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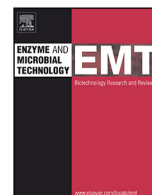

In situ extractive fermentation for the production of hexanoic acid from galactitol by *Clostridium* sp. BS-1

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ABSTRACT

Clostridium sp. BS-1 produces hexanoic acid as a metabolite using galactitol and enhanced hexanoic acid production was obtained by *in situ* extractive fermentation with *Clostridium* sp. BS-1 under an optimized medium composition. For medium optimization, five ingredients were selected as variables, and among them yeast extract, tryptone, and sodium butyrate were selected as significant variables according to a fractional factorial experimental design, a steepest ascent experimental design, and a Box–Behnken experimental design. The optimized medium had the following compositions in modified *Clostridium acetobutyricum* (mCAB) medium: 15.5 g L^{-1} of yeast extract, 10.13 g L^{-1} of tryptone, 0.04 g L^{-1} of $FeSO_4{\cdot}7H_2O$, 0.85 g L^{-1} of sodium acetate, and 6.47 g L^{-1} of sodium butyrate. The predicted concentration of hexanoic acid with the optimized medium was 6.98 g L^{-1}, and this was validated experimentally by producing 6.96 g L^{-1} of hexanoic acid with *Clostridium* sp. BS-1 under the optimized conditions. *In situ* extractive fermentation for hexanoic acid removal was then applied in a batch culture system with the optimized medium and 10% (v/v) alamine 336 in oleyl alcohol as an extractive solvent. The pH of the culture in the extractive fermentation was maintained at 5.4–5.6 by an acid balance between production and retrieval by extraction. During a 16 day culture, the hexanoic acid concentration in the solvent increased to 32 g L^{-1} while it was maintained in a range of 1–2 g L^{-1} in the medium. The maximum rate of hexanoic acid production was 0.34 g L^{-1} h^{-1} in *in situ* extractive fermentation.

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1. Introduction

Fossil fuel depletion and global warming have heightened the need for the development of alternative energy and chemical materials [1,2]. One such alternative is biological conversion of biomass carbohydrates into biofuels and biomaterials. Seaweed (Ceylon moss) has a high content of carbohydrates, galactose (23%) and glucose (20%), and thus its potential as a biomass resource is comparable to that of land plants [3]. One of the major constituents of seaweed, D- and L-galactose, could be converted to galactitol by an enzymatic reaction catalyzed by aldose reductase or a hydrogenation reaction with a chemical catalyst. Both form of D- and L-galactose could be utilized for the production of bioenergy or biochemicals, if a microorganism utilized galactitol. Recently, a strict anaerobic bacteria metabolizing galactitol was reported, and this anaerobe produced volatile fatty acids including hexanoic acid as a metabolic end product [4].

Hexanoic acid is a saturated fatty acid that has six carbons and one carboxylic group, and is a light colorless or yellow oily liquid with an acrid odor [5]. It is often found in oils and animal fats and has been used in diverse industrial applications such as perfumes, medicine, food additives, lubricating grease, tobacco flavor, rubber, and dyes [6,7]. In addition, hexanoic acid can be converted into other useful materials such as hexyl hexanoate and hexanol *via* esterification and hydrogenation [8,9].

Several bacterial species have been reported as hexanoic acid producers, and diverse approaches are being applied for the production of hexanoic acid by bacterial fermentation. For utilization of cellulose in hexanoic acid production, *Clostridium kluyveri*, producing hexanoic acid from ethanol plus either acetate or succinate, was co-cultured with ruminal cellulolytic bacteria,

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Fibrobacter succinogenes and *Ruminococcus flavefaciens* that produce acetate and succinate [10]. Another hexanoic acid producer *Megasphaera elsdenii* increased hexanoic acid production after removal of the hexanoic acid with an anionic exchange resin and the increment of cell number with immobilization in a fed-batch culture [11].

The production of metabolites by microbes could be increased in optimized culture conditions, and methods for medium optimization, such as a fractional factorial experimental design and a Box–Behnken experimental design, have been applied for the production of biofuels and biomaterials such as butanol and butyric acid [12,13]. The Box–Behnken experimental design is an experimental strategy to find the optimum conditions for a multivariable system, and also an efficient tool for optimization of multiple variables with a minimum number of experiments [14]. A group of mathematical and statistical procedures in the Box–Behnken experimental design can be used to study relationships among a number of independent variables and one or more responses [15].

Production of organic acid by microorganisms is negatively regulated by decreased pH and increased intracellular organic acid accumulation [16]. Various methods for removal of inhibitory microbial metabolites have been investigated by a number of researchers. Liquid–liquid partitioning for recovery of metabolites is the conventional solvent extraction method used for the separation of metabolites such as alcohols, carboxylic acids, and amino acids from fermentations [17,18].

Extraction was applied to diverse organic acid fermentation to increase productivity. Butyric acid production by *Clostridium butyricum* was increased by extractive fermentation with 20% (w/w) Hostarex in oleylalcohol [19], and butyric acid production by immobilized cells of *Clostridium tyrobutyricum* was attempted with 10% (v/v) alamine 336 in oleyl alcohol as the extractant [20]. Extraction was also applied for the removal of hexanoic acid using tri-*n*-butyl phosphate in solvents such as benzene, toluene, hexanol, octanol, and decanol [21,22]. However, there have been no reports applying extraction for the biologically produced hexanoic acid.

In a previous study, we reported *Clostridium* sp. BS-1 producing 2.9 g L^{-1} of hexanoic acid using galactitol [4]. In this work, enhanced production of hexanoic acid by *Clostridium* sp. BS-1 was studied with a medium optimization, for which a fractional factorial experimental design, a steepest ascent experimental design, and a Box–Behnken experimental design were applied. *In situ* extractive fermentation was also performed, where the pH was maintained for continual production of hexanoic acid and a solvent solution was used for retrieval of hexanoic acid.

