

Enhanced Biotransformation Productivity of Gamma-Decalactone from Ricinoleic Acid Based on the Expanded Vermiculite Delivery System

Shimin Guan, Shaofeng Rong, Mengze Wang, Baoguo Cai, Qianqian Li, and Shuo Zhang*

Department of Biological Engineering, Shanghai Institute of Technology, Shanghai 201418, P.R. China

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*Corresponding author

Phone: +86-021-60877260;

E-mail: zhangshuo0430@163.com

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Natural gamma-decalactone (GDL) produced by biotransformation is an essential food additive with a peach-like aroma. However, the difficulty of effectively controlling the concentration of the substrate ricinoleic acid (RA) in water limits the biotransformation productivity, which is a bottleneck for industrialization. In this study, expanded vermiculite (E-V) was utilized as a carrier of RA to increase its distribution in the medium. E-V and three commonly used organic compounds were compared with respect to their effects on the biotransformation process, and the mechanism was revealed. Scanning electron microscopy, Fourier transform infrared spectroscopy and thermogravimetric analysis indicated that RA was physically adsorbed onto the surface of and inside E-V instead of undergoing a chemical reaction, which increased the opportunity for interactions between microorganisms and the substrate. The highest concentration of GDL obtained in the medium with E-V was 6.2 g/l, which was 50% higher than that in the reference sample. In addition, the presence of E-V had no negative effect on the viability of the microorganisms. This study provides a new method for producing natural GDL through biotransformation on an industrial scale.

Keywords: Biotransformation, gamma-decalactone, ricinoleic acid, expanded vermiculite

Introduction

Lactones are molecules resulting from hydroxy acid cyclization, which comprises a carbon cycle with one oxygen atom. Lactones are attractive flavor additives for food and pharmaceutical products due to their fruit aroma [1]. Among these compounds, gamma-decalactone (GDL) is the most important, with a characteristic peach-like flavor detectable at low concentrations below 5 mg/l [2]. Traditionally, GDL is obtained by direct extraction from fruits and plants or by chemical synthesis. However, the aromatic quality of synthetic GDL is lower than that of natural GDL. The yield from the extraction method is limited and influenced by geographical and climatic conditions, resulting in a high price (US_\$3,000/kg). In recent years, the utilization of microorganisms and enzymes for the production of flavor compounds has received a great deal of attention. The main driving force is that the flavor compounds produced by this biotechnological method can be labelled “natural” with much lower cost [2].

In the biotransformation process, several microorganisms have been selected for their potential to produce aroma substances, including *Pseudomonas*, *Sporobolomyces*, *Pichia*, *Candida*, *Rhodotorula*, and *Yarrowia lipolytica* [2]. Among these microorganisms, *Y. lipolytica* is reported to have the strongest productivity [3]. Castor oil is commonly utilized as a substrate for the production of GDL by microorganisms [3–5]. However, the castor oil should be hydrolyzed to ricinoleic acid (RA), which accounts for 86% of castor oil as the main component, prior to entering the bioconversion cycle [6]. Inside the cells of microorganisms, RA enters the mitochondria. After four β -oxidation cycles, RA degrades to 4-hydroxy-decanoyl-CoA, which then cyclizes to GDL [3]. Moradi et al. reported that the highest concentration of GDL was 62.4 and 52.9 mg/l from 1.5% RA and 2.5% castor oil, respectively. [7] Rong et al. proved that L-carnitine shortened the biotransformation period by approximately 10 h and increased GDL production by 19.5% when RA was utilized by *Saccharomyces cerevisiae* MF013 [8].

However, the low solubility of RA in water limits the

probability that microorganisms will come into contact with the substrate, which results in a low yield of GDL. To increase the solubility of the substrate, many surfactants have been added to the media. Nelma Gomes reported much more accumulation of GDL, approximately 1.8 g/l, after the addition of Tween 80 [9]. Aguedo discovered that two tested ionic surfactants could increase the solubility and transport rate of RA [10, 11]. However, removing surfactants from the final products in the extraction phase is difficult. In addition, some surfactants have been reported to change the permeability of the cell membrane during biotransformation, which may exert a toxic effect on the cell membrane at certain concentrations [12]. Therefore, seeking a new approach to increase the dispersion of the substrate without inhibiting cellular activity is essential to improve the production of GDL.

