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Distribution of the molecular weight of levans produced by *Zymomonas mobilis* growth in sugar cane juice media

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ABSTRACT

The distribution of molecular mass of levan from *Zymomonas mobilis* CP4 in sugar cane juice was investigated for the effect of pH, growth time and initial sugar concentration using response surface methodology. The experimental runs were carried out according to a 2^3 statistical design, with further expansion to orthogonal block runs. The distribution of molecular mass of levans from different cultures was estimated by gel permeation chromatography. Statistical analysis and interpretation as well as the evaluation of chromatographic profiles suggest that the production of high molecular mass levan (greater than 6.7×10^5) was achieved in pH 5.0 after 24 hours, with no significant effect of initial sugar concentration.

KEYWORDS

Levan;
Zymomonas mobilis;
Molar mass;
Gel permeation chromatography (GPC);
Response-surface methodology;
Sugar cane juice.

INTRODUCTION

Levan consists of β-(2,6)-linked fructose residues, and may contain β-(2,1) branches[1,2]. Molecular weights have been reported to be 2×10^6 Da in levan from *Bacillus polymyxa*[3], 12.7×10^7 Da in *Streptococcus salivarius*[4] and higher than 10^7 Da in *Zymomonas mobilis*[2]. Levan has potential applications as prebiotic agent, as thickner, emulsifier, stabilizer and encapsulating agent[5]. Levan also has several applications in medicine, including its use as an antitumor agent[1,7,8]. Evidences suggest that glucans with higher molecular weights have higher antitumor activity than the ones with lower degree of polymerisation[6]. Antitumor activity of levan from *Zymomonas mobilis* is related to a specific molecular weight range, and the maximal values for this activity were found in polymers with $4.6x10^5$[8,9].

Biopolymers have a wide range of applications, and new molecules with commercial potential are one of the targets in industrial research. However, exploring their structural features and physical properties further than current theoretical knowledge is a challenge to be overcome[10]. Several applications of exopolysaccharides are related to their pattern of branching and degree of polymerisation[11]. Reports on the factors that affect the chemical structure of levans are scarce,

although studies with other polysaccharides have shown that molecular weight may vary according to the microorganism and culture conditions. Medium pH has been reported as the most influential factor in molecular weight of exopolysaccharides from several microrganisms[6,12]. Likewise, time[13], initial sugar concentration[12], temperature[12], nitrogen source[14,15], increased ionic strength[4], and levan addition[4,16] have been shown to be relevant in exopolysaccharide chain polymerisation and can be altered by changing culture conditions.

The carbon source is a markedly important factor in EPS biosynthesis and the use of alternative substrates in biotechnological processes can be an option for decreasing costs and providing nutrients that are necessary for microbial metabolism. Amongst the substrates reported in literature, sugar cane molasses[17], beet sugar[18,19] and sugar cane juice[19] can be named. Sugar cane juice is a broadly available, low-cost agroindustrial byproduct that can be purchased throughout the year in Brazil. It has high titers of sucrose and salts needed for microbial growth, as well as adequate osmolarity for levan biosynthesis.

Response surface methodology (RSM) comprises a group of statistical techniques for empirical model building and model exploitation. By careful design and analysis of experiments, it seeks to relate a response to the levels of a number of predictors, that affect it. The RSM has proved valuable in the solution of a large variety of problems, and the more important applications are: (1) approximate mapping of a surface within a limited experimental region, (2) choice of operating conditions to achieve desired specifications, and (3) search for optimal conditions.Box and Draper[27] discuss the requirements and properties of a response surface design. The relative importance of checking fit, blocking, and obtaining an independent estimate of error will differ in different circumstances, and the minimum value of experimental runs will thus correspondingly differ. But this minimum design will in any case only be adequate if variance is below some critical value. This is the case for factorials and (central) composite response surface designs[27].

Levan has potential industrial applicatons, and the use of local available substrates for production of this EPS could decrease its production costs as well as add value to agroindustrial byproducts. In this respect, the objective of this work was to use response surface methodology to the effects of pH, growth time and sugar concentration in the molecular weight distribution of levan from *Zymomonas mobilis*, using sugar cane juice.