2. Materials and methods

2.1. Media and culture conditions

Clostridium sp. BS-1 (KCCM 10991P), isolated from a sludge sample of a waste water treatment plant, was used for the production of hexanoic acid with galactitol [4]. *Clostridium* sp. BS-1 was maintained in a modified *Clostridium acetobutyricum* (mCAB) medium containing the following components in grams per liter of distilled water: yeast extract, 4; tryptone, 1; KH_2PO_4, 1.5; K_2HPO_4, 1.5; L-asparagine, 0.5; $MgSO_4·7H_2O$, 0.1; $MnSO_4·H_2O$, 0.1; $FeSO_4·7H_2O$, 0.015; NaCl, 0.1; and L-cysteine–HCl, 0.25. For optimized medium, yeast extract, tryptone, $FeSO_4·7H_2O$, sodium acetate, and sodium butyrate were selected as variables, and optimized amounts of these five variables were added into a basal medium containing the following components in grams per liter of distilled water: galactitol, 20; 2-(N-morpolino)ethanesulfonic acid (MES), 19.5; KH_2PO_4, 1.5; K_2HPO_4, 1.5; L-asparagine, 0.5; $MgSO_4·7H_2O$, 0.1; $MnSO_4·H_2O$, 0.1; NaCl, 0.1; and L-cysteine–HCl, 0.25. MES was added for all of the experimental conditions to prevent acidification, and the initial pH was adjusted to 6.5 ± 0.2 with 6 N NaOH. A sufficient amount of galactitol (100 g L^{-1}) was used for the supply of carbohydrate in the batch culture *in situ* extractive fermentation. The anaerobic condition for the culture corresponded with that in previously reported methods [23]. The grown seed culture of *Clostridium* sp. BS-1 was inoculated (5%, v/v) into appropriate media for each cultivation. The cells were cultivated in an anaerobic condition and incubated at 40 °C and 150 rev min^{-1} after inoculation.

Table 1
Variables and experimental design levels for the factorial fractional design.

Independent variable (g L^{-1})	Coded symbol	Range and level		
		−1	0	1
Yeast extract	X_1	1	5	9
Tryptone	X_2	1	5	9
$FeSO_4·7H_2O$	X_3	0	0.04	0.08
Sodium acetate	X_4	0	3	6
Sodium butyrate	X_5	0	3	6

2.2. Experimental designs

A fractional factorial experimental design, a steepest ascent experimental design, and a Box–Behnken experimental design was used in order to obtain the optimized medium for hexanoic acid production by *Clostridium* sp. BS-1. A fractional factorial experimental design was used to evaluate preliminary significance of the five selected variables affecting hexanoic acid production. A first-order model was suggested on the basis of the following equation (1):

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where Y is the response (hexanoic acid production); β_0 is the model intercept; β_i is the coefficient of linear equation; and X_i is the dimensionless coded level of the independent variable. Each coded variable represents two levels, low (denoted as −1) and high (denoted as +1), according to the following equation (2):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (2)$$

where x_i is the dimensionless coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the central point, and ΔX_i is the step change value.

Among the five variables, yeast extract, tryptone, and $FeSO_4·7H_2O$, and their highest level values were selected on the basis of the mCAB medium, which was used for the isolation of *Clostridium* sp. BS-1. Sodium acetate and its high level value were selected on the basis of the two media; one is used for the production of hexanoic acid by *C. kluyveri* ATCC 8527 [10] and the other is used for growth and butyrate production by *M. elsdenii* [24]. Selection of sodium butyrate was based on its hypothetical involvement in the production of hexanoic acid [25,26] and its highest level value was based on the amount of sodium acetate selected in this study. The other components were the same as in the mCAB medium.

As a preliminary experiment, a one-factor-at-a-time test, where only one factor or variable is changed at a time while keeping the others fixed [27], was performed with each variable in a range of 0–5 g L^{-1}. Zero concentration of several components influenced the product pattern; therefore, zero concentration was included as a low level.

The three levels of each variable, used in the fractional factorial design experiment, are shown in Table 1. Five ingredients in the medium were studied in 2^{5-1} fractional factorial designs (16 experiment runs), a half fraction of the full 2^5 designs. The five central point (level 0) replications were included in the design to estimate the experimental variance (Table 2).

The data from the fractional factorial experimental design were used for a further analysis with the method of steepest ascent. The method of steepest ascent is a procedure for sequentially moving along the steepest ascent path [28], and this method was used to obtain the concentration ranges of variables for a Box–Behnken experimental design.

As the significant factor, three independent variables selected from the fractional factorial experimental design were further analyzed using a Box–Behnken experimental design [14]. Seventeen experimental runs, 5 replication runs at the central point and 12 experimental runs with three significant variables, were performed. The experimental results acquired from the Box–Behnken experimental model were used for the prediction of the maximum concentration of hexanoic acid by the following second-order equation, a quadratic model:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (3)$$

where Y is the predicted response (hexanoic acid productivity); β_0 is an offset term; β_i is the linear effect; β_{ii} is the squared effect; β_{ij} is the interaction effect; and x_i is the dimensionless coded value of the real variable.

The experimental results obtained with the Box–Behnken experimental model were statistically analyzed for their reliability with an analysis of variance (ANOVA) in Design-Expert 7.0 (Stat-Ease, MN, USA). The significance of statistical data for each variable was also evaluated with ANOVA. All data in this medium optimization study were analyzed by Design-Expert 7.0. The fits of the first-order equation model and the second-order equation model were validated using the coefficient of determination R^2 and the adjusted R^2. The significant variables were screened on the basis of a F test and p value at the 95% significant level for the fractional factorial experimental design and the Box–Behnken experimental design.

Table 2
Fractional factorial experimental design (2^{5-1}) matrix and results.

Run	Real value ($g\,L^{-1}$)					Coded value					Hexanoic acid ($g\,L^{-1}$)
	Yeast extract	Tryptone	$FeSO_4{\cdot}7H_2O$	Sodium acetate	Sodium butyrate	X_1	X_2	X_3	X_4	X_5	
Factorial fractional design											
1	1	1	0	0	6	−1	−1	−1	−1	1	0.41
2	9	1	0	0	0	1	−1	−1	−1	−1	3.60
3	1	9	0	0	0	−1	1	−1	−1	−1	2.10
4	9	9	0	0	6	1	1	−1	−1	1	4.96
5	1	1	0.08	0	0	−1	−1	1	−1	−1	0.97
6	9	1	0.08	0	6	1	−1	1	−1	1	4.49
7	1	9	0.08	0	6	−1	1	1	−1	1	4.30
8	9	9	0.08	0	0	1	1	1	−1	−1	3.78
9	1	1	0	6	0	−1	−1	−1	1	−1	0.57
10	9	1	0	6	6	1	−1	−1	1	1	2.58
11	1	9	0	6	6	−1	1	−1	1	1	0.88
12	9	9	0	6	0	1	1	−1	1	−1	3.71
13	1	1	0.08	6	6	−1	−1	1	1	1	0.50
14	9	1	0.08	6	0	1	−1	1	1	−1	3.31
15	1	9	0.08	6	0	−1	1	1	1	−1	2.91
16	9	9	0.08	6	6	1	1	1	1	1	5.60
Central points											
17	5	5	0.04	3	3	0	0	0	0	0	5.15
18	5	5	0.04	3	3	0	0	0	0	0	5.05
19	5	5	0.04	3	3	0	0	0	0	0	4.82
20	5	5	0.04	3	3	0	0	0	0	0	5.15
21	5	5	0.04	3	3	0	0	0	0	0	5.15

