J Biochem Tech (2012) 3(5): S161-S166
ISSN: 0974-2328

Standardization of biosurfactant enrichment process by factorial design and elucidating its physico-chemical and structural characteristics

Karadi R V, Lokesh K N*, Channarayappa, M V Venkataranganna, Bindu Sadanandan, Siva Kiran R R

Received: 27 August 2012 / Received in revised form: 07 November 2012, Accepted: 8 November 2012, Published online: 18 April 2013

Abstract

Biosurfactant recovery by *Flavobacterium* sp. was standardized by factorial design $3^{(k-p)}$. The extraction of biosurfactant was carried out by organic solvent extraction, ammonium sulphate precipitation and acid precipitation. The organic solvent extraction was performed with varied proportion (3 levels) of chloroform and methanol i.e. (X*:1) designated as F1 (varied proportion of chloroform) and (1: X**) referred as F2 (varied proportion of methanol) respectively, similarly ammonium sulphate (F3) and acid precipitation (F4) was performed with 3 varying experimental level. The statistical data interpretation *viz* ANOVA, Pareto chart of standardized effect, Half normal probability plot inferred organic solvent extraction as a efficient method for recovery of biosurfactant, than other counter parts of extraction .The surface plot between significant factors, given the standardized proportion of organic solvents for extraction of biosurfactant, which was found to be 1:1. Surface tension and CMC value of recovery biosurfactant was found to be 33 mN/m and its CMC was 400- 500mg respectively, it has shown maximum emulsification index of 94% for soyabean oil. The presence of glycolipid moiety in the recovery biosurfactant was elucidated by IR and NMR spectroscopic studies

Keywords: Factorial design, organic solvent, CMC, Emulsification index

Karadi R V

KLE's University, College of Pharmacy, Hubli- 580031, India

Lokesh K N*, Channarayappa, Bindu Sadanandan, Siva kiran R R

Department of Biotechnology, M.S Ramaiah Institute of technology, Bangalore – 560054

*Email: knlokes@gmail.com

M V Venkataranganna

Preclinical Pharmacology and toxicology, Connexios Life *Sciences,* Pvt Ltd, Bangalore, India

Introduction

Biosurfactants are amphiphilic compounds derived from microbial sources, they are surface bound, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively (Karanth et al. 1999).

All surfactants are chemically synthesized. Nevertheless, in recent years, much attention has been directed towards biosurfactants due to their broad range of functional properties and diverse synthetic capabilities of microbes (Singh et al. 2007). Moreover, they are ecologically safe and biosurfactants can be used as bioemulsifier in bioremediation of hydrocarbons in oil reservoirs (Amedea Perfumo et al. 2010). Biosurfactants used in biofloatation method for recovery of coal (Mohammad Hassan Fazaelipoor 2010). Moreover, its possible application in food processing and bio-medical sectors has been explored (Singh et al. 2007; Gharaei-Fathabad 2011).Even if optimum production is obtained using optimal media and culture conditions, the production process is still incomplete without an efficient and economical means for recovery of the products. For many biotechnological products, the downstream processing costs account for~60% of the total production costs. Several conventional methods for the recovery of biosurfactants, such as acid precipitation, solvent extraction, crystallization, ammonium sulphate precipitation and centrifugation and foam fractionation have been widely reported (Desai J D and Banat I M 1997; Thitima Sarachat et al. 2010). Often a single downstream processing technique is not enough for product recovery and purification. In such cases, a multi-step recovery strategy, using a sequence of concentration and purification steps, is more effective in such a multi-step recovery for biosurfactants; it will be possible to obtain the product at any required degree of purity (Reiling H E 1986).

In the present investigation, the enrichment methodologies for recovery of crude biosurfactant, obtained from *Flavobacteium* sp MTCC 2495 were standardized by means of factorial design. The recovery methods such as organic solvent extraction, ammonium sulphate precipitation and acid precipitation were employed for recovery of biosurfactant which were standardized factorial design $3^{(k-p)}$ and also the physicochemical and structural properties of crude biosurfactant were also elucidated by IR and NMR studies.