Adsorption is recognized as a promising technique due to its ease of operation, easy availability, high efficiency and relative low cost of application in the biotransformation process. Expanded vermiculite (E-V) is a natural, multilayer-structure magnesium aluminium silicate inorganic mineral with many crystal interpacket spaces acting as pores; the volume of the material can be expanded by 8 to 30 times depending on the texture and the thermal conditions used [13]. Charges located between the sheets contribute to the good ion-exchange properties of E-V. Owing to its remarkable abundance and cheap price, E-V is widely applied in the agricultural, industrial and environmental fields as a micro-porous, non-toxic, absorbent and sustained-release material [14, 15]. Because E-V is an inert inorganic material, toxic effects on cells and the phenomenon of emulsification can be avoided.

In this study, we utilized E-V as a carrier of RA to increase the distribution and controlled release of the substrate in media and compared its effect on biotransformation with three commonly utilized hydrophilic organic solvents: dimethylsulfoxide (DMSO), ethylene glycol and acetone [16]. Changes in cell proliferation, pH, and the concentrations of RA and GDL were monitored every 12 h. Scanning electron microscopy (SEM), gas chromatography (GC) and high-performance liquid chromatography (HPLC) were utilized to reveal the adsorption mechanism of the E-V-RA complexes and the relationship between the RA release rate and GDL productivity. To the best of our knowledge, most studies have focused on increasing GDL productivity using surfactants or immobilization of microorganisms; only a few studies have focused on the utilization of an RA-controlled delivery system to disperse the substrate. Our research is the first attempt to use E-V

with the purpose of efficiently producing GDL at the industrial level.

Materials and Methods

Microorganisms and Reagents

Y. lipolytica (CGMCC 2.2087), used for GDL biotransformation, was obtained from the China General Microbiological Culture Collection Center (China). The RA substrate was obtained from Zibo Zhoucun Mingdong Chem, Ltd. (China). E-V was obtained from Suzhou Yilufa Environmental Protection Technology, Ltd. (China). The GDL standard was provided by Adamas (China). Analytical-grade ethanol, DMSO, ethylene glycol, acetone and other chemicals were purchased from Sinopharm Chemical Reagent, Ltd. (China).

Cultivation of Microorganisms

Y. lipolytica (CGMCC 2.2087) was cultured for 30 h on agar slant culture medium (20 g/l agar, 10 g/l glucose, 10 g/l peptone, and 12 g/l yeast extract) at 28°C and inoculated in a 250 ml baffled Erlenmeyer flask containing 50 ml of YPD medium (10 g/l glucose, 10 g/l peptone, and 12 g/l yeast extract). Flasks were shaken at 200 rpm and 28°C for 24 h until the cultures reached the late-logarithmic growth phase. These suspensions were used to inoculate the biotransformation media.

Preparation of RA and E-V Complexes

RA was embedded in E-V through the following steps. A 1:1 mass ratio of RA to E-V was stirred for 30 min in a 50°C water bath. The embedded complexes were added to the biotransformation medium and were subjected to wet sterilization at 121°C for 15 min. The adsorption capacity of E-V was estimated as follows:

$$\text{Adsorption capacity (g/g)} = \frac{\text{Weight of embedded material (g)} - \text{Weight of E-V (g)}}{\text{Weight of E-V (g)}}$$

Bioconversion of RA to GDL

Two different strategies of GDL production were compared: the addition of E-V and organic solvents. For the medium with E-V, 15% *Y. lipolytica* cell suspensions were inoculated directly into 30 ml biotransformation medium containing 80 g/l E-V-RA complex (containing 40 g/l RA), 7 g/l MgSO₄, 7 g/l K₂HPO₄, 15 g/l glycerol, and 0.1 g/l carnitine in ultrapure water at the natural pH of the medium. For the medium with organic solvents, after 1% DMSO, ethylene glycol, and acetone were added individually to medium containing 40 g/l RA, 7 g/l MgSO₄, 7 g/l K₂HPO₄, 15 g/l glycerol, and 0.1 g/l carnitine in a sterile environment, 15% cell suspensions were inoculated. The culture was agitated at 250 rpm at 28°C for 84 h. A medium without E-V or organic solvents was prepared as a reference. In all cases, samples were collected aseptically every 12 h.

