JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

OPTIMAZTION OF GAMMA-DECALACTONE PRODUCTION BY YEAST *YARROWIA LIPOLYTICA* USING THE TAGUCHI METHOD

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doi: 10.15414/jmbfs.2016.6.1.685-688

ARTICLE INFO

Received 17. 10. 2014
Revised 13. 3. 2016
Accepted 5. 4. 2016
Published 1. 8. 2016

Regular article

OPEN ACCESS

ABSTRACT

The γ-decalactone is one of the lactones with peachy aroma which has been approved as food additive by FDA. The aim of this study was to optimize media composition and conditions for microbial biotransformation of ricinoleic acid and castor oil as substrates to γ-decalactone using the yeast *Yarrowia lipolytica*. The *Y. lipolytica* DSM 3286 strain was used as biotransformation agent in different trails designed by Taguchi method. The highest concentration of γ-decalactone was 62.4 and 52.9 mg/L from 1.5% ricinoleic acid and 2.5% castor oil, respectively. Nitrogen sources exhibited significant effect on the biotransformation. The maximum γ-decalactone production occurred at pH 6. The results showed that the composition of biotransformation medium composition is important for γ-decalactone production.

Keywords: γ-Decalactone, *Yarrowia lipolytica*, Ricinoleic acid, Castor oil, Biotransformation, Taguchi method

INTRODUCTION

Lactones are attractive molecules as additive for food and pharmaceutical products due to their pleasant flavor. The γ-decalactone is one of the lactones with peach flavor which has been approved as food additive by FDA. Microbial production of flavors as an alternative route to extraction from plants or chemical synthesis has received great deal of attention. A variety of microorganisms can be used 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" **(Longo & Sanromán, 2006; Scharder, 2007; Schrader, Etschmann, Sell, Hilmer, & Rabenhorst, 2004**). Lactones are ubiquitous flavor and aroma constituents of many essential oils and plant volatiles (**Başer & Demirci, 2007**). The 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 the accumulation of γ-decalactone during the growth of a *Candida* species on ricinoleic acid (**Okui, Uchiyama, & Mizugaki, 1963; Romero-Guido *et al.*, 2011**).
The medium composition is of great importance in microbial production of γ-decalactone. Microorganisms can use ricinoleic acid as suitable substrate for production of this aroma compound (**Lee & Chou, 1994**). Castor oil which contains 85% of ricinoleic acid could be used as readily available, inexpensive substrate for γ-decalactone production (**Dufosse *et al.*, 1998; Neto, Pastore, & Macedo, 2004**).
Environmental conditions such as temperature and pH are important and influence the γ-decalactone production. The nitrogen sources effect on this fermentation is complex. The highest yields were obtained using complex nitrogen sources which is probably due to supplying trace nutrients and minimizing the toxic effects of fatty acids by sequestering them (**Maume & Cheetham, 1991**).
The non-conventional yeast *Yarrowia lipolytica* have good potential to production of γ-decalactone. In the previous studies, this yeast has been used for γ-decalactone production from ricinoleic acid and castor oil as substrates (**Aguedo, Wache, Belin, & Teixeira, 2005; N. Gomes, Teixeira, & Belo, 2010; Moradi, Asadollahi, & Nahvi, 2013**).
Since control of bioconversion parameters was shown to significantly affect γ-decalactone production, a large number of experiments are needed to optimize bioconversion conditions (**Lee & Chou, 1994**). To circumvent this problem, Taguchi method was used for optimization of bioconversion conditions. Taguchi method requires only a small number of experiments to study the entire parameters involved in process. Furthermore, this method allows studying effects of multiple factors on the process yield in a fast and economic way using an orthogonal array design (**Montgomery, 1991**). Taguchi method shows the importance of distinct values to improve the process and product quality (**Fraley, Oom, Terrien, & Alewsk, 2007**). This study therefore aimed to optimize important factors affecting γ-decalactone production from ricinoleic acid and castor oil as substrates by the Taguchi method.