Materials and Methods

Bacterial strain and culture growth conditions

Flavobacterium sp. MTCC 2495 was maintained in Modified Wakimoto Medium which has following composition: Ca $(NO_3)_2.4H_2O$, 0.5 g/l; Na_2PO_4. H_2O, 2.0 g/l; Peptone, 5.0 g/l; sucrose, 15.0 g/l; $FeSO_4$, 0.5 g/l; agar, 15g/l , maintained at 30° C and was sub cultured every 2 weeks.

Production medium for biosurfactant

In our previous investigation (unpublished data) we optimized the medium constituent's for production of biosurfactants by the strain *Flavobacterium* sp MTCC 2495, using Mineral salt medium (MSM), which consists of critical medium components such as 10.85 g/l sucrose and 3.11 g/l peptone and ferrous sulphate as trace element, was inculcated with 2% V/V of seed culture and incubated at 30°C , pH of 7.1, at 300 rpm in lab scale fermentor (Sartorious B Lite Model) of capacity 2 liters, for 48 hrs.

Recovery and enrichment method

The fermented broth was harvested by centrifuged at 15,300 X g for 10 min at 10 °C; the cell free supernatant was subjected for enrichment operation. The enrichment operations, such as organic solvent extraction, ammonium sulphate precipitation and acid precipitation were used for recovery of biosurfactant.

Experimental designs

Response surface methodology is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery et al. 1997

Factorial designs

In order to identify which component(s) of the medium has a significant effect on cellular growth a first optimization step was developed. In a factorial design the influences of all experimental variables, factors, and interaction effects on the response or responses are investigated (Montgomery et al. 1997). The standardization of enrichment methods for recovery of biosurfactant were carried out by using the response surface methodology, 3 level factorial design $3^{(k-p)}$, 27-trial experimental design, where each variable was tested in three different coded levels: low (−1), middle (0) and high (+1). The corresponding coded values correspond for enrichment operations are shown as in Table no. 1.

A second-order polynomial model (Eq.1) was fitted to the response data obtained from the design.

$$Y = \beta 0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{22} X_{22} + \beta_{33} X_{23} + \beta_{44} X_{24} \quad \text{(Eq.1)}$$

Table 1: Coded factors for factorial design $3^{(3-0)}$ design.

Factor	Coded	-1	0	1	Units
Solvent Extraction (X*:1)	X1	1	4	7	--
Solvent Extraction (1:X**)	X2	1	4	7	--
Ammonium sulphate	X3	20	50	80	% w/v
Acid precipitation	X4	1	3	5	% v/v

(X*: 1); Varied parts of chloroform, (1: X**); Varied parts of methanol

Where Y is the predicted response, β_0 is the model constant; X_1, X_2, X_3 and X_4 are Independent variables; β_1, β_2, β_3 and β_4 are linear coefficients and β_{11}, β_{22} β_{33} and β_{44} are the quadratic coefficients. Validation experiments were carried out to verify the validity and the accuracy of the models.

Data analysis

Experimental designs matrix for factorial design were generated by using statistical software package, Stat Soft®, STATISTICA version 6.0. The data analysis of experimental design was also performed by using this software. The quality of the first order polynomial model was expressed by the coefficient of determination (R^2 value) and its significance was validated by ANOVA. The 2D interaction contour surface plot of significant variable was generated.

Physicochemical characterization of Biosurfactant

The physico-chemical characterizations of flavolipid biosurfactant were carried out different methods such as, surface tension, critical micelle concentration (CMC) and emulsification activity

Determination of Surface tension

The surface tension of the methanolic extract at different biosurfactant concentrations was measured by using a du No¨uy ring-type tensinometer. The surface tension measurement was carried out at 27 ± 1°C after dipping the platinum ring in the solution for a while in order to attain equilibrium conditions. The measurement was repeated three times and an average value was obtained. The critical micelle concentration (CMC) was then determined form the plot between break point of the surface tension versus biosurfactant Log concentration curve.