Analytical Methods

a) Cell concentration

The free cell concentration was estimated by UV spectrophotometry. Each sample taken from the medium was diluted 15 times for triplicate measurements.

b) Characterization of embedded material

Images of the E-V, embedded complex, and adhered *Y. lipolytica* were obtained through SEM (Hitachi S-3400N, Japan) at an accelerating voltage of 15.0 kV. Samples were fixed on a specimen holder with aluminium tape and sputtered with gold under high-vacuum conditions.

Thermogravimetric (TG) analysis (NETZSCH, Germany) of the E-V, RA, and embedded material was performed. Nitrogen was used as the shielding gas during the analysis at a flow rate of 20 ml/min, and the temperature ranged from 30–700°C at a rate of 10°C/min.

Differential thermogravimetric (DTG) analysis was performed by calculating the first derivative of the TG curve or the rate of mass change rate with respect to temperature and time. The mathematical expression is:

$$\text{DTG } (\%/^{\circ}\text{C}) = \frac{dW/dt}{dT/dt}$$

where W is the mass fraction, T is the programmed temperature, and t is time.

Fourier transform infrared (FTIR) spectroscopy (Thermo Fisher Scientific Inc., USA) was used to analyze the E-V, RA, and embedded material in the 4,000–400 cm^{-1} range. Samples were blended with KBr to form pellets.

c) pH Measurement

Samples were collected for pH analysis with a pH meter (PHS-3C, Leici, China) every 12 h in triplicate.

d) Analysis of components in fermentation broth

Samples were collected at intervals to determine the concentrations of GDL and RA. Pure chemical compounds ($\geq 98\%$) were used as standards. Each sample was mixed with certain volumes of ethanol, gently shaken for 5 min, and then centrifuged at 4,500 $\times g$. The supernatant was collected for testing. GDL was analyzed by using a GC instrument (Agilent 6890N, Agilent Technologies, Ltd., USA) equipped with a 19091J-433 HP-5 chromatographic column (30 m \times 0.25 mm \times 0.25 μm). The split ratio was 30:1, and N_2 was the carrier gas. The operating conditions for the analysis were as follows: a 0.2 μl sample was injected at 250°C; the detector temperature was 300°C; and the oven temperature was maintained at 120°C for 2 min, increased to 205°C at a rate of 30°C/min, raised to 215°C at a rate of 4°C/min, raised to 280°C at a rate of 20°C/min and held constant for 3 min.

The concentration of RA was monitored using an HPLC instrument (Agilent 1260, Agilent Technologies, Ltd., USA) equipped with an XDB-C18 column (250 mm \times 4.5 mm). Approximately 2 μl sample volumes were injected into the column and eluted at a flow rate of 1 ml/min with 5% v/v of 0.1%

v/v phosphoric acid/water and 95% v/v acetonitrile, and a detection wavelength of 205 nm was used.

The conversion rate of the product was estimated as follows:

$$\text{Conversion rate } (\%) = \frac{\text{Concentration of GDL/MW of GDL}}{\text{Concentration of converted RA/MW of RA}} \times 100$$

where MW is the molecular weight.

Results

Effect of E-V and Organic Solvents on the Biotransformation Process

The effects of E-V, DMSO, ethylene glycol and acetone on biotransformation were compared with the reference results. The cell viability, pH, RA consumption and GDL yield were evaluated (Fig. 1). Regarding cell viability, there was a slight decrease in all the cultures with additions, except for the reference culture, in which the OD increased from 0.426 to 0.453 during biotransformation. However, the concentration of cells in the culture with ethylene glycol declined the most, from 0.426 to 0.328 (Fig. 1A). This indicates that ethylene glycol may have a stronger toxic effect on the proliferation of microorganisms. In terms of pH, there was not much variation during biotransformation, except in the medium with ethylene glycol, in which the pH decreased from 6.36 to 4.59 (Fig. 1B).

