PRODUCTION OF LIPOPEPTIDE BIOSURFACTANT BY INDIGENOUS ISOLATE OF Bacillus *sp.* BMN 14 IN A BATCH BIOREACTOR

Liesbetini Hartoto and Djumali Mangunwidjaja

Research Center for Biotechnology. Bogor Agricultural Univ., Darmaga , Bogor 16610 Indonesia

ABSTRACT: The ability of *Bacillus sp* BMN 14, an indigenous strain isolated from palm oil contaminated soil, to grow and produce lipopeptide biosurfactant on various sugar (glucose, fructose and sucrose with concentration of 1.0; 2.0; 3.0; 4.0; 5.0 and 6.0 % was firstly studied on shake flask cultures. Among those various sugar tested, 4.0 % glucose in mineral salts medium supported maximum growth and highest biosurfactant yield. The production of biosurfactant was then investigated in a batch 2- L stirred tank bioreactor at temperature 30° C and pH 6.5-7.0 The highest performance of bioreactor was obtained at an aeration rate of 0.5 vvm. In this process, the maximum biomass of 4.19 g/L, biosurfactant concentration of 0.87 g/L, and lowest surface tension of 28.3 mN/m were obtained.

Key Words: Biosurfactant, Aeration, Batch bioreactor, *Bacillus* sp BMN 14

INTRODUCTION

Biosurfactants are microbially produced compounds that have hydrophobic and hydrophilic moieties and capable to decrease surface and interfacial tension. These compounds have important advantages, such as low toxicity and biodegradable, high specifity, effective physicochemical properties and temperature stability. These biological compounds are grouped as glycolipids, lipopeptides, phospholipids, fatty acids and neutral lipids (Mulligan and Gibbs, 1993). Interest in biosurfactant has increased considerably in recent years, as they are potential candidates for many commercial applications in petroleum, pharmaceuticals and cosmetics, pulp and paper, and food processing industries (Desai and Desai, 1993)

The strain *Bacillus* sp produces different lipopeptides biosurfactant named iturin A, iturin C, bacillomycin D and L, bacillomycin F, mycosubtilin and surfactin. These substances differ mainly by their peptidic moiety (Jacques, et al. 1994) Surfactin, a cyclic lipopeptide produced by *Bacillus subtilis* is one of the most effective biosurfactant knows so far. It is capable to lowering the surface tension from 72.0 to 27.9 mN/m at a concentration s low as 0.005 % (Desai and Desai, 1993). Horowitz and Griffin (1991) found that surfactant BL-86 is a mixture of lipopetides with the mayor components ranging in size from 979 to 1091 daltons with varying in increments of 14 daltons. There are seven amino acids per molecule, while lipid portion is composed of 8 to 9 methylene groups and mixture of linear and branch tail.

Unfortunately, up to now, biosurfactant are unable to compete economically, with the chemically synthesized compounds on the market, due to high production cost. These are primarily due to insufficient bioprocessing, methodology, poor strain productivity and the use of expensive substrates (Fiechter, 1992). In this paper, the production of biosurfactant by an indigenous isolate of *Bacillus sp.* BMN 14 on glucose, fructose and sucrose as substrate are presented. In addition, the effect of aeration rate for the production of biosurfactant in a batch stirred bioreactor was investigated.

METHODOLOGY

Bacillus sp. BMN 14 isolated from palm-oil contaminated soil by Richana (1997) and was found to be lipopeptide biosurfactant producing strain. It was maintained at 4oC in nutrient agar, was used in all experiments. The basic mineral salt medium for bacterial growth (Cooper, et al.,1981) modified by Sen, et al. (1997) contained (in g/L) : NH_4NO_3 4.002; $MgSO_4.H_2O$ 0.1972; $CaCl_2.2H_2O$ 0.001; EDTA 0.0017; $FeSO_4.7H_2O$ 0.004;, $MnSO_4$ 0.275; KH_2PO_4 4. 0827; Na_2HPO_4 7.12. The inoculum was prepared and cultivated at 37°C and 140 rpm in a shaking incubator for 30 h. The inoculum was added to the cultivation media by the volume ratio of 10.0 % (v/v). The production of biosurfactant was conducted in a 2.0 L Bioreactor (Biostat M-B. Braun, Germany) at 37°C, pH 6.5-7.0 and agitation rate 200 rpm. The aeration rate was varied on 0.5; 1.0 and 1.5 vvm. The bioreactor was equipped with a collection vessel in its air-exhaust line to trap the foam overflow. Samples of cultivation culture

were taken at 3-6 h intervals for: biomass, biosurfactant, surface tension, and residual sugar determinations. The biomass was determined gravimetrically by the weight of cells (Scragg, 1991;Sheppard and Cooper, 1991). Crude biosurfactant was isolated from the culture broth by acid precipitation and analyzed by HPLC using the method of Juwarkar *et al.* (1994). Surface tension of the culture broth was measured with Du Nouy tensiometer CSC 70545 (Babu *et al.*, 1994). Residual glucoses were analyzed by dinitro-salicylic acid method of Miller (1959) with modifications.