Measurement of emulsification activity

The emulsification activity of the biosurfactant was measured by adding about 3.0 ml of different oil samples (Soybean, Olive oil, Palm oil, Groundnut oil, Paraffin oil) to 2.0 ml of the biosurfactant in a graduated test tube and vortexes at high speed for 2 min. Distilled water was used as control in the determination. Measurement of the emulsification index (E_{24}) was made 24 hr later as the height of the emulsion layer, divided by the total height of the mixture and multiplied by 100 (D G Cooper and Goldenberg 1987).

Structural characterization of biosurfactant

The structural characteristics of the isolated biosurfactant sample were determined by using Fourier transform infrared (FT-IR) spectroscopy. Moreover, nuclear magnetic resonance (NMR) analysis was employed to identify the chemical structures of the components in the biosurfactant. Infrared (IR) spectroscopy of the biosurfactant was carried out on a Genesis - 2 SPIR. IR spectra were collected between 500 and 4000 wave numbers /cm. The ^{1}H NMR spectra of each fraction obtained from the fractionation step were achieved from FT NMR 500 MHz spectrometer (JEOL, JNM-A500) using deuterated chloroform as a solvent.

Results and Discussions

Our previous investigation has shown that biosurfactant production from *Flavobacterium* sp MTCC 2495 is growth associated, there was a liner relation ship existed between biomass and biosurfactant

production (unpublished data).Due to economical considerations use of most the biosurfactants would have to involve either whole cell culture broths or other crude and partially purified preparations (Sanket J Joshi and Anjana J Desai 2010).

Standardization of biosurfactant yield by RSM

The factorial design enables the identification of the significant enrichment operation for biosurfactant recovery. Table 2 presents the result of $3^{(3-0)}$ factorial design for recovery of biosurfactant by different methods (Solvent, acid, salt precipitation, acid precipitation).

Table 2: Experimental design and results of the factorial design $3^{(3-0)}$

Run No.	X1	X2	X3	X4	Biosurfactant yield g/l	Observed (g/l)	Predicted (g/l)
1	1	1	20	1	3.43	3.43	3.531
2	1	1	50	5	3.11	3.11	3.469
3	1	1	80	3	3	3	3.543
4	1	4	20	5	2.76	2.76	2.536
5	1	4	50	3	2.32	2.32	2.423
6	1	4	80	1	2.96	2.96	2.744
7	1	7	20	3	2.98	2.98	2.593
8	1	7	50	1	2.87	2.87	2.728
9	1	7	80	5	2.99	2.99	2.852
10	4	1	20	5	2.23	2.23	1.65
11	4	1	50	3	1.92	1.92	1.538
12	4	1	80	1	2.43	2.43	1.859
13	4	4	20	3	0.32	0.32	0.604
14	4	4	50	1	0.21	0.21	0.739
15	4	4	80	5	0.99	0.99	0.863
16	4	7	20	1	0.24	0.24	0.909
17	4	7	50	5	0.54	0.54	0.847
18	4	7	80	3	1.05	1.05	0.921
19	7	1	20	3	1.5	1.5	1.686
20	7	1	50	1	2.21	2.21	1.82
21	7	1	80	5	1.21	1.21	1.944
22	7	4	20	1	0.87	0.87	0.887
23	7	4	50	5	1.09	1.09	0.824
24	7	4	80	3	1	1	0.899

Based on experimental data, the Pareto chart of standardized effects were plotted for identifying the factors that are important in biosurfactant production. The selected factors main effects are ranked in order according to their level of significance. It is evident from the Pareto chart Fig. 1, that the most important variables are X1 and X2 (both linear and quadratic form) having standardized effects of (absolute value) 8.41, 5.62, 3.91 and 3.15 respectively