MATERIAL AND METHODS

Microorganism and culture conditions

Y. lipolytica DSM 3286 was cultured on YPD medium at 29 ˚C, and maintained at 4 ˚C on YPD-agar (**Barth & Gaillardin, 1996**). Basal main medium for γ-decalactone contained ricinoleic acid or castor oil, yeast extract, peptone. The flasks were incubated in shaker-incubator at 200 rpm and 29 ˚C **(Moradi *et al.*, 2013**).

Design of experiments

The Taguchi fractional factorial experiment design approach has been used for optimization of production variables. It is a robust methodology against uncontrollable environmental changes (**Patil, Sachin, Wakte, & Shinde, 2014**). The Qualitek-4 software was used for Taguchi experimental design in this research. In the previous study, it was shown that substrate (ricinoleic acid or castor oil), yeast extract concentration, peptone concentration, and pH are the four main factors affecting production of γ-decalactone by *Y. lipolytica* (**Moradi *et al.*, 2013**). Therefore, these four main factors were used each at four levels as shown in Tables 1-3.

Lactone extraction and detection

For extraction and analysis of γ-decalactone, 50 mg/L of γ-valerolactone (internal standard) was added to 2 mL of filtered sample of the culture medium (**Alchihab *et al.*, 2009**). Afterwards, diethyl ether was used as organic phase to lactone extraction **(Aguedo, Wache, Coste, Husson, & Belin, 2004; Groguenin *et al.*, 2004**). The ether layer was recovered and the analysis was performed with HP6890 gas chromatograph (Agilent) equipped to an FID detector. The analytes were separated on a HP-5 capillary column with helium as carrier gas at a flow

rate of 3 mL/min. A split/splitless injector was used in the split mode (split ratio 1:30). The injector and FID temperatures were 200 and 250 ˚C, respectively. The oven temperature was increased from 60 to 195 ˚C at a rate of 20 ˚C /min and then at a rate of 10 ˚C /min to 270 ˚C (**An, Joo, & Oh, 2013; Moradi *et al.*, 2013)**.

RESULTS

In order to investigate the best conditions for optimum γ-decalactone production from ricinoleic acid or castor oil as substrates by *Y. lipolytica* DSM 3286, trails were done according to experiments proposed by the Qualitek-4 software (Fig. 1 and Fig. 2).

In the case of ricinoleic acid as substrate, maximum γ-decalactone production of 62.4 mg/L was obtained at concentrations of ricinoleic acid 15 mL/L, peptone 3 g/L, and yeast extract 9 g/L at pH 6 (Trail 10).

The main effects of each factor on γ-decalactone production from ricinoleic acid by *Y. lipolytica* DSM 3286 were determined by Qualitek-4 software (Fig. 3). Lower γ-decalactone production rate was observed at higher ricinoleic acid concentrations and pH whereas increasing yeast extract concentration had positive effect and enhanced production rate of γ-decalactone. Peptone concentration had variable effects on γ-decalactone production.

Optimum conditions for γ-decalactone production from castor oil were obtained as 25 mL/L of castor oil, 9 g/L of peptone and yeast extract, and pH 6 in which 52.9 mg/L of γ-decalactone was produced. However, this combination had not been used in any of the experiments designed by the software.

The main effects of each factor on γ-decalactone production from castor oil by *Y. lipolytica* DSM 3286 were determined by Qualitek-4 software (Fig. 4). If increase the amounts of castor oil and yeast extract, the production of γ-decalactone will be increased. Peptone had no significant effect on γ-decalactone production. Acidic pH is better than neutral pH for production of γ-decalactone by *Y. lipolytica* DSM 3286 (Fig. 4).