The highest yield of GDL was 6.2 g/l in the culture with E-V at 60 h, which was 50% higher than that in the control (Fig. 1C). The second highest yield was obtained in the culture with acetone, which was 5.32 g/l. The yields in the medium with DMSO and the reference were almost the same, with values of 4.34 g/l and 4.14 g/l, respectively. The lowest yield was obtained from the medium with ethylene glycol, which was 1.125 g/l. Obviously, the effect of ethylene glycol on biotransformation is negative. From 48 h to 60 h, a high production rate of GDL was obtained in all the cultures, and this rate gradually slowed after 60 h. Among these media, the highest production rate was achieved in the culture with E-V, which was 0.517 g/l•h. (Table 2).

Fig. 1D indicates that the highest consumption of RA was achieved in the medium with E-V, which was 14.24 g/l. The reason for this phenomenon was speculated to be that the sustained release effect of E-V increased the utilization of RA. The second highest consumption of RA was obtained in the medium with acetone, which was 13.15 g/l. The lowest consumption of RA was obtained in the medium with ethylene glycol, which was 5.92 g/l. The concentrations of RA in the medium with DMSO and the

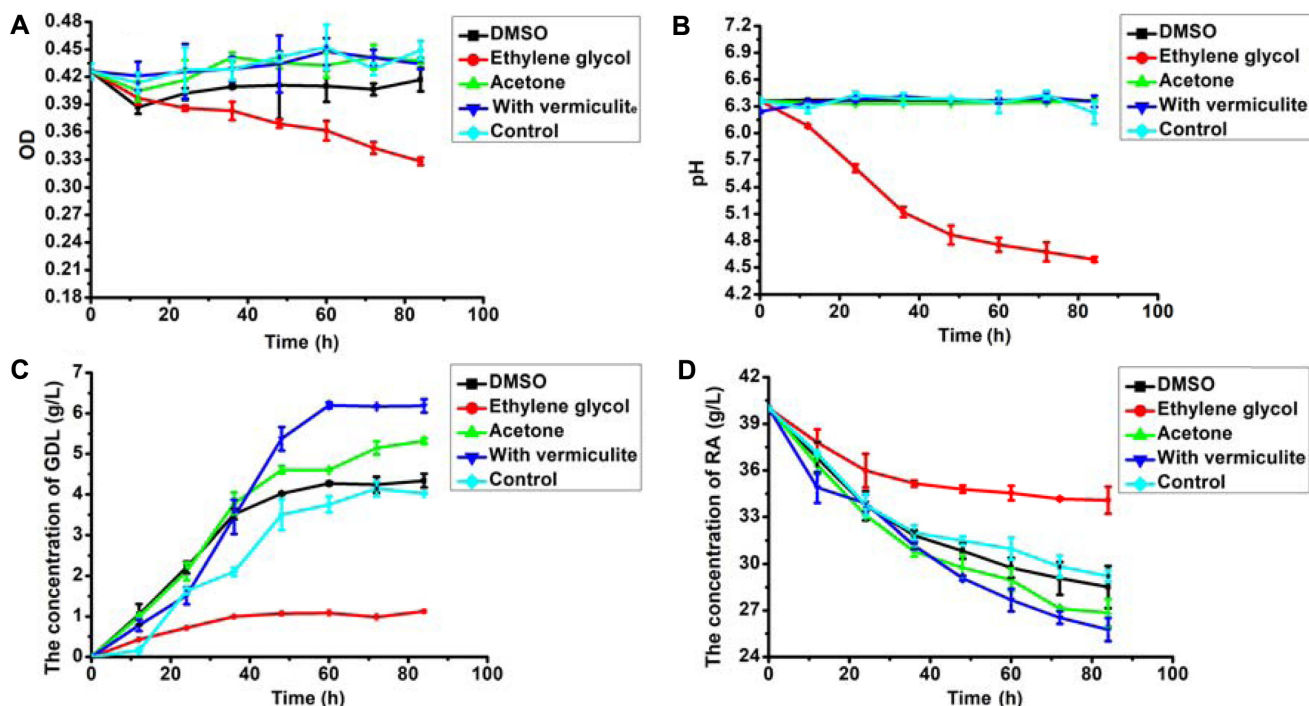


Fig. 1. Changes in yeast cell growth (A) pH (B) GDL concentration (C) and RA concentration (D) during the cultivation. Error bars show standard errors of means of three replicates.