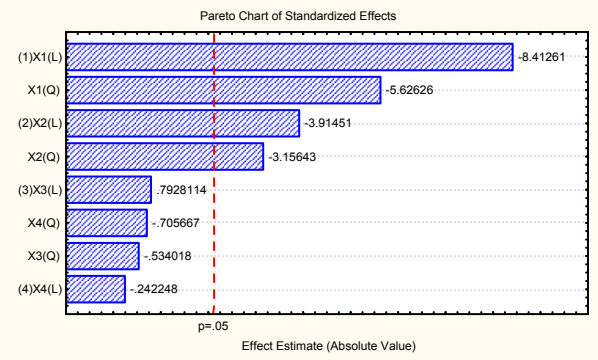

Figure 1: Pareto chart of standardized effects of independent variables on biosurfactant yield (g/l) obtained from *Flavobacterium* sp MTCC 2495.

Further confirmation about the significant factors was obtained from ANOVA. The observed lowest P-value (0.0000) and highest F-value (70.77) that is (F>P value) was observed with X1(linerar) followed, X1(quadratic) and X2 (both linear and quadratic) as shown in Table 3.

Table 3: ANOVA results of factorial design $3^{(3-0)}$ design

Factors	SS	df	MS	F	p
X1(L)	12.97102	1	12.97102	70.77200	0.000000
X1(Q)	5.80167	1	5.80167	31.65483	0.000024
X2 (L)	2.80845	1	2.80845	15.32336	0.001016
X2(Q)	1.82602	1	1.82602	9.96304	0.005461
X3 (L)	0.11520	1	0.11520	0.62855	0.438213
X3 (Q)	0.05227	1	0.05227	0.28518	0.599863
X4 (L)	0.01076	1	0.01076	0.05868	0.811327
X4 (Q)	0.09127	1	0.09127	0.49797	0.489428
Error	3.29902	18	0.18328		
Total SS	26.97567	26			

The Half-Normal probability plot of effects is very useful for separating random noise from 'real effects based on their distribution on the plot as shown in Fig.2. It is evident from that among selected factors of solvents extraction method with varied proportion of X1 and X2 were positioned outlier with better factor confidence levels.

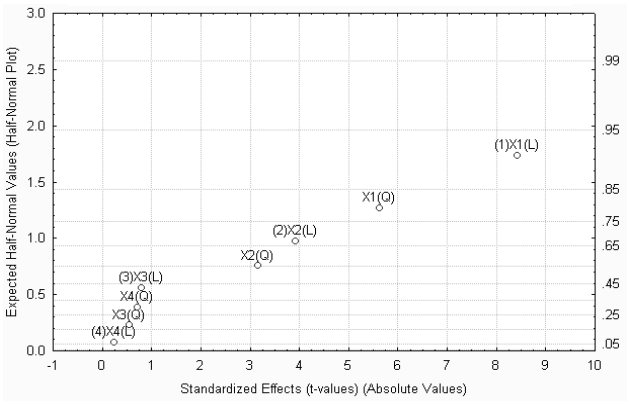

Figure 2: The Half normal probability plot of biosurfactant yield (g/l), used to identify factors which has larger standardized effects with better confidence level

The Second order regression equation provided the levels of biosurfactant production as a function of initial values of sucrose, peptone, ferrous sulphate which can be predicted by the following equation Eq (2).

$$Y = 5.419 - 1.15\,X_1 + 0.11\,X_1^2 - 0.62\,X_2 + 0.66\,X_2^2 \quad (2)$$

Where, Y represents biosurfactant yield g/l and X_1, with varied proportion of chloroform (1- 7 parts with 4 as central value), X_2, varied proportion of methanol (1- 7 parts with 4 as central value) .The model adequacy was checked and it was found to be adequate, the goodness of fit of the model was expressed by the coefficient of determination R^2, which was calculated to be 0.87, indicating that 87% of the variability in the response could be explained by the model. Fig. 3 shows the surface response plot of the model equation. From equations derived by differentiation of Eq. (2), the model has predicted the solution at minimum, as 1 part of chloroform with 1 part of methanol and the predicted solution was 0.32 g/l. In order to confirm the predicted results of the model, the recovery of biosurfactant was carried out by using chloroform and methanol, in

1:1 ratio, a value of 0.39 g/l yield was reported, with deviation of ± 0.05 corresponding to the predicted value.