Since maximum γ-decalactone titer was observed at the highest concentration of castor oil (25 mL/L), the effect of higher concentrations of castor oil on the γ-decalactone production was examined. However, it was found that higher concentrations of castor oil did not further improve γ-decalactone production (Fig. 5).

Table 1 Levels of factors for ricinoleic acid as substrate

Factors	Level 1	Level 2	Level 3	Level 4
Ricinoleic acid (mL/L)	5	10	15	20
Peptone (g/L)	0	3	6	9
Yeast extract (g/L)	0	3	6	9
pH	4	5	6	7

Table 2 Levels of factors for castor oil as substrate

Factors	Level 1	Level 2	Level 3	Level 4
Castor oil (mL/L)	10	15	20	25
Peptone (g/L)	0	3	6	9
Yeast extract (g/L)	0	3	6	9
pH	4	5	6	7

Table 3 The L16 orthogonal array for trails

Trial number	Ricinoleic acid or castor oil (mL/L)	Peptone (g/L)	Yeast extract (g/L)	pH
1	Level 1	Level 1	Level 1	Level 1
2	Level 1	Level 2	Level 2	Level 2
3	Level 1	Level 3	Level 3	Level 3
4	Level 1	Level 4	Level 4	Level 4
5	Level 2	Level 1	Level 2	Level 3
6	Level 2	Level 2	Level 1	Level 4
7	Level 2	Level 3	Level 4	Level 1
8	Level 2	Level 4	Level 3	Level 2
9	Level 3	Level 1	Level 3	Level 4
10	Level 3	Level 2	Level 4	Level 3
11	Level 3	Level 3	Level 1	Level 2
12	Level 3	Level 4	Level 2	Level 1
13	Level 4	Level 1	Level 4	Level 2
14	Level 4	Level 2	Level 3	Level 1
15	Level 4	Level 3	Level 2	Level 4
16	Level 4	Level 4	Level 1	Level 3

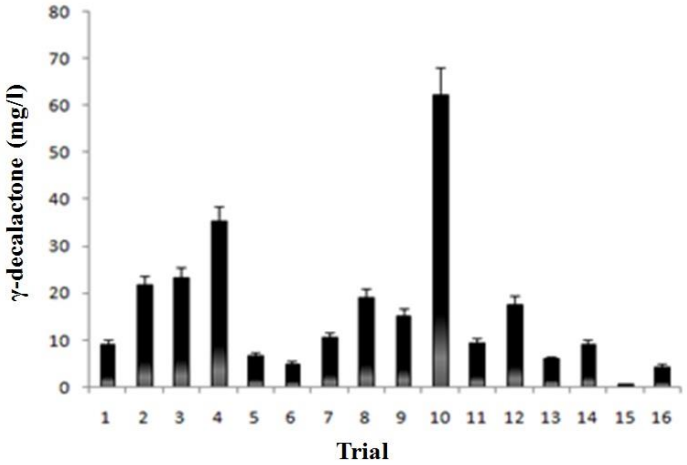

Figure 1 The results of different trails proposed by Qualitek-4 software for γ-dacalactone production on ricinoleic acid as substrate by *Y. lipolytica* DSM 3286.

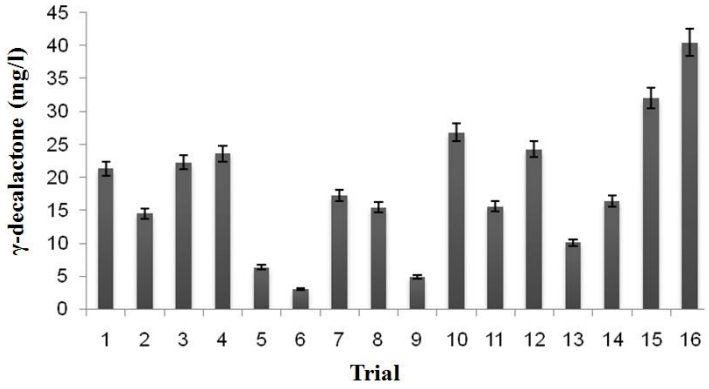

Figure 2 The results of different trails proposed by Qualitek-4 software for γ-dacalactone production on castor oil as substrate by *Y. lipolytica* DSM 3286.