2.3. Extractive fermentation

A solvent solution of 10% (v/v) alamine 336 (Cognis) in oleyl alcohol (Ecogreen, DHW) was used for extractive fermentation. After mixing, the solvent solution was washed two times for 1 h by distilled water to remove trace elements and unknown chemicals that may inhibit the growth of *Clostridium* sp. BS-1. The mixture was then autoclaved at 121 °C for 15 min before being used for the extraction. The optimized medium composition from the Box–Behnken experimental design was used for extractive fermentation. The experiment was conducted in a 500 ml serum bottle with 100 ml of a solvent solution and 100 ml of the optimized medium. In this extractive fermentation of *Clostridium* sp. BS-1, the initial concentration of galactitol increased up to 100 $g\,L^{-1}$ for an adequate supply of galactitol. *Clostridium* sp. BS-1 with 5% (v/v) seed in the mid-exponential phase was inoculated into the medium and was cultured for one day before the extractive fermentation to prevent increase of initial pH by the extraction of produced carboxylic acid and to reduce toxic effects of the solvent against bacterial cells. After one day of cultivation the solvent solution was injected by a syringe onto the cultured broth. Biphasic status of the solvent and aqueous culture broth was maintained for a 16 day incubation period during the extractive fermentation in the batch culture, even though the bottle was shaken horizontally (150 rpm).

2.4. Analytical methods

The experiments for medium optimization were conducted in duplicate and the means are shown with standard deviations presented by error bars. The culture broth of *Clostridium* sp. BS-1 was taken periodically for measuring cell growth, pH, and content of metabolites. The carboxylic acids in the broth were analyzed by a gas chromatogram (GC; Agilent 6890N, USA) equipped with a flame ionized detector, according to a previously described procedure [23]. Cell growth was measured using a UV-spectrophotometer (Shimazu mini-1240, Japan) at 600 nm, and galactitol concentration was measured using an HPLC (Agilent 1200 series, USA) with a refractive index detector and a cation-exchange column (Aminex® HPX-87h; 300 mm × 7.8 mm, Bio-rad, USA), respectively. In the HPLC analysis, the sample injection volume, elution solution, flow rate, and temperature were 10 μl, 5 mM H_2SO_4, 0.5 ml min^{-1}, and 60 °C, respectively, and galactitol (TCI, Tokyo) was used as an external standard. For measurement of the extracted metabolites in the solvent layer, the upper solvent layer was taken from the biphasic fermentation culture, and then diluted 20 times with methanol and analyzed with a GC. N content of cells in the medium was predicted from a stoichiometric equivalent (1.42 g COD/g $C_5H_7O_2N$) using the COD value [29].

2.5. Kinetics of hexanoic acid production

Kinetics of hexanoic acid production by *Clostridium* sp. BS-1 in the batch cultures with and without *in situ* extractive fermentation were studied using the following modified Gompertz equation [30]:

$$P_i = P_{max,i} \times \exp\left\{-\exp\left[\left(R_{max,i} \times \frac{e}{P_{max,i}}\right) \times (\lambda_i - t) + 1\right]\right\} \quad (4)$$

where i represents hexanoic acid; P_i is the production of i formed per liter of working volume at cultivation time t; $P_{max,i}$ is the potential maximum i production (g) per liter of working volume; $R_{max,i}$ is the maximum rate of i production ($g\,L^{-1}\,day^{-1}$); e is 2.718281828; and λ_i is the lag-phase time (h).

3. Results and discussion

3.1. Fractional factorial experimental design

The optimized medium for the production of hexanoic acid with galactitol by *Clostridium* sp. BS-1 was based on mCAB, and five ingredients for hexanoic acid production were selected for a fractional factorial experimental design. Yeast extract and tryptone were selected because these two components contain essential sources for cell viability such as nitrogen and vitamins [31]. $FeSO_4{\cdot}7H_2O$ was selected for its involvement in H_2 metabolism as a co-factor of [Fe–Fe] hydrogenase. Hydrogenase is an essential enzyme related to acetic acid and butyric acid synthesis pathways in *Clostridium* sp. [32]. Sodium acetate and sodium butyrate were selected because they are metabolic intermediates of hexanoic acid production in anaerobic fermentation and may be used as electron acceptors in the hexanoic acid production pathway. Therefore, we hypothesized that the production of hexanoic acid by *Clostridium* sp. BS-1 may be positively influenced by the external addition of acetic acid and/or butyric acid.

The ranges of concentration of yeast extract, tryptone, $FeSO_4{\cdot}7H_2O$, sodium acetate, and sodium butyrate in grams per liter were 1–9, 1–9, 0–0.08, 0–6, and 0–6, respectively. The fractional factorial experimental design and results of all factor levels are displayed in Tables 1 and 2, respectively, and the estimates of the fractional factorial experimental design are expressed in Table 3. As shown in Table 3, p values of yeast extract, tryptone, $FeSO_4{\cdot}7H_2O$, sodium acetate, and sodium butyrate represented as X_1, X_2, X_3, X_4, and X_5, respectively, appeared to influence hexanoic acid production, as their p values were lower than 0.05. A first-order equation could be obtained as follows (5):

$$\begin{aligned}\text{hexanoic acid} = {} & 2.79 + 1.2X_1 + 0.74X_2 + 0.44X_3 - 0.28X_4 \\ & + 0.17X_5 - 0.23X_1X_2 + 0.23X_2X_5 + 0.32X_3X_5 \\ & - 0.29X_4X_5 \end{aligned} \quad (5)$$

Table 3
Results of the statistical analysis of the fractional factorial experimental design on hexanoic acid production.