MATERIALS AND METHODS

Microorganism and culture conditions

Zymomonas mobilis CP4 (ATCC 31821) were maintained as 4°C in a culture medium contained [g/L]: sucrose, 20; yeast extract, 2.5; KH_2PO_4, 1.0; $(NH_4)SO_4$, 1.0; $MgSO_4(7H_2O)$, 0.5 and renewed each 4 weeks. The inoculum culture contained [g/L]: sucrose, 100; yeast extract, 2.0; KH_2PO_4, 2.0; $(NH_4)SO_4$, 1.0; $MgSO_4(7H_2O)$, 0.5. The cells concentration was standardized in 0.2 g/L

The batch fermentation medium contained yeast extract, 2.0; $MgSO_4(7H_2O)$, 2.0; initial sugar cane juice concentrantion containing 150 g/L sucrose. Reducing sugar (glucose and fructose) less than 0.5%. Commercial sucrose was added until reach the concentrations determined by the statistical design. The cultures were maintained in static conditions at 28°C. The initial pH of the growth medium was adjusted using NaOH 2N and H_2SO_4 1N. The values of pH, culture time and sugar cane juice concentration were adjusted according to the statistic design in TABLE 1.

Analytical methods

The fermentation culture was centrifuged at 4300 x g for 20 min at 4°C and re-suspended in saline 0.9% and the biomass was measured by turbidimetry in 605 nm. The reducing sugars were quantified according to Somogy[22] and Nelson[23], using glucose as standart. Total sugars were determined according to Dubois et al.[24] using glucose as standart. Levan was separated by precipitation with ethanol 95% at 4°C, centrifuged at 8700 x g for 20 min at 4°C and estimated by phenol-sulfuric acid[24] using fructose as standart.

Levan samples for molecular weight estimation by gel permeation chromatography (GPC), was treated with cold ethanol 95% (1:3) (v/v) and incubated at 4°C for 24 h. After this time, it was centrifuged and the pellet was re-dissolved in distilled water. 0.5 mL of the sample were applied to the column at 4mg/ml. The

molecular mass of levan was determined by a gel permeation chromatography (GPC) with a 40 cm x 1.6 cm column of Sepharose 6B (Sigma) using 50 mM phosphate buffer pH 7.0 as elution buffer, at the flow rate of 14 ml/h. The system was equipped with a peristaltic pump. Fractions of 3.5 mL were collected and analyzed for total sugars (TS) by phenol-sulfuric acid method[24]. *Leuconostoc mesenteroides* dextran standarts (Sigma) from 5.0 x 10^4 to 1.0 x 10^6 were used to construct a calibration curve.

Design of experiments

A central composite design (CCD) with 2 orthogonal blocks was used[27]. The first block containing 8 factorial runs and suitable central runs was used for selecting the most important factors. The second block containing the star points ($\alpha = 1.8$), added to the same number of central runs allowed the use of a second order model (TABLE 1).

The software STATISTICA 5.1 (1998) was used for obtaining the analysis of variance (ANOVA), regression coefficients and response surface plots. Evaluated factors were: X_1 (pH); X_2 (fermentation time); X_3 (initial sugar cane juice concentration). The response Y (levan with molecular weight greater than 6.7×10^5) was transformed to $Z = Y^2$, based on result of Box-Cox test for increasing the goodness of fit.

RESULTS AND DISCUSSION

Variable screening

Statistical analysis of block 1 (TABLE 1), which corresponds to a 2^3 factorial design, showed that pH and time were relevant factors in production of high molar weight levan. However, binary interaction X_1X_2 had significant effect on response Y (levan with molecular weight greater than 6.7×10^5). Curvature test indicated the necessity of a second order model for fitting the experimental data (TABLE 2).

Response modeling

Results of block 1 lead to the development of a second analysis, with convenient variation amplitude ($\alpha = \pm 1.8$) for ensuring block orthogonality (TABLE 1 - Block 2). The effect of block was not significant ($p > 0.3379$) even considering orthogonal blocking, therefore both blocks can be analysed as one design.

TABLE 1 : Factorial design 2^3 (block 1) and star design (block 2) for investigation of the effect of pH, culture time and initial sugar concentration in biomass, levan production and high molecular weight levan (HMWL) responses.