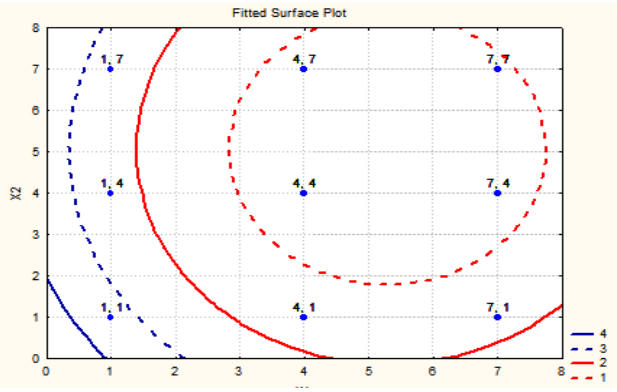

Figure 3: Contour graphs showing interaction between variables X1 and X2 on biosurfactant yield**.**

Physicochemical characterization

Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions, stabilization or destabilization of emulsions, and hydrophilic-liphophilic balance (HLB). Surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer. The surface tension of distilled water is 72 mN/m, and addition of surfactant lowers this value to 30 mN/m. When a surfactant is added to air/water or oil/water systems at increasing concentrations, a reduction of surface tension is observed up to a critical level, above which amphiphilic molecules associate readily to form supramolecular structures like micelles, bilayers, and vesicle. This value is known as the critical micelle concentration (CMC). CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant. Microbial culture broth or biosurfactants are diluted several fold, surface tension is measured for each dilution, and the CMC is calculated from this value (D G Cooper and D A Paddock 1983). As shown in Fig.4, the log concentration of biosurfactant has lowered the surface tension of as low as 33 mN/m and its CMC was 400- 500mg.

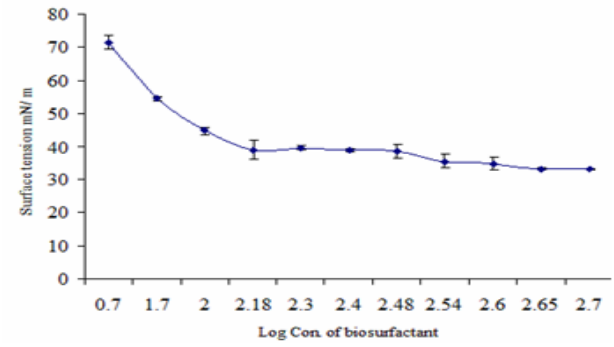

Figure 4: CMC value of biosurfactant obtained by *Flavobacterium* sp MTCC 2495. The logarithmic concentration of biosurfactant at which surface tension of the broth remain stationary, regarded as CMC.

Emulsification index (E_{24})

An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase. Biosurfactants may stabilize (emulsifiers) or destabilize (deemulsifiers) the emulsion. The emulsification activity is assayed by the ability of the surfactant to generate turbidity due to suspended hydrocarbons such as a hexadecane–2-methylnaphthalene mixture etc., in an aqueous assay system (Rosenberg et al. 1979). The ability of biosurfactants to form stable emulsions with vegetable oils and fats suggests potential application as cleaning and emulsifying agent in the food industry (Nitschke and Pastore, 2006).With different hydrocarbon and vegetable oils suggests considerable potential applications in the petroleum, food and pharmaceutical industries. In addition to surface and interfacial tension, stabilization of an oil and water emulsion is commonly used as a surface activity indicator (Cooper and Goldenberg 1987). As shown in Table 4, all the hydrocarbons tested served as substrates for emulsification by the biosurfactant, soyabean and olive oil are having best (E_{24}) value of 94 ± 0.8 and 92 ± 1.2 respectively.