Source	Coefficient estimate	*F* value	*p* value
Intercept	2.79		
X_1[a]	1.21	157.95	<0.0001
X_2	0.74	58.58	<0.0001
X_3	0.44	20.86	<0.0001
X_4	−0.28	8.72	<0.0014
X_5	0.17	3.24	0.3379
X_1X_2	−0.23	5.65	0.0415
X_1X_5	0.23	5.71	0.0406
X_2X_5	0.23	5.77	0.0398
X_3X_5	0.32	10.78	0.0095
X_4X_5	−0.29	9.11	0.0145
Model		28.64	<0.0001
Curvature		132.23	<0.0001
Lack of fit		12.27	0.0154

[a] X_1, yeast extract; X_2, tryptone; X_3, $FeSO_4 \cdot 7H_2O$; X_4, sodium acetate; and X_5, sodium butyrate.

Even though the *p* value was less than 0.05, the concentrations of $FeSO_4 \cdot 7H_2O$ and sodium acetate were fixed in the steepest ascent experimental design. In the case of $FeSO_4 \cdot 7H_2O$, cell growth and hexanoic acid production showed sufficient performance even only with 0.04 g L^{-1} of $FeSO_4 \cdot 7H_2O$ in the medium, and an increase of the amount of $FeSO_4 \cdot 7H_2O$ did not influence hexanoic acid production in the steepest ascent experimental design (data not shown). Therefore, the concentration of $FeSO_4 \cdot 7H_2O$ was fixed at a zero level, 0.04 g L^{-1}. Sodium acetate presents a negative coefficient value in Table 3. The growth of *M. elsdenii* is stimulated by the addition of acetate; however, it was also reported that increased acetate utilization leads to increased concentration of butyric acid but decreased hexanoic acid production [24]. This phenomenon of hexanoic acid production by addition of acetic acid was observed in hexanoic acid production by *Clostridium* sp. BS-1; therefore, it is necessary to fix sodium acetate at a proper concentration. A one-factor-at-a-time test with diminishing amount of sodium acetate was performed, and the optimal concentration of sodium acetate was determined as 0.85 g L^{-1} at the central point of the other four variables.

Sodium butyrate, denoted as X_5, did not appear to influence hexanoic acid production, as its *p* values were more than 0.05, and hexanoic acid concentration was not increased when a 0.17 coefficient of X_5 was applied in the method of steepest ascent. However, X_5 showed some interaction effects with other variables, as indicated by the in *p* values, and the combined models containing X_5, such as X_2X_5, X_3X_5, and X_4X_5, displayed more significant effects than other combined models in terms of *p* value (Table 3). A maximum of 5.6 g L^{-1} of hexanoic acid was produced when 6 g L^{-1} of sodium butyrate (code value +1) was used at run 16 in the factorial fractional experimental design. Therefore, sodium butyrate was selected as a variable in the steepest ascent experimental design. The amount of sodium butyrate was increased according to the coefficient value of the combined models, showing <0.05 *p* value in Table 3, and a three times greater X_5 coefficient value to obtain 6 g L^{-1} of sodium butyrate (code value +1). The coefficient values of yeast extract and tryptone in the first order equation of the fractional factorial design were used as levels of variables for the method of steepest ascent.

As shown in the results of the method of steepest ascent (Table 4), the concentration of hexanoic acid increased gradually to 6.7 g L^{-1}, up to run 4. After run 4, the concentration of hexanoic acid did not increase. Therefore, the condition of variables in run 4 was used as the central point and runs 3 and 5 were used for −1 and +1 in the Box–Behnken experimental design.

Table 4
Steepest ascent experimental design matrix and experimental responses.

Run	X_1 (g L^{-1})[a]	X_2 (g L^{-1})	X_5 (g L^{-1})	Hexanoic acid (g L^{-1})
1	5.00	5.00	3.00	3.94
2	9.00	7.44	4.26	5.65
3	13.00	9.88	5.52	6.25
4	17.00	12.32	6.78	6.73
5	21.00	14.76	8.04	6.00
6	25.00	17.20	9.30	5.20
7	29.00	19.64	10.56	4.48
8	33.00	22.08	11.82	3.01
9	37.00	24.52	13.08	1.79
10	41.00	26.96	14.34	0.88

[a] X_1, yeast extract; X_2, tryptone; and X_5, sodium butyrate.

3.2. Box–Behnken experimental design

The Box–Behnken experimental design was used for a second order model. The range for the experiment was decided from the results of the steepest ascent experimental design. Therefore, the best result from the steepest ascent experimental design was used as the central point and the side conditions of the central point were used as conditions of −1 and +1 (Table 5). The three significant factors, yeast extract, tryptone, and sodium butyrate, were evaluated to approach the optimum region. A second order model, calculated from Eq. (3) by adopting experimental results, was obtained as the following equation:

$$Y = 6.85 - 0.24X_1 - 0.72X_3 - 0.32X_1^2 - 1.48X_3^2 \quad (6)$$

where *Y* denotes the predicted hexanoic acid concentration, and X_1 and X_3 represent the coded values of yeast extract and sodium butyrate, respectively. One of the three variables, tryptone, did not significantly influence hexanoic acid production in the range of tryptone used in the Box–Behnken experimental design; tryptone therefore was not included in Eq. (6).

A statistical analysis using ANOVA was performed in order to evaluate the appropriateness of the model. The predicted model was tested by a *F*-test and coefficient of determination, R^2. The *F* and *p* values of the model were 41.21 and 0.0001, respectively (Table 6). Therefore, the model showed appropriateness at the 95% level of significance. Moreover, the values of R^2 and adjusted R^2 were 0.93 and 0.90, respectively, indicating that the second order polynomial equation is appropriate for describing the results depicted in the Box–Behnken experimental design and the results in Table 5. It was reported that as the R^2 value approaches 1.00, the model becomes accordingly more appropriate [33]. In the model term, *p* values of probability below 0.05 indicate that the model terms are significant; that is, the factors significantly influence hexanoic acid production by *Clostridium* sp. BS-1. According to the *p* values, X_1, X_3, X_1^2, and X_3^2 were significant (Table 6), but X_2, X_2^2, X_1X_3, X_2X_3, and X_1X_2 showed probability values > 0.05, indicating that they are not significant. A 3D contour plot shows that the optimum point is located on the plot (Fig. 1). According to the second order polynomial equation, the optimized medium conditions were acquired as the following components in gram per liter: yeast extract, 15.5; tryptone, 10.13; $FeSO_4 \cdot 7H_2O$, 0.04; sodium acetate, 0.85; and sodium butyrate, 6.47. The predicted concentration of hexanoic acid calculated from Eq. (6) with this optimized medium composition was about 6.98 g L^{-1}, the highest value in Fig. 1. In the optimized medium, the optimal amounts of both yeast extract and tryptone are higher than the amounts of any compound required for anabolic metabolism. It is possible that only some components of yeast extract or tryptone influence the production of hexanoic acid by *Clostridium* sp. BS-1. Therefore, identification of the factors in yeast extract or tryptone may be necessary for economic production of hexanoic acid by *Clostridium* sp. BS-1 in future study.