RESULTS AND DISCUSSION

Effect of Various Sugar on Biosurfactant Production

In order to establish the most suitable carbon source for the production of biosurfactant by Bacillus sp BMN 14 strain, different sugars, i.e glucose, fructose, sucrose was observed from 1.0 to 6.0 %. The experiments revealed that among three sugars used, glucose seemed to be was the best as a carbon source for growth and biosurfactant production. Among the six concentrations (1.0;2.0;3.0;4.0;5.0; and 6.0%) tried, a 4.0% was found to be the optimum for the cell growth as well as biosurfactant production At glucose concentration of 4.0 %, the highest biomass and biosurfactant, the maximum specific growth, and lowest surface tension obtained were 6.35 g/l, 2.23 g/l, 0.065 h^{-1} and 29.0 mN/m, respectively. The bacterial growth was observed at 30 h cultivation (Table 1)

Table 1. Cultivation parameters for the biosurfactant production by *Bacillus sp.* BMN 14 using glucose as a carbon source.

Glucose (%)	Biomass (g/l)	Surface tension (mN/m)	Biosurfactant (g/l)	μ_{max} (h^{-1})
1	3.56	30.8	1.24	0.046
2	4.10	29.9	1.33	0.049
3	5.79	30.2	2.02	0.063
4	6.35	29.0	2.23	0.065
5	5.70	30.2	1.57	0.063
6	5.46	31.8	1.45	0.061

Table 2 shows the effect of fructose as carbon source on the bacterial growth and biosurfactant production. As shown in this table, the maximum growth rate (0.062 h^{-1}) and highest biosurfactant (1.48 g/L) were obtained when 3.0% fructose used as a carbon source. In this condition, the lowest surface tension of 29.4 mN/m was observed.

Table 2. Cultivation parameters for the biosurfactant production by *Bacillus sp.* BMN 14 using fructose as a carbon source

Fructose (%)	Biomass (g/l)	Surafce Tension (mN/m)	Biosurfactant (g/l)	μ_{max} (h^{-1})
1	2.26	31.0	0.79	0.046
2	3.09	30.2	1.16	0.060
3	3.23	29.4	1.48	0.062
4	3.06	30.8	0.94	0.058
5	2.85	31.1	0.77	0.059
6	2.43	32.0	0.64	0.052

To investigate the possible utilization of sucrose for biosurfactant production the carbon source of medium was replaced by sucrose varied from 1.0 to 6.0 % (Table 3). From the results of the Table 3, 4.86 g/L dry biomass, 1.58 g/L biosurfactant, the growth rate of 0.062 h^{-1} and lowest surface tension of 29.0 mN/m were observed from cultivation media containing 2.0% of sucrose. The results obtain revealed the importance contribution of glucose as carbon source for the production biosurfactants.

Table 3 . Cultivation parameters for the iosurfactant production by *Bacillus sp.* BMN 14 using sucrose as a carbon source

Sucrose (%)	Biomass (g/l)	Surface tension (mN/m)	Biosurfactant (g/l)	μ_{max} (h^{-1})
1	3.42	30.4	1.40	0.046
2	4.86	29.0	1.58	0.062
3	4.41	29.2	1.14	0.057
4	3.98	29.4	0.92	0.057
5	3.69	30.8	0.81	0.056
6	3.30	32.0	0.72	0.056

Production of Biosurfactant in a Batch Bioreactor

The cultures obtained under optimal carbon source were then tested to produce biosurfactant in different aeration rate. The process was conducted in a 2.0 L batch stirred bioreactor (working volume 1.3 L) at 37°C, agitation rate 200 rpm , and pH 6.5-7.0. An agitation rate of 200 rpm was selected since high biosurfactant yield was achieved at this rate. The aeration rate of 0.5; 1.0; and 1.5 vvm were applied