Table 4: Emulsification index of biosurfactant with different hydrocarbon oil

Oil	**Emulsification index (E_{24}) %**
Soybean oil	94 ± 0.8
Olive oil	92 ± 1.2
Palm oil	88 ± 1.4
Groundnut oil	86 ± 1.2
Paraffin oil	90 ± 0.9

Structural characterization

The chemical composition of each component fractionated from the crude biosurfactant was preliminarily investigated by using FTIR technique as shown in Fig 5. The important adsorption bands located at 3421, 2927, 2855, 1744, and 1300–1100 cm $^{-1}$ indicate that all of them have chemical structures identical to those of Glycolipid. The broad band appearing at 3421 cm^{-1} should be assigned to the O–H stretching vibrations of hydroxyl groups in the chemical structures of the biosurfactants. The strong adsorption peaks present at 2927 and 2855cm^{-1} are expected to be the C–H stretching vibrations of the hydrocarbon chain positions. The characteristic peak displayed at 1744 cm^{-1} relates to the C=O stretching vibrations of the carbonyl groups while the C– O stretching bands at 1300–1000 cm^{-1} confirm the presence of the bonds formed between carbon atoms and hydroxyl groups in the chemical structures of biosurfactant.

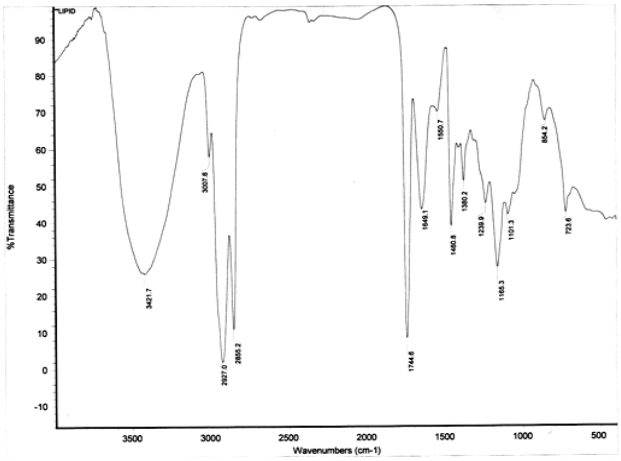

Figure 5: (A-C): Fourier transforms infrared absorption spectra of crude biosurfactant obtained from *Flavobacterium* sp MTCC 2495. The absorption bands used for qualitative analysis of biosurfactant

The characteristic chemical shifts present in the ^{1}H NMR spectra confirm that all of the isolated fractions have the molecular structures of the glycolipid species as shown in Fig. 6. The presence of long hydrocarbon chains is indicated by the appearance of the

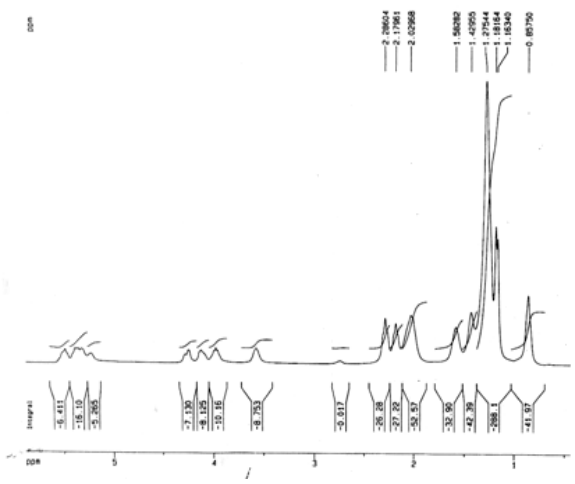

Figure 6: ^{1}H NMR absorption spectra of crude biosurfactant obtained from *Flavobacterium* sp MTCC 2495. characteristic chemical shifts in the region of 0.8–1.4

With the use of ^{1}H NMR analyses, the chemical structures of all the isolated fractions were revealed. The characteristic chemical shifts present in the 1H NMR spectra confirm that all of the isolated fractions have the molecular structures of the rhamnolipid species (types of glycolipids). The presence of long hydrocarbon chains and rhamnose rings is indicated by the appearance of the characteristic chemical shifts in the region of 0.8–1.4 and 3.3–5.5 ppm, respectively. The preliminary spectroscopic studies by IR and NMR has given one common conclusion that the recovered biosurfactant is belongs to class of glycolipid.