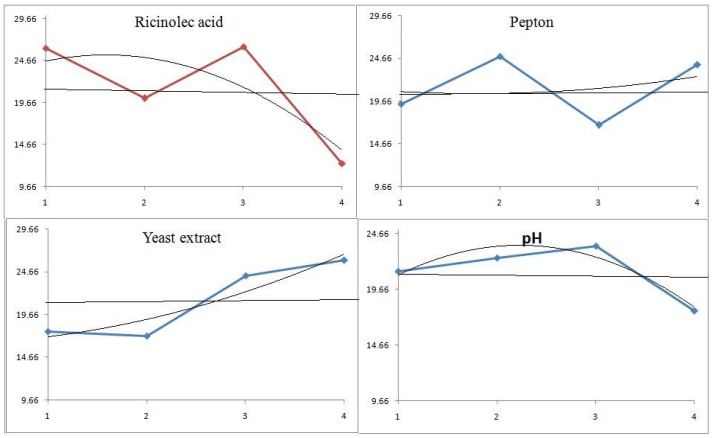

Figure 3 The main effects of each factor on γ-decalactone production from ricinoleic acid by *Y. lipolytica* DSM 3286.

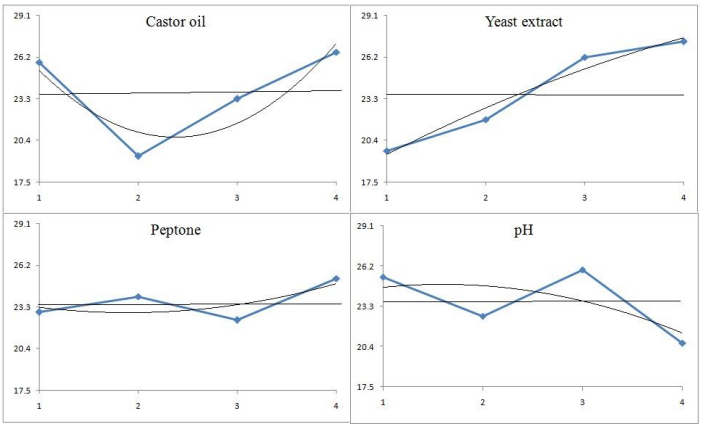

Figure 4 The main effects of each factor on γ-decalactone production from castor oil by *Y. lipolytica* DSM 3286.

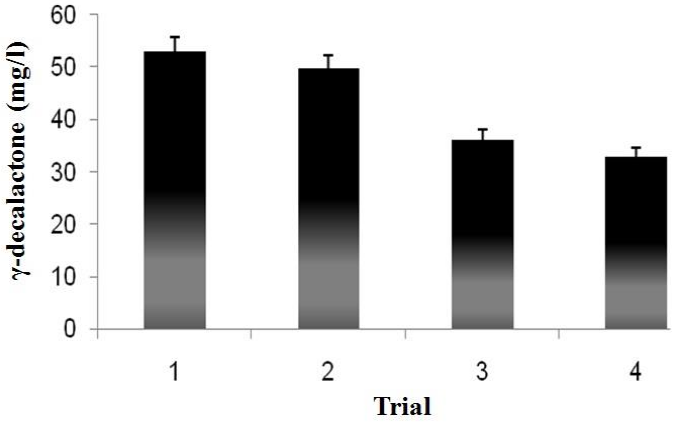

Figure 5 Confirmatory tests for γ-decalactone production from castor oil by *Y. lipolytica* DSM 3286. Trail (1) is a confirmatory test for proposed experiment by the software; Trails (2,3,4) contain 30, 35 and 40 mL/L of castor oil, respectively, along with other suggested factors for optimization including peptone 9 g/L, yeast extract 9 g/L and pH 6.