Table 5
Box–Behnken experimental design matrix and experimental responses.

Run	Real value level (g L^{-1})			Coded value			Hexanoic acid (g L^{-1})
	Yeast extract	Tryptone	Sodium butyrate	X_1	X_2	X_3	
Box-Benken model design							
1	13	9.88	6.78	−1	−1	0	6.70
2	21	9.88	6.78	1	−1	0	6.35
3	13	14.76	6.78	−1	1	0	7.00
4	21	14.76	6.78	1	1	0	5.80
5	13	12.32	5.52	−1	0	−1	5.85
6	21	12.32	5.52	1	0	−1	5.55
7	13	12.32	8.04	−1	0	1	4.55
8	21	12.32	8.04	1	0	1	4.46
9	17	9.88	5.52	0	−1	−1	6.60
10	17	14.76	5.52	0	1	−1	5.70
11	17	9.88	8.04	0	−1	1	4.40
12	17	14.76	8.04	0	1	1	4.50
Central points							
13	17	12.32	6.78	0	0	0	6.75
14	17	12.32	6.78	0	0	0	6.85
15	17	12.32	6.78	0	0	0	6.89
16	17	12.32	6.78	0	0	0	6.85
17	17	12.32	6.78	0	0	0	7.15

3.3. Verification of the predicted model

In order to confirm whether the value from the predicted model coincides with a practical value, an experiment for validation was performed by the solution suggested from the Box–Behnken experimental design. The test culture was performed for 3 days in batch, and the concentration of the final points was similar to that of the predicted maximum value of hexanoic acid. The cell density reached the highest level after one day of cultivation, the galactitol and butyric acid content were continuously reduced, pH was maintained at around pH 6.5, the hexanoic acid content was continuously increased, and the acetic acid content slightly increased over its initial level during three days' batch culture (Fig. 2). *Clostridium* sp. BS-1 produced 6.96 g L^{-1} of hexanoic acid in the optimized medium with consumption of 15 g L^{-1} of galactitol; this means that *Clostridium* sp. BS-1 produced two-fold more hexanoic acid with the optimized medium compared with the previous result using mCAB [4]. Interestingly, added butyric acid was consumed down to 2.5 g L^{-1} within one day. When the concentration of hexanoic acid was close to 7 g L^{-1}, cell growth showed a stationary phase, and no more hexanoic acid was produced after three days of cultivation (data not shown).

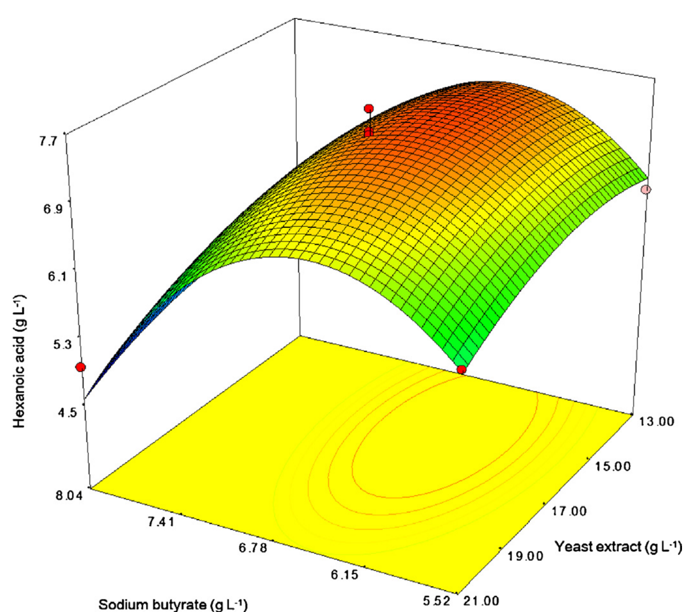

Fig. 1. Predicted model: 3D contour plot showing the effect of the amounts of yeast extract and sodium butyrate on the response of hexanoic acid by *Clostridium* sp. BS-1.

The growth of most bacteria that can produce organic acids such as acetic acid and butyric acid is inhibited by decreased pH induced by the accumulation of organic acids in the culture broth. Therefore, it appears that the growth of *Clostridium* sp. BS-1 was inhibited by the accumulation of hexanoic acid in the culture broth. In addition, although *Clostridium* sp. BS-1 survived at 7 g L^{-1} of hexanoic acid, hexanoic acid had a bactericide effect against some bacteria, and 1% (w/v) of hexanoic acid inhibited the growth of *Escherichia coli* and *Staphylococcus aureus* [34].

Production of hexanoic acid can be increased when produced organic acids are removed during fermentation. In the case of *M. elsdenii* ATCC 25940, the production of hexanoic acid was increased up to 6–8 g L^{-1} when the hexanoic acid in culture broth was removed with an anionic exchange resin [11]. Therefore, extractive fermentation was performed for enhanced production of hexanoic acid by *Clostridium* sp. BS-1.

3.4. Effect of in situ extractive fermentation on the hexanoic acid production by Clostridium sp. BS-1 and modeling

A batch culture with *in situ* biphasic extractive fermentation was performed to determine whether *Clostridium* sp. BS-1 is able to produce hexanoic acid continuously. A mixture of alamine 336 and oleyl alcohol was used as a solvent solution for the extraction of hexanoic acid. Oleyl alcohol is known as an excellent extractor by a distribution effect and also has low toxicity against bacteria [35]. In

Table 6
Results of the statistical analysis of the Box–Behnken experimental design on hexanoic acid production.