reference medium were 28.51 g/l and 29.21 g/l, respectively, which were almost the same. The highest conversion rate of RA to GDL was achieved in the medium with E-V, which was 88.3% at 60 h (Table 1). For the media with DMSO and acetone and the reference medium, the conversion rates were 72.9%, 76.9%, and 78.9%, respectively, at 48 h. The lowest conversion rate was obtained in the medium with ethylene glycol, which was 36.1%. During the

biotransformation process, the conversion rates in the cultures with DMSO, acetone, and E-V and the reference culture showed an increasing trend before slightly declining, except for the culture with ethylene glycol, in which the conversion rate consistently declined. Hence, the addition of ethylene glycol could strongly inhibit biotransformation productivity. The addition of E-V had a much less negative effect on the metabolism of *Y. lipolytica*,

Table 1. The conversion rate of RA to GDL.

Time (h)	0	12	24	36	48	60	72	84
Control (%)	0	19.6 ± 2.3	46.0 ± 3.3	56.0 ± 0.4	72.9 ± 5.4	71.9 ± 1.8	71.4 ± 1.8	68.3 ± 2.0
DMSO (%)	0	57.9 ± 4.4	61.9 ± 5.3	75.3 ± 0.1	76.9 ± 3.6	73.1 ± 3.8	64.9 ± 3.1	66.5 ± 5.3
Ethylene glycol (%)	0	36.1 ± 10.5	32.3 ± 8.4	35.9 ± 0.2	35.9 ± 0.7	34.8 ± 0.02	29.6 ± 2.3	33.5 ± 3.4
Acetone (%)	0	48.6 ± 5.8	53.4 ± 5.2	71.9 ± 2.7	78.9 ± 4.8	73.3 ± 4.5	69.9 ± 1.5	67.0 ± 3.8
With E-V (%)	0	26.8 ± 0.3	43.2 ± 2.2	69.7 ± 6.7	86.3 ± 3.5	88.3 ± 4.3	80.4 ± 2.2	76.2 ± 2.0

Table 2. The production rate of GDL.

Time (h)	0	12	24	36	48	60	72	84
Control (g/l-h)	0	0.013 ± 0.0035	0.136 ± 0.0059	0.175 ± 0.0082	0.292 ± 0.032	0.313 ± 0.0165	0.345 ± 0.0153	0.335 ± 0.0012
DMSO (g/l-h)	0	0.0871 ± 0.022	0.184 ± 0.012	0.293 ± 0.011	0.335 ± 0.002	0.356 ± 0.004	0.353 ± 0.016	0.362 ± 0.014
Ethylene glycol (%)	0	0.036 ± 0.004	0.060 ± 0.001	0.083 ± 0.003	0.089 ± 0.006	0.09 ± 0.008	0.082 ± 0.008	0.094 ± 0.004
Acetone (g/l-h)	0	0.083 ± 0.002	0.175 ± 0.018	0.315 ± 0.022	0.384 ± 0.008	0.384 ± 0.001	0.429 ± 0.014	0.443 ± 0.006
E-V (g/l-h)	0	0.065 ± 0.012	0.126 ± 0.018	0.288 ± 0.035	0.448 ± 0.024	0.517 ± 0.006	0.514 ± 0.001	0.515 ± 0.014

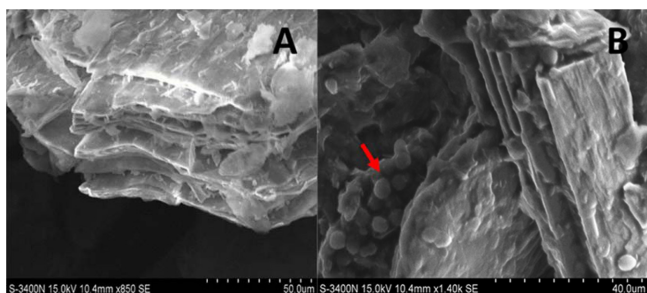


Fig. 2. SEM images of the E-V (A) and E-V-RA (B).

which could improve the utilization of substrate in the fermentation broth.