Runs	Coded Variables				Responses		
	X1	X2	X3	Block	Biomass g/L	Levan g/L	HMWL %
1	-1	-1	-1	1	0,695	1,107	43,320
2	1	-1	-1	1	1,197	2,287	40,816
3	-1	1	-1	1	0,990	2,535	45,771
4	1	1	-1	1	1,575	4,749	53,029
5	-1	-1	1	1	0,464	0,540	45,545
6	1	-1	1	1	0,886	3,977	34,639
7	-1	1	1	1	0,815	0,124	41,129
8	1	1	1	1	1,508	5,574	56,357
9	0	0	0	1	1,157	5,759	54,637
10	0	0	0	1	1,293	7,873	52,224
11	-1,8	0	0	2	0,282	0,151	23,828
12	1,8	0	0	2	1,616	1,965	46,461
13	0	-1,8	0	2	0,197	0,148	10,344
14	0	1,8	0	2	1,368	2,822	47,286
15	0	0	-1,8	2	1,272	2,810	53,869
16	0	0	1,8	2	1,251	4,767	54,426
17	0	0	0	2	1,282	5,456	52,527
18	0	0	0	2	1,375	6,164	54,623

		Original levels				
		-1,8	-1	0	1	1,8
X_1	pH	3,2	4	5	6	6,8
X_2	Culture time (h)	2,4	12	24	36	45,6
X_3	Initial sugar concentration (g/L)	110	150	200	250	290

TABLE 2 : Analysis of variance showing the effects of pH and culture time in the production of high molecular weigth levan (Z) by *Zymomonas mobilis* CP4 in first block.

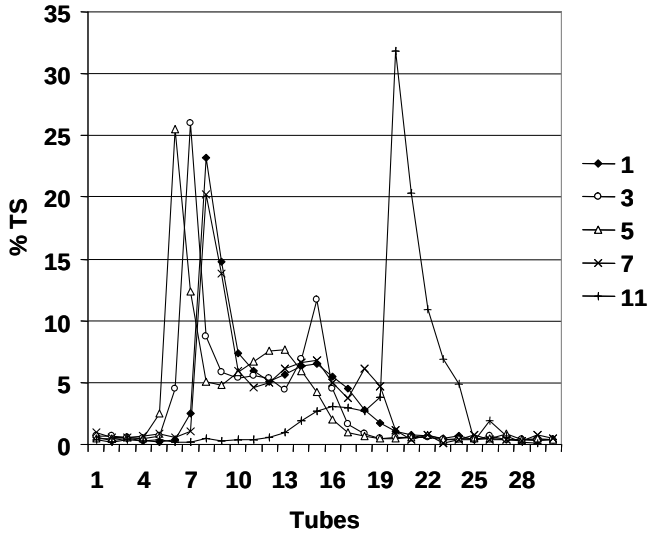
Source of variation	Sum of squares	Degrees of freedom	Mean Square	F-test	p
X_1 (L)	10,2995	1	10,2995	6,4086	0,126989
X_2 (L)	127,7302	1	127,7302	79,4766	0,01235
X_1X_2	161,0731	1	161,0731	100,2233	0,009831
Residual	41,0838	6	6,8473		
Total	340,1866	8			

Levan samples assayed by gel permeation chromatography (GPC) produced chromatograms shown in Figures 1, 2, 3 and 4. The elution profile of the samples showed revealed the existence of

predominantly two classes of average molecular weights (Mw): HMWL - high molecular weight levan (greater than 6.7×10^5) and LMWL - low molecular weight (less than 5.0×10^4).

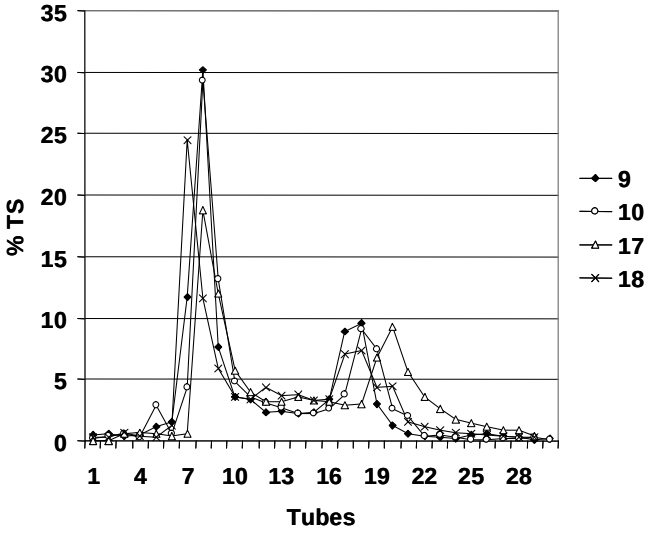
The high molecular weight class was measured as % of total levan extracted from culture. The values of sugars eluted in this first peak were added and their percentage is shown in TABLE 1. This fraction predominates over the low molecular weight class, and therefore was chosen for modelling response Y.