Source	Coefficient estimate	*F* value	*p* value
Intercept	6.85		
X_1 [a]	−0.24	5.27	0.0406
X_3	−0.72	47.07	<0.0001
X_1^2	−0.32	4.93	0.0464
X_3^2	−1.48	104.71	<0.0001
Model		41.21	<0.0001
Lack of fit		5.42	0.0600

[a] X_1, yeast extract and X_3, sodium butyrate.

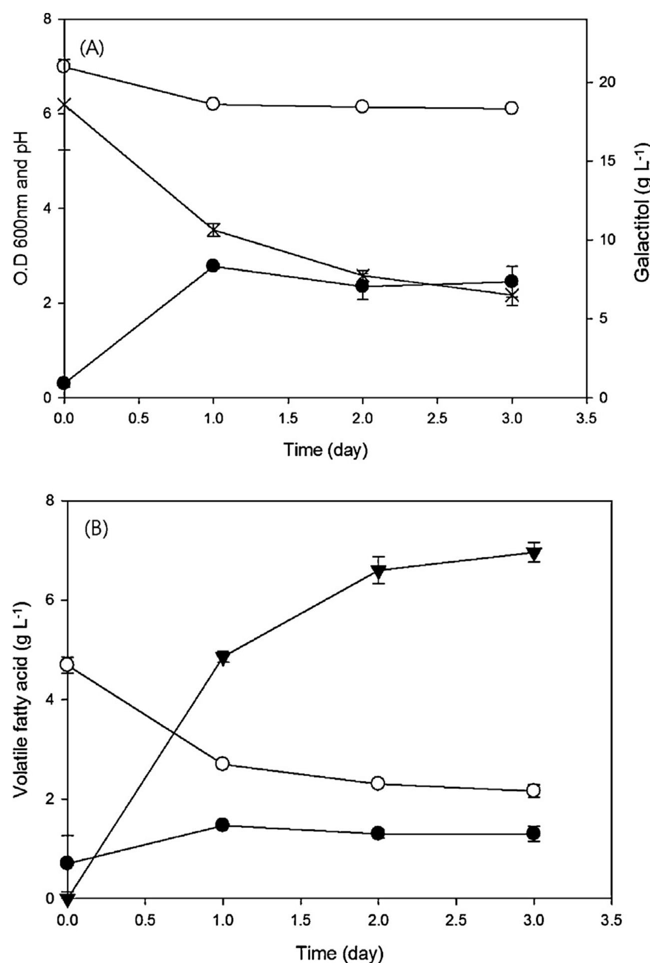

Fig. 2. Growth of *Clostridium* sp. BS-1 and production of volatile fatty acid in the optimized medium. (A) x, galactitol (g L^{-1}); ○, pH; and ●, O.D. at 600 nm. (B) ▼, hexanoic acid (g L^{-1}); ○, butyric acid (g L^{-1}); and ●, acetic acid (g L^{-1}).

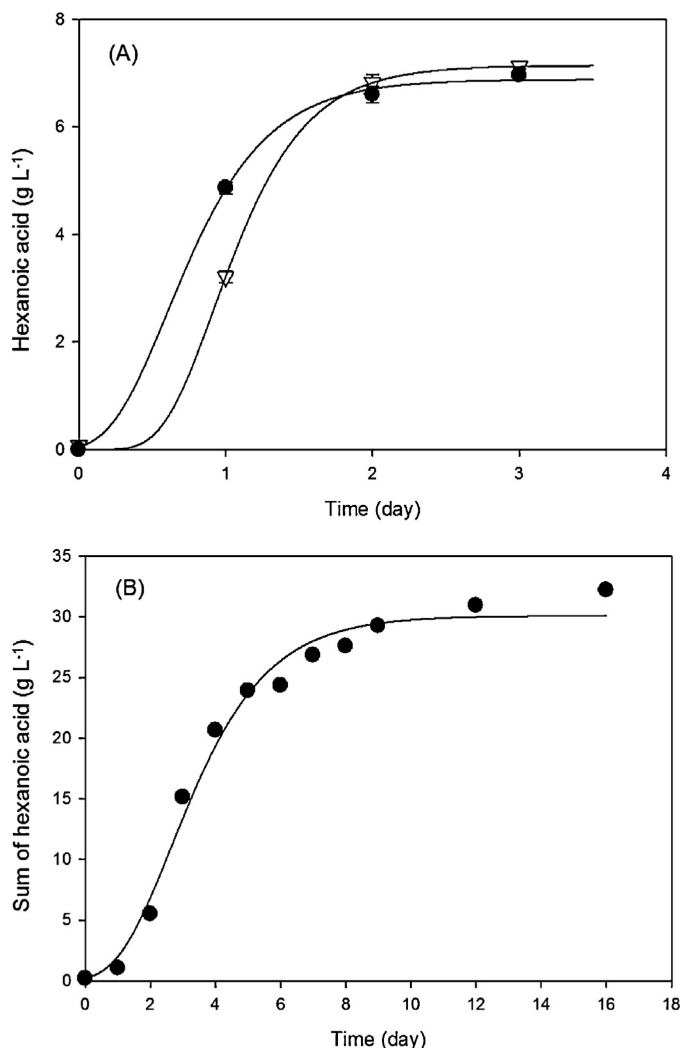

Fig. 3. Modified Gompertz plot for the production of hexanoic acid by *Clostridium* sp. BS-1 in batch culture and *in situ* extractive fermentation. (A) Hexanoic acid in batch culture at optimal condition: ▽, medium containing 20 g L^{-1} galactitol; ●, medium containing 100 g L^{-1} galactitol. (B) Sum of hexanoic acid in aqueous phase and solvent phase during *in situ* extractive fermentation with optimal medium containing 100 g L^{-1} galactitol.

addition, alamine 336 has shown an excellent removal effect over carboxylic acids, although it has a considerable toxic effect against bacterial cells [36]. Wu and Yang reported that a mixture of these chemicals has a synergetic effect on the extraction of butyric acid produced by *C. tyrobutyricum* [20].