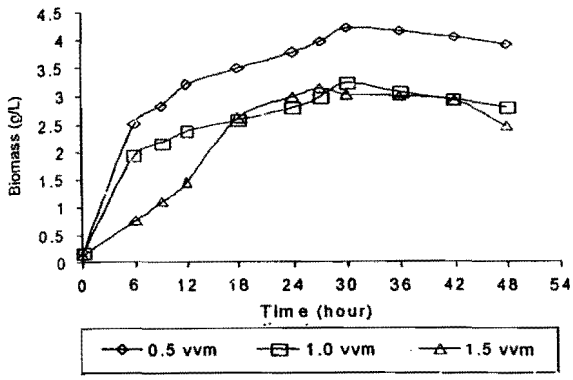

Figure 1. The pattern of biomass production by *Bacillus sp.* BMN 14

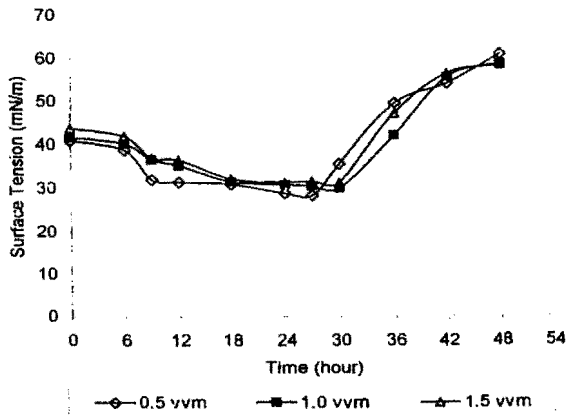

Figure 3. The pattern of surface tension of culture broth

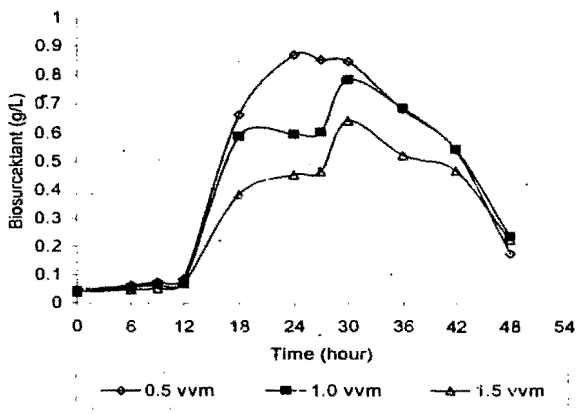

Figure 2. The pattern of biosurfactant production by *Bacillus sp.* BMN 14

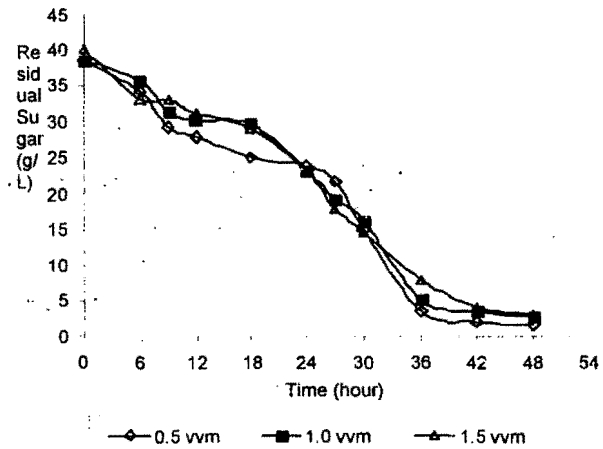

Figure 4. The pattern of residual sugar of culture broth

The pattern of biomass and biosurfactant production, surface tension and residual sugar depicted in Figure 1, 2, 3 and 4. The highest biomass, biosurfactant , the maximum specific growth, and lowest surface tension at 0.5 vvm were 4.19 g/l, 0.87 g/l, 0.07 h^{-1} and 28.3 mN/m, respectively

The best yield of biosurfactant was obtained using 0.5 vvm aeration rate compared higher aeration rate. This suggest that biosurfactant formation by the cells under different degrees of aeration was best under low aeration rate or oxygen limitation. It led to speculate that the high aeration rate promoted foam production in short time.

Table2. Cultivation parameters for the biosurfactant production by *Bacillus* sp. BMN 14 with various aeration rate.

Aeration (vvm)	Biomass (g/l)	Surface Tension. (mN/m)	Biosurfactant (g/l)	μ_{Maks} (h^{-1})	Yp/x	Yp/s	Yx/s	Product Formation (g/l.jam)	Substrate Utilization (g/l.jam)	q_p (h^{-1})	q_s (h^{-1})
0.5	4.19	28.3	0.869	0.07	0.553	0.054	0.102	0.035	0.657	0.039	0.722
1.0	3.21	29.9	0.782	0.06	0.642	0.038	0.062	0.028	0.712	0.039	1.026
1.5	3.12	31.3	0.642	0.09	0.224	0.026	0.116	0.022	0.778	0.020	0.769

Cultivation parameters for the production of biosurfactant by *Bacillus sp.* BMN 14 with various aeration rate is presented in Table 4.