Characterization of the E-V-RA complexes

Micro-morphology analysis. Fig. 2 shows SEM images of E-V and E-V coated with the substrate (RA) and *Y. lipolytica*. As shown in Fig. 2A, E-V has a regular, expandable lamellar structure, which gives it a larger specific surface area. The RA was adsorbed onto the interlayer lamellar structure and the surface of E-V, which increased the contact opportunity between *Y. lipolytica* and RA as they aggregated on the E-V and was conducive to a higher output, as indicated by the red point in Fig. 2B.

FTIR spectra analysis. Fig. 3 shows the FTIR spectra of E-V, RA and the E-V-RA complex, which provide structural information. The peaks of the E-V-RA complex (Fig. 3a) showed that the asymmetrical stretching vibrations of Si-O-Si and Si-O-Al and the bending vibrations of Si-OH appeared at 987 and 710 cm^{-1} , respectively, which were identical to the characteristic peaks of E-V (Fig. 3b). These results suggested that the structure of E-V was unaltered. Due to the alkene C-H bending vibration of RA (Fig. 3c), the fingerprint peaks at 1,430 and 1,280 cm^{-1} were also

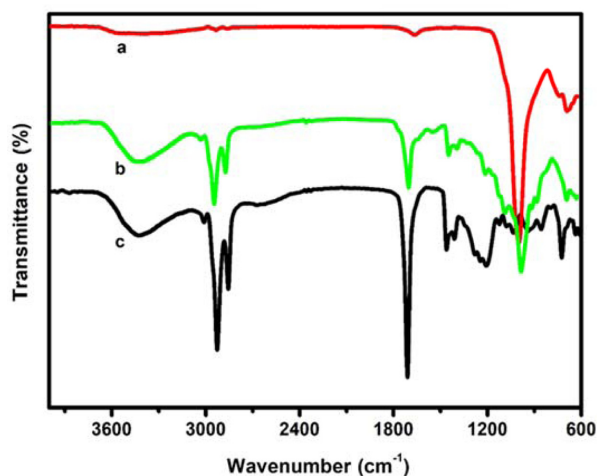


Fig. 3. FTIR of E-V-RA(a), E-V(b) and RA(c).

present in Fig. 3b, suggesting the adsorption of RA onto the E-V surface. However, the stretching vibrational peaks of =C-H, -C=O and -CH₂ of RA at 3,010, 1,710, and 2,926 cm^{-1} , respectively, in Fig. 3b were weaker than those in Fig. 3c. No new characteristic peaks appeared, which indicated the formation of adsorption complexes between RA and E-V instead of a chemical reaction.

Thermogravimetric (TG) analysis and differential thermogravimetric (DTG) analysis. Thermogravimetric (TG) analysis was carried out to provide supporting evidence for the FTIR results. As shown in Fig. 4, no obvious variation in the curve of E-V was observed in the examined temperature range. RA was stable at temperatures below 200°C. However, when the temperature increased from 200°C to 377°C, 96% weight loss occurred at a DTG temperature of 362°C. There was also a distinct weight loss for the E-V-RA complex from 250°C to 407°C, which represents the degradation of RA (Fig. 4A) at a DTG

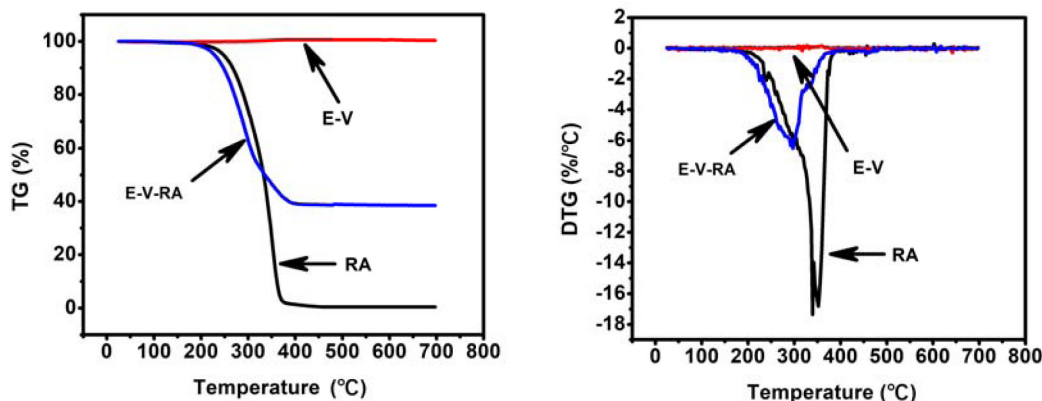


Fig. 4. TG and DTG curves of E-V, E-V-RA and RA.