The elution profiles of samples in pH 4.0 (Figure 1) showed the presence of only one class of levan with high molecular weight. In pH 5.0 a high percentage of HMWL and a slight peak of LMWL can be observed (Figure 2). Elution of levans from cultures in pH 6.0 clearly showed the presence of a second peak corresponding the LMWL (Figure 3). The presence of levans with molecular weights even lower can be observed in pH 6.8 (run 12), although the standard curve used does not allow to precisely detect differences within that range (Figure 3). These results demonstrated that increasing the pH led to the formation of a low molecular weight class of levans for *Zymomonas mobilis* CP4. These results are in agreement with Shu et al.[6], who have reported that exopolysaccharides (EPS) isolated from *Antrodia camphorata* showed higher molecular weight in cultures grown in pH 4.0, whereas the low molecular weight EPS were produced in pH 6.0.



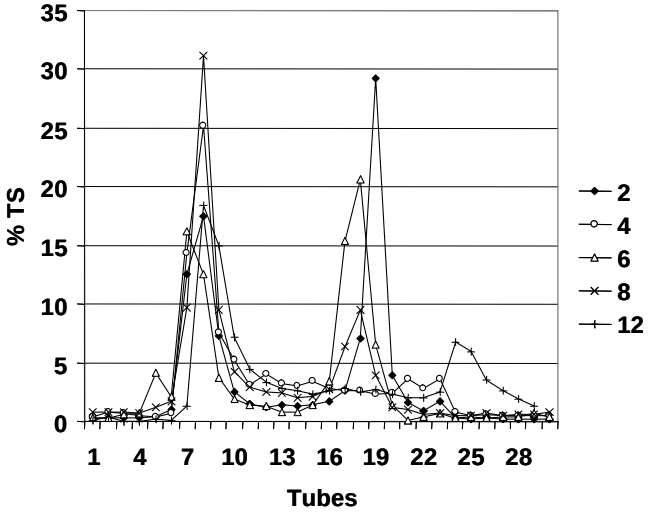
TS: % of total sugars eluted. Tubes: 3mL/15'

Figure 1 : Chromatograms of *Zymomonas mobilis* levans in sugar cane juice cultives at pH 4.0 (runs 1, 3, 5, and 7) and pH 3.2 (runs 11).



TS: % of total sugars eluted. Tubes: 3mL/15'

Figure 2 : Chromatograms of *Zymomonas mobilis* levans in sugar cane juice cultives at central points (runs 9, 10, 17 and 18) - TABLE 1. The pH was 5.0, time culture 24h and sugar cane juice concentration 200 g/L.



TS: % of total sugars eluted. Tubes: 3mL/15'

Figure 3 : Chromatograms of *Zymomonas mobilis* levans in sugar cane juice cultives at pH 6.0 (runs 2, 4, 6 and 8) and pH 6.8 (run 12).

However, Kim et al.[12] observed in cultures of *Leuconostoc mesenteroides* for production of dextrans that a higher pH range (5.5 to 6.0) favoured the formation of high molecular weight fractions; whereas a low molecular weight fraction was formed in pH lower than 4.5. Therefore, pH has a marked effect in production and molecular weights distribution of EPS, but the responses may vary according to the microorganism and pH ranged tested.

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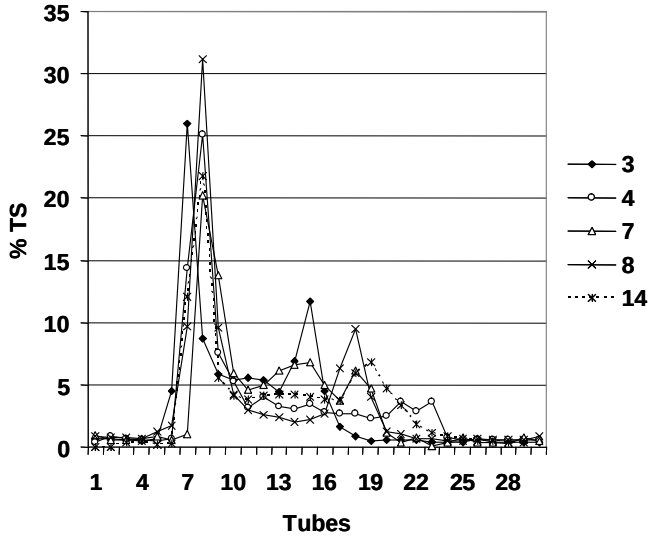
The growth time that was most favourable for production of HMWL was 24 hours, and this fraction comprised 53.47 % of the sample (TABLE 1). In 36 hours (Figure 4) this fraction has decreased to 49.03 % and in 45.6 hours (run 14) only 47.28 % of the sample were HMWL (Figure 4). These results are probably related to degradation of levan by levanase produced by *Zymomonas mobilis*[26] secreted in later growth time. According to Han[5], this hydrolytic activity could be responsible for the formation of short and heterogeneous chains instead of long and uniform polymers. Other works have demonstrated the participation of EPS-degrading enzymes in later growth time, such as in EPS from *Anthrodia camphorata*[6], *Lactobacillus rhamnosus*[13] and pullulan from *Aureobasidium pullulans*[26].