Clostridium sp. BS-1 showed tolerance against 10% (v/v) alamine 336 and oleyl alcohol, and the production of hexanoic acid by *Clostridium* sp. BS-1 was not inhibited in biphasic extractive fermentation with 10% (v/v) alamine 336 in oleyl alcohol (data not shown). The optimal medium conditions except galactitol followed the results of the Box–Behnken experimental design, and were used for *in situ* biphasic extraction. In a pre-test of extractive fermentation with the optimized medium, 20 g L^{-1} of galactitol was completely metabolized in three days (data not shown); therefore additional supply of galactitol is needed for further hexanoic acid production. The solubility of galactitol in water is substantially lower (31 g L^{-1} at 15 °C) than that of other carbohydrate monomers such as glucose (910 g L^{-1} at 25 °C), fructose (3750 g L^{-1} at 20 °C), galactose (683 g L^{-1} at 25 °C), and sorbitol (182 g L^{-1} at 20 °C) [37]. Additional supplementation of solubilized galactitol to the extractive fermentation broth would thus lend a considerable dilution effect to the culture system of *Clostridium* sp. BS-1, and would increase the volume of the medium considerably. Therefore, 100 g L^{-1} of galactitol was added to the optimized medium for the extractive fermentation. At the time of seeding, the insolubilized galactitol particles precipitated in the bottle, and during the progress of the extractive fermentation by *Clostridium* sp. BS-1 galactitol, particles were solubilized and insoluble particles disappeared. The saturated concentration of hexanoic acid did not show metabolite inhibition by *Clostridium* sp. BS-1 without extraction (Fig. 2(B)).

The performances of *Clostridium* sp. BS-1 for the production of hexanoic acid in a batch culture with 20 g L^{-1} or 100 g L^{-1} of galactitol and with the *in situ* extractive fermentation were evaluated using the Gompertz model. The parameters from the modified Gompertz equation are summarized in Table 7, and curves are fitted in Fig. 3. Compared with data obtained from 20 g L^{-1} of galactitol, P_{max} (the potential maximum production of hexanoic acid), R_{max} (the maximum rate of hexanoic acid production), and λ_i (the lag-phase time) were increased at the optimized medium containing100 g L^{-1} of galactitol. This demonstrates that the increased concentration of galactitol had an inhibitory effect on the initial growth rates of *Clostridium* sp. BS-1, but slightly increased the production of hexanoic acid.

In the *in situ* biphasic extractive fermentation, P_{max} increased up to about 30 g L^{-1} and R_{max} increased up to 6.68 g L^{-1} day $^{-1}$. Also, λ_i was lengthened to 0.98. This was attributed to the added solvent and increased galactitol having simultaneous negative effects

Table 7
Hexanoic acid production calculated using a modified Gompertz equation.

Culture condition[a]	P_{max} (g L^{-1})	R_{max} (g L^{-1} day^{-1})	λ_i (day)	R^2	Adj R^2
20 g L^{-1} of galactitol	6.88	6.60	0.22	0.9864	0.9833
100 g L^{-1} of galactitol	7.14	7.50	0.50	0.9998	0.9994
In situ extractive fermentation[b]	30.09	6.68	0.99	0.9992	0.9977

[a] Cells were cultured at batch cultures with the optimized medium containing 20 g L^{-1} or 100 g L^{-1} of galactitol.
[b] 10% alamine 336 in oleyl alcohol used as an extraction solvent, and 100 g L^{-1} of galactitol are incorporated in the optimized medium.

on hexanoic acid production. However, as shown in Fig. 3(B), *Clostridium* sp. BS-1 appeared to overcome solvent toxicity and osmotic pressure by galactitol for the production of hexanoic acid.

It was reported that acetic acid and butyric acid formed during the early stage of acetone-butanol fermentation by *Clostridium acetobutylicum* are taken up and activated to their respective CoA thiolesters *via* an acetoacetyl-CoA: acetate (butyrate) CoA-transferase [38]. Butyric acid is involved in the initiation of solventogenesis in *C. acetobutylicum* fermentation, and butanol production starts when butyric acid is raised to a certain concentration in the broth [39]. During the extractive fermentation, the amount of butyric acid was stably maintained at around 2 or 3 g L^{-1} in the aqueous medium phase and the solvent phase. However, the initial amount of butyric acid was reduced by half: therefore, it is posited that butyric acid can be used as a carbon source or a trigger for the production of hexanoic acid by *Clostridium* sp. BS-1.

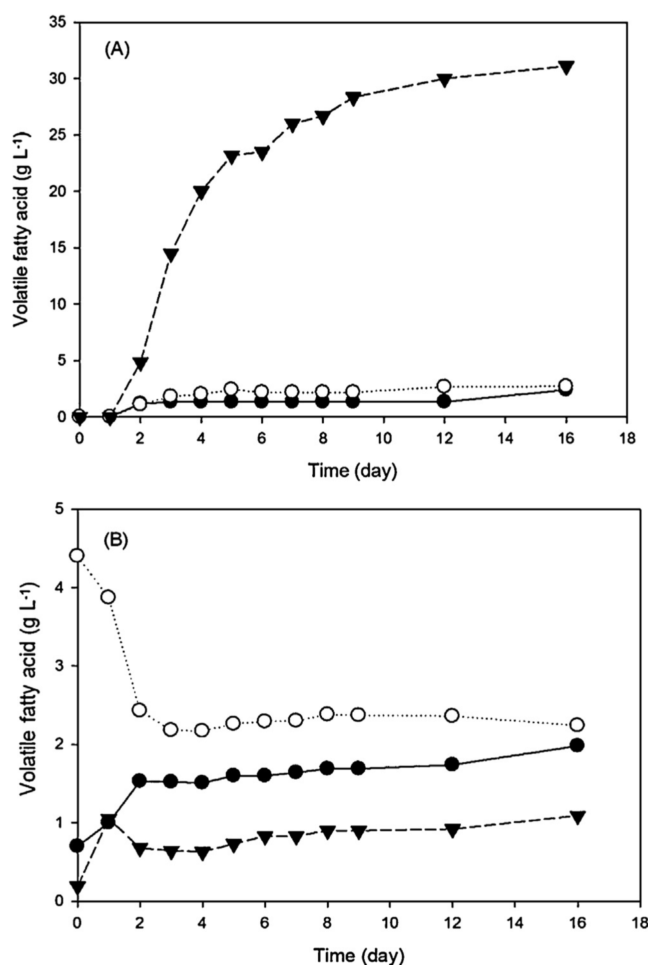

Fig. 4. Change of volatile fatty acid in solvent phase and aqueous medium phase during *in situ* extractive fermentation. (A) Solvent phase; (B) aqueous medium phase. ▼, hexanoic acid; ○, butyric acid; and ●, acetic acid.