temperature of 297°C. It could be speculated that the weight loss in the adsorption complex was mostly attributed to the decomposition of RA. In addition, an approximately 62.49% weight loss was observed for the E-V-RA adsorption complex. This result indicates that the composite structure did not influence the thermostability of RA and E-V. Only physical adsorption occurred between RA and E-V, instead of the formation of chemical bonds. The calculated proportions of the weight losses suggested that the adsorption capacity of E-V was 1.5 g/g.

Discussion

From the comparative results for the culture with E-V and the three organic solvents above, we conclude that E-V has a better effect on the biotransformation of GDL. In this biotransformation process, E-V acted as a controlled delivery agent to constantly release RA and thus improved its utilization efficiency. The bioconversion rate of RA to GDL was higher in the medium with E-V, and the highest yield of GDL was 6.2 g/l, achieved at 60 h, which was 50% higher than the reference yield. As shown in Figs. 2, 3, and 4, an adsorption complex between RA and E-V was formed. The mechanism behind this behavior could be interpreted as follows: The layered structure of E-V provides a feasible material for the adsorption of RA due to its accordion-like volume expansion and high specific surface [17–19]. The complexes could be formed by physical adsorption or through covalent or hydrogen bonds between the carboxyl groups in RA and the hydroxyl groups in the inner sphere and outer sphere of E-V. It is supposed that the complexes adsorbed on the outer sphere are readily released. However, the complexes bound in the interlayer space might be stronger and more protected against desorption. This adsorption mechanism determines the effective control of the RA release rate and the recycling of E-V.

In addition, *Y. lipolytica* could adhere to the surface and interlayer of E-V, which was more conducive to GDL biotransformation. The productivity was compared with that of cell immobilization methods reported previously. Many different materials used to produce GDL were compared, and the maximum yield was 1.597 ± 34 mg/l, obtained from *Y. lipolytica* immobilized on polymethacrylate (DupUM) after 264 h [20]. The highest yield of GDL was 4.17 g/l, which was obtained from *Y. lipolytica* immobilized on a mixture of sodium alginate and attapulgitite [21]. However, due to their poor mechanical properties, immobilized carriers are liable to crack. In addition, the

mass transfer rate is deeply influenced by the pore structure of the carrier. The productivity of the process is not as high as that in free cells despite the enhanced maximum aroma concentration based on the cell recycling usage. Compared with the cell immobilization method, utilizing an E-V delivery system to produce GDL has much greater advantages.

Regarding the organic solvents, the yields of GDL were all lower than that in the medium with E-V. Among these other media, the highest yield of GDL was obtained in the medium with acetone, which was 5.32 g/l. This value was 14% lower than that in the medium with E-V but 28.5% higher than the control. However, the lowest cell viability, yield of GDL, pH and consumption of RA were all obtained in the medium with ethylene glycol. Some studies have indicated that low concentrations of organic solvents have a hormesis effect [22], promote the production of some lipases and release secondary metabolites [23, 24]. Moreover, hydrophilic organic solvents can improve the permeability of the cell membrane by destroying the hydrophobicity of the lipids and increasing the flow of lipids or oil-like substrates in the cell membrane [25, 26]. DMSO could promote the production of GDL, but it was toxic towards *Y. lipolytica*. It has been reported that 1% DMSO can induce apoptosis in human lens cells [27], and DMSO has been proven to provoke a c-myc-dependent decrease in ornithine decarboxylase activity, followed by a depletion of intracellular polyamine levels, associated with programmed cell death and cell growth arrest [28].

The toxicity of organic solvents to cells depends mainly on their concentration in the cell membrane. Furthermore, the addition of organic solvents would cause inconvenience in separation and purification due to the intersolubility of acetone and GDL. Compared with the addition of organic solvents, E-V has no negative effect on cells and forms a stable delivery system. Applying E-V as a delivery system in the biotransformation of GDL to increase the GDL yield is a potential method of improving natural GDL production on an industrial scale.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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