Conclusion

For recovery of biosurfactant the solvent extraction method chloroform: methanol, (1:1) method was found to be most appropriate than the other methods of recovery. The crude biosurfactant which was recovered by *Flavobacterium* sp MTCC 2495 has potent phenomenon of changing interfacial tensions and has promising emulsifying index. The recovered crude biosurfactant has functional groups, resembling glycolipid, therefore, as it is derived from *Flavobacterium* sp., referred as Flavo-glycolipid or simply Flavolipid.

Acknowledgments

Authors are grateful to the dean, Dr. F V Manvi and Principal Dr A D Taranhalli of KLE's University College of Pharmacy, Belgaum for providing necessary facilities for the research work. Authors want to extend their warm regards to M.S. Ramaiah institute of technology for giving valuable inputs on design of experiments and also acknowledge valuable suggestions.

References

Ashiuchi M, Kamei T, Baek D H et al (2001) Isolation of *Bacillus subtilis* (chungkookjang), a poly-gamma-glutamate producer with high genetic competence. Appl Microbiol Biotechnol 57:764-769

Bajaj I B, Lele S S, Singhal R S (2009) A statistical approach to optimization of fermentative production of poly(gamma-glutamic acid) from *Bacillus licheniformis* NCIM 2324. Bioresour Technol 100:826-832

Chauhan B, Gupta R (2004) Application of statistical experimental design for optimization of alkaline protease production from *Bacillus sp. RGR-14*. Process Biochem 39:2115-2122

Cromwick A M, Gross R A (1995) Investigation by NMR of metabolic routes to bacterial γ -poly(glutamic acid) using 13C-labeled citrate and glutamate as media carbon sources. Can J Microbiol 41:902-909

Cromwick M, Birrer G A, Gross R A (1996) Effects of pH and aeration on g-poly(glutamic acid) formation by *Bacillus licheniformis* in controlled batch fermentor cultures. Biotechnol Bioeng 50:222-227

Deng S B, Bai R B, Hu X M et al (2003) Characteristics of a bioflocculants produced by *Bacillus mucilaginosus* and its use in starch wastewater treatment. Agric Biol Chem 60:588-593.

Du G, Yang G, Qu Y et al (2005) Effects of glycerol on the production of poly(c-glutamic acid) by *Bacillus licheniformis*. Process Biochem 40:2143-2147

Dubois M, Gilles K A, Hamilton J K et al (1956) Colorometric methods for determination of sugars and related substances. Anal Chem 28:350-356

Gong W X, Wang S G, Sun XF et al (2008) Bioflocculant production by culture of Serratia ficaria and its application in wastewater treatment. Bioresour Technol 99:4668-4674

Goto A, Kunioka M (1992) Biosynthesis and hydrolysis of poly (g-glutamic acid) from *Bacillus subtilis* IFO3335. Biosci Biotechnol Biochem 56:1031-1035

Hanlon G W, Hodges N A, Russell A D (1982) The influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by Bacillus licheniformis. J Gen Microbiol 128:845-851

Houghton J I, Quarmby J (1999) Biopolymers in wastewater treatment. Curr Opin Biotechnol 10:259-262

Ko YH, Gross R A (1998) Effects of glucose and glycerol on gamma-poly(glutamic acid) formation by Bacillus licheniformis ATCC 9945a. Biotechnol Bioeng 57:430-437