DISCUSSION

Yeasts are excellent biocatalysts in the field of alkane and fatty acids transformation into dicarboxylic acids and lactones (**Waché, 2013**). The γ-decalactone production can be improved by cell density, oil concentration and oxygen transfer rate in batch and step-wise fed-batch cultures of *Y. lipolytica* (**Braga & Belo, 2015**).

Some of yeasts such as *Y. lipolytica* can produce γ-decalactone from ricinoleic acid as key precursor and substrate **(Darvishi Harzevili, 2014**). Castor oil is natural and nontoxic oil, biodegradable, and a renewable resource obtained from the seeds of the castor plant *Ricinus communis* (**Puthli, Rathod, & Pandit, 2006**). The castor oil is rich in ricinoleic acid and therefore could be used as cheap source for γ-decalactone production (**Braga & Belo, 2013; Nelma Gomes, Braga, Teixeira, & Belo, 2013; N. Gomes *et al.*, 2010**). *Y. lipolytica* is able to hydrolyze castor oil to ricinoleic acid and gradually use it, because a high concentration of this acid has inhibitory effect on the growth of yeast cells. Presence of castor oil at the beginning of the fermentation and then adding it at a later stage could enhance the γ-decalactone production level by *Y. lipolytica* because the production of γ-decalactone needs continuous induction of the β-oxidation pathways or fatty acid uptake and export systems (**Feron, Blin-Perrin, Krasniewski, Mauvais, & Lherminier, 2005; Maume & Cheetham, 1991**). Yeast extract and peptone were used as nitrogen sources for the production of γ-decalactone in this research according to previous studies (**Alchihab *et al.*, 2009; Moradi *et al.*, 2013**).

Four factors with four levels were chosen for optimization of γ-decalactone production by the Taguchi method as fractional factorial experiment design. First factor was carbon source (ricinoleic acid or castor oil) which have been investigated as substrate and an activator for enzymes in the pathway of γ-decalactone synthesis in *Y. lipolytica*. Second and third factors were nitrogen sources including yeast extract and peptone that are important to increase cell growth and biotransformation. Last factor was pH which should be optimized to support maximum cell growth and γ-decalactone production.

Maximum production of γ-decalactone was 62.4 and 52.9 mg/L by *Y. lipolytica* DSM 3286 on ricinoleic acid and castor oil as substrates, respectively. According to the previous studies, the nitrogen source effect was complex on this bioconversion. The γ-decalactone yield was low in the media with less than 2 g/L nitrogen content. Furthermore, a little additional γ-decalactone was produced in media containing greater than 20 g/L of nitrogen concentrations. The function of these complex protein sources is important to increase cell growth and minimize toxic effect in biotransformation (**Maume & Cheetham, 1991; Patil *et al.*, 2014**).

The optimum pH was 6 to γ-decalactone production for both substrates. The bioconversion of ricinoleic acid into 4-HDA and then γ-decalactone was obtained at acid pH (**Lee, Lin, & Chou, 1995**). Other researchers used this pH (**Aguedo, Ly, et al., 2004; Alchihab *et al.*, 2009; Wache, Aguedo, LeDall, Nicaud, & Belin, 2002; Wache, Aguedo, Nicaud, & Belin, 2003**) but in this work pH was optimized step by step from acidic to neutral.

CONCLUSION

In conclusion, biotransformation medium composition is important for γ-decalactone production. In particular, interaction of carbon sources and yeast extract as nitrogen source is important. Furthermore, nitrogen sources are important to increase cell growth and biotransformation. Microbial fermentation was used as a potential tool to the production of natural lactones and attractive subject for researchers in this filed. The production of lactone will be established on the industrial scale because it is an extracellular product and can be produced easily with higher yields by culturing yeast in a bioreactor.

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