary, the overall productivities and yields of lactones relative to the substrate are displayed on Table 1.

Although the productivity of γ-decalactone was considerably higher in the batch mode, the level of substrate conversion to both lactones was higher in the intermittent fed-batch, leading to higher aroma concentrations, which may have an important impact on a subsequent purification process. Nevertheless, the productivity of γ-decalactone obtained in the intermittent fed-batch technique was similar to the one obtained by Alchihab et al. (2010), which was 45 mg l^{-1} h^{-1} with another yeast strain and castor oil.

Concerning 3-hydroxy-γ-decalactone, the highest productivity was obtained in the fed-batch with constant feeding rate technique, contrary to γ-decalactone which showed the lowest productivity level under those conditions. In terms of yields relative to the substrate, the intermittent fed-batch was also the strategy more profitable for 3-hydroxy-γ-decalactone production, suggesting that if we could reduce the accumulation of this compound in further studies, by optimizing operating conditions, the yields of our product of interest, γ-decalactone, would be greatly increased.

Conclusion

γ-Decalactone production by intermittent fed-batch technique using *Y. lipolytica* is a process with great potential for industrial application since the conversion of ricinoleic acid into γ-decalactone was improved through this process and high values of aroma concentration were reached. However, further studies are required to optimize the process, namely the optimization of the rate of substrate addition during the biotransformation. Nevertheless, considering that this is the first report on the production of γ-decalactone from methyl ricinoleate, by *Y. lipolytica*, under fed-batch conditions, the results herein presented are very promising and give new insights for developing an innovative process with a potential for producing the aroma at industrial level.

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