As shown in Fig. 4(A), the amount of hexanoic acid in the solvent increased rapidly up to 26 g L^{-1} in the 5 day cultivation; the productivity of hexanoic acid thereafter began to decrease gradually. The hexanoic acid concentration in the aqueous medium layer was stably maintained between 1 and 2 g L^{-1} during the 16 days' fermentation (Fig. 4(B)). The initial nitrogen content in the optimized medium was 2.7 g L^{-1}, which is calculated from the sum of nitrogen in the yeast extract (about 10%) and tryptone (about 15%); and the final nitrogen content in the 16 day culture broth was 0.87 g L^{-1}, which is indirectly measured using the COD equivalent. Thus, the medium contained enough nitrogen for *Clostridium* sp. BS-1 to use during cultivation. The extractive fermentation pH was maintained at around 5.5 during the 16 day batch culture. The stable pH may be related with the constant total amount of carboxylic acids in the medium layer (Fig. 4(B)).

In the extraction of acetic and propionic acid, only undissociated acids are extracted to solvents [40]. At neutral pH the organic acids are deprotonated in the fermentation broth; therefore, reduction of pH under pK_a of the target organic acid is usually necessary before solvent extraction for 2–4 carbon number organic acid. In the case of butyric acid extraction, most solvents work well only with a pH value much lower than the pK_a value of butyric acid, and the extraction efficiency decreases when the pH is higher than the pK_a value of butyric acid [20,36]. Even though the pK_a values of butyric acid and hexanoic acid are similar, 4.82 and 4.88, respectively; the practical extraction efficiency of hexanoic acid was much higher than that of butyric acid. The higher extraction efficiency of hexanoic acid may be related with its lower miscibility in water (10.82 g L^{-1}). There is a significant difference in the solubility between butyric acid and hexanoic acid, and the solubility diminishes as the number of methylene groups in the alkyl chain increases [41]. Therefore, we speculated that the hydrocarbon group of hexanoic acid would positively influence the extraction efficiency. As shown in Fig. 4(B), over 90% (w/w) hexanoic acid was extracted into the solvent solution, 10% (v/v) alamine 336 in oleyl alcohol.

The specific productivity and yield of hexanoic acid production in this study were compared with previously reported results (Table 8). *M. elsdenii* and *C. kluyveri* are well known as hexanoic acid producing anaerobes. Recently, *C. kluyveri* strain 3231B was reported as an isolate from the bovine rumen. This isolate represented the highest hexanoic acid production (12.8 g L^{-1}) together with butyric acid (3.0 g L^{-1}) without adaptation in a batch culture, with ethanol and acetate serving as an electron donor and an acceptor, respectively [42]. For industrial application of biomolecule production, the retrieval of products is one of the major steps in the whole production process; therefore, economic retrieval methods should be considered together with the productivity of the target molecule. The extractive fermentation results of *Clostridium* sp. BS-1 presented an increased amount of hexanoic acid production by BS-1 and relatively easy retrieval of the produced hexanoic acid without cellular damage. *In situ* extractive fermentation with the optimized medium by *Clostridium* sp. BS-1 showed the excellent performance of hexanoic acid production in terms of both final concentration and productivity.

Clostridium sp. BS-1 was originally selected for utilization of galactitol, because if a microbe utilizes sugar alcohol it means

Table 8
Comparison of biological hexanoic acid production.

Strains	Methods	Hexanoic acid ($g L^{-1}$)	Specific productivity ($g L^{-1} h^{-1}$)	Yield	Reference
M. elsdenii ATCC25940	Fed batch with free resin	19.3	0.13	0.27 $g g^{-1}$ glucose	[11]
C. kluyveri ATCC 8527	Co-culture	4.6	0.10	–	[10]
C. kluyveri 3231B	Batch culture using ethanol and acetate	12.8	0.18	0.45 $g g^{-1}$ ethanol and acetate	[42]
Clostridium sp. BS-1	Batch culture	2.9	0.04	0.3 $g g^{-1}$ galactitol	[4]
Clostridium sp. BS-1	Batch culture using optimized media	6.9	0.28	0.46 $g g^{-1}$ galactitol	This study
Clostridium sp. BS-1	Batch culture using optimized media and extractive fermentation	32.0	0.34	0.42 $g g^{-1}$ galactitol	This study

both forms of carbohydrate monomer from hydrolysis of a polymer can be metabolized. Although galactitol is more expensive than glucose at present, if a sufficient amount of seaweed can be supplied for bioenergy and biomaterial production, *Clostridium* sp. BS-1 may provide a source of economically producible biological hexanoic acid. As future studies for economic hexanoic acid production, substitution of yeast extract and tryptone to more economic nitrogen sources and/or growth factors should be investigated. Furthermore, direct production of hexanoic acid from crude galactitol, which is produced by hydrolysis and hydrogenation of seaweed, may be necessary for industrial utilization of *Clostridium* sp. BS-1.

4. Conclusion

The medium composition for hexanoic acid production by *Clostridium* sp. BS-1 was optimized with statistical methods, a fractional factorial experimental design, and a Box–Behnken experimental design. The experimental designs were tested in batch cultures. The data were analyzed by Design-Expert. Yeast extract, tryptone, $FeSO_4·7H_2O$, sodium acetate, and sodium butyrate were selected for medium optimization, and among them, yeast extract and sodium butyrate prominently affected hexanoic acid production. The concentrations of acetic acid and $FeSO_4·7H_2O$ were fixed to minimum concentrations, because they affected hexanoic acid production sufficiently despite that only small amounts were added to the medium. The model suggested solutions and the predicted maximum concentration of hexanoic acid. The solution was verified as the following composition in gram per liter: yeast extract, 15.5; tryptone, 10; $FeSO_4·7H_2O$, 0.04; sodium acetate, 0.85; and sodium butyrate, 6.5. When the solution was verified practically, hexanoic acid increased to 6.96 $g L^{-1}$. In order to recover produced hexanoic acid, an *in situ* biphasic extractive system using alamine 336 and oleyl alcohol was applied. Using the Gompertz equation, lag time, productivity, and potential hexanoic acid production amount were evaluated as parameters and compared with conditions without a solvent. The biphasic extraction system lengthened lag time but productivity and product concentration increased considerably. As a result, *Clostridium* sp. BS-1 produced up to 32 $g L^{-1}$ of hexanoic acid due to effective HA extraction and the maintenance of a low (1–2 $g L^{-1}$) concentration of HA in the culture broth.

Acknowledgements

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