Kumar C G, Malik R K, Tiwari M P et al (1999) Optimal production of Bacillus alkaline protease using a cheese whey medium. Microbiologie des Alimentes et Nutrition 17:39-48

Kunioka M, Goto A (1994) Biosynthesis of poly(g-glutamic acid) from L-glutamic acid, citric acid, and ammonium sulfate in *Bacillus subtilis IFO3335*. Appl Microbiol Biotechnol 40:867-872

Kurane R, Toeds K, Takeda K et al (1986) Culture conditions for production of microbial flocculant by *Rhodococcus erythropolis*. Agric Biol Chem 50:2309-2013

Li Y, He N, Guan H et al (2003) A novel polygalacturonic acid bioflocculant REA-11 produced by Corynebacterium glutamicum: a proposed biosynthetic pathway and experimental confirmation. Appl Microbiol Biotechnol 63:200-206

Lian B, Chen Y, Zhao J et al (2008) Microbial flocculation by Bacillus mucilaginosus: Applications and mechanisms. Bioresour Technol 99:4825-4831

Mahmoud D A R (2006) Isolation of polyglutamic Acid flocculant producing bacteria from extreme Egyptian environments. J Appl Sci Res 2:608-612

Malathi S, Chakraborty R (1991) Production of Alkaline Protease by a New Aspergillus flavus Isolate under Solid-Substrate Fermentation Conditions for Use as a Depilation Agent. Appl Environ Microbiol 57:712-716

Priest F G (1977) Extracellular enzyme synthesis in the genus Bacillus. Bacteriol Rev 41:711-753

Puri S, Beg Q K, Gupta R (2002) Optimization of alkaline protease production from Bacillus sp. by response surface methodology. Curr Microbiol 44:286-290

Reddy LV, Wee YJ, Yun J S et al (2008) Optimization of alkaline protease production by batch culture of Bacillus sp. RKY3

through Plackett-Burman and response surface methodological approaches. Bioresour Technol 99:2242-2249

Salehizadeh H, Vossoughi M, Alemzadeh I (2000) Some investigations on bioflocculant producing bacteria. Biochem Eng J 5:39-44

Shih I L, Van Y T (2001) The production of poly-(gamma-glutamic acid) from microorganisms and its various applications. Bioresour Technol 79:207-225

Shih I L, Van Y T, Chang YN (2002) Application of statistical experimental methods to optimize production of poly(g-glutamic acid) by *Bacillus licheniformis* CCRC 12826. Enzyme Microb Technol 31:213-220

Shih I L, Van Y T, Yeh LC et al (2001) Production of a biopolymer flocculant from Bacillus licheniformis and its flocculation properties. Bioresour Technol 78:267-272

Suh H H, Kwon G S, Lee C H et al (1997) Characterization of bioflocculant produced by *Bacillus* sp. DP-152. J Ferment Bioeng 84:108-112

Suo C, Mei L H, Huang J et al (2007) Selection of γ-poly glutamic acid high yield strain by 60Co γ-irradiation and the optimization of its culture medium. J Chem Eng (Chinese Universities) 21:820-825

Takeda M, Koizumi J I, Matsuoka H et al (1992) Fators affecting the activity of a protein bioflocculant produced by *Nocardia amarae*. J Ferment Bioeng 74:408-409

Tobisch S, Zuhlke D, Bernhardt J et al (1999) Role of CcpA in regulation of the central pathways of carbon catabolism in Bacillus subtilis. J Bacteriol 181:6996-7004

Toeda K, Kurane R (1991) Microbial flocculant from *Alcaligenes cupidas* KT 201. Agric Biol Chem 55:2793-2799

Wu J Y, Ye H F (2007) Characterization and flocculating properties of an extracellular biopolymer produced from a *Bacillus subtilis* DYU1 isolate. Process Biochem 42:1114-1123

Yokoi H, Natsuda O, Hirose J et al (1995) Characteristics of a biopolymer flocculant produced by *Bacillus sp. PY-90*. J Ferment Bioeng 79:378-380