The significant amount of biosurfactant began to appear in the culture broth could be observed after 12 h cultivation, and increased during the exponential growth phase up to 0.9 g/L, and then decreased after the onset of stationary growth phase (Figure 1 and 2). The result led to suggest that biosurfactant produced by Bacillus sp BMN 14 strain was synthesized during the exponentiai growth phase. It was also supported the parallel relationship between bacterial growth and surface tension of culture broth (Figure 3). From this result, we concluded that fermentative production of biosurfactant by Bacillus sp BMN 14 strain was found to be growth – associated.

The main technical problem in the production of extracellular biosurfactant under aerobic condition is the extensive formation of foam.The increase of foam production resulted in overflow of culture broth through the air exhaust line and decrease the culture broth volume (Kim et al., 1997). During our experiments, it was observed that biosurfactant were the main composition of the foam.

CONCLUSION

The study revealed that the optimal glucose concentration was 4.0 %, while above 4.0 % there is an effect of substrate inhibition which was indicated by the decrease of maximum specific growth rate. Compared to other sugar (fructose and sucrose), glucose was the best carbon source for growth and biosurfactant production by an indigenuous *Bacillus sp.* BMN 14 strain.

The best yield of biosurfactant was obtained in a batch stirred bioreactor at 37°C, pH 6.5-7.0, agitation rate of 200 rpm and aeration rate of 0.5 vvm. In this process, the maximum growth rate of 0.07 h^{-1} and biosurfactant yield of 0.87 g/L and the lowest surface tension of 28.3 mN/m were obtained.

ACKNOWLEDGEMENT

This research was supported by the Project University Research for Graduate Education (URGE). Ministry of National Education, Republic of Indonesia. Thanks are due to Mr Chong Gun Hean and Mr Mohd Noriznam Mokhtar of the Department of Food and Process Engineering Faculty of Engineering, Universiti Putra Malaysia for their assistance in the preparation of this manuscript.

REFERENCES

Babu, P.S., Deshpande,M.,, Juwarkar, A., and Khanna, P. Characterization and properties of the microbial biosurfactant produced by *Bacillus licheniformis* Strain BS 1. *J. Austral. Biotechnol.* 4:5-8 (1994)

Cooper, D.G. and Zajic. J.E., Surface active compound from microorganism,. *Adv. Appl. Microbiology* 26:229-253 (1990)

Cooper, D.G. MacDonald, C.R., Duff, S.J.B., and Kosaric,N., Enhanced production of surfactin *from Bacillus subtilis* by continuous product removal and metal cation addition. *Appl. Environ. Microbiol* 42: 408-412(1981)

Desai, J.D and Desai.A.J., *Production of Biosurfactant.* In Kosaric. N (Ed.). . Biosurfactant : Production – Properties – Application. Dekker New York pp (1993)

Fiechter, A., Biosurfactants : Moving towards industrial application *TIBTECH.* 10:208-217 (1992)

Horowittz, S. and W.M. Grifgfin. 1991. Structural analysis of *Bacillus licheniformis* 86 Surfactant. *J. Ind. Microbiol.* 7: 45-52

Jacques, Ph., Hbid, C., Vanhentenryck,F., Destain,J., Bare, G., Razafindalamo,H.,Paquaot,M., and Thonart,Ph. Quantitative and Qualitative Study of the Production of Broadspectrum Antifungal Lipopeptide *Bacillus* subtilis S499. *Proc. Europ. Cong. Biotechnol.* Berlin, Germany. Pp 1067 – 1070 (1994).

Juwarkar, A. Deshpande, M..Babu, P and P. Khanna,P., Lipopeptide Biosurfactant from *Bacillus licheniformis* strain BS1 and Its Application. *Proc.Inter Symp on Bioproduct Processing.* Kuala Lumpur, Malaysia.(1999).

Kim, H.S., Yoon, B.D, Lee,C.H , Suh, H.H., Oh, H.M., Katsuragi,T and Tani. T., Production and properties of lipopeptide biosurfactant from *Bacillus subtilis* C9. *J. of. Ferment. Bioeng.* 84(1): 41-46 (1997)

Makkar, RS and Comeotra. S.S., Production of biosurfactant at mesophilic and thermophilic conditions by a strain of *Bacillus subtilis. J. of Ind Microbiol. Biotechnol.* 20(5): 48-52(1998).

Richana, N., Makagiansar, H.Y., Romli,M., Irawadi,T.T., and Mangunwidjaja,D. The Isolation of a Lipopeptide

Biosurfactant Producing Bacillus sp. Proc.Int. Conf on Biotechnol. Jakarta Indonesia, pp 42- 49 (1997)

Sen R. and Swaminathan, T., . Application of response-surface methodology to evaluate the optimum environmental conditions for the enhanced production of surfactin. *App. Microbiol. Biotechnol.* 47 : 358-363 (1997).