TS: % of total sugars eluted. Tubes: 3mL/15'

Figure 4 : Chromatograms of *Zymomonas mobilis* levans in sugar cane juice cultives at 36h (runs 3, 4, 7 and 8) and 45.6h (run 14) – TABLE 1.

Sucrose concentration was the least influential factor in production of high molar mass levan (TABLE 2 and TABLE 3). Within the range studied, no correlation could be observed between total sugar concentration in sugar cane juice and molar mass distribution. However, other works have shown that higher concentrations of sugars leads to the production of low molar mass EPS in *Leuconostoc mesenteroides*[12] and *Aureobasidium pullulans*[15].

Transformation and analysis of responses allowed to create the model represented by Equation 1 with Adjusted $R^2 = 0.891$ and not significant lack-of-fit ($p > 0.098$). The transformed response Z was obtained from original response Y ($Z = Y^2$).

$$Z = 2935.284 + 274.876\ X_1 - 448.818\ X_1^2 + 468.932\ X_2 - 507.99 X_2^2 + 410.859\ X_1X_2 \quad (1)$$

TABLE 3 : Analysis of variance showing the effects of pH and culture time in the production of high molecular weight levan (Z) by *Zymomonas mobilis* CP4.

Source of variation	Sum of squares	Degrees of freedom	Mean Square	F-test	p
X_1 (L)	1094060	1	1094060	13,28208	0,00336
X_1(Q)	3339740	1	3339740	40,545	0,000036
X_2 (L)	3184118	1	3184118	38,65574	0,000045
X_2 (Q)	4278567	1	4278567	51,94252	0,000011
X_1X_2	1350443	1	1350443	16,3946	0,001613
Residual	988454	12	82371		
Total	12924319	17			

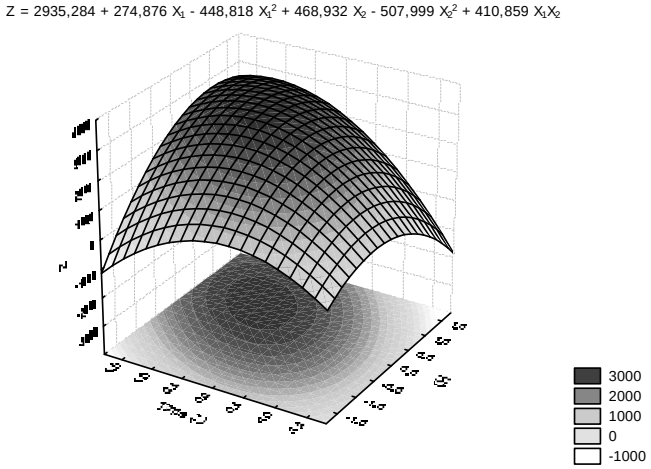


Figure 5 : Response-surface to high molecular weight levan (HMWL) production by *Zymomonas mobilis*. Concentration was fixed in 200 g/L.

The ANOVA table (TABLE 3) shows the significance of effects. Initial sugar concentration was not significant ($p > 0.5963$) and hence not included in the equation (1) for formation of peak 1. The fitted surface obtained from the model is shown in Figure 5.

Analysis of data in TABLE 1 and fitted surface revealed that the values observed in the central runs are close to the optimum. Data obtained in two experimental runs previously carried out in the same conditions of the central point runs (pH 5.0; culture time 24 h and initial sugar cane juice concentration 200 g/L) were used for experimental validation of the model. The value predicted for response Y = vZ was 54.18 % and the

confidence interval (95 %) is between 52.59 % and 55.74 %. Validation experiments yielded the values 54.10 %, within the confidence interval and 51.62 %, nearing the lower limit of the confidence interval. The average value, 52.878 % (Z = 2796.127) of levans with molecular weights greater than $6.7.10^5$ Da, is within the confidence interval, which indicates a good prediction capability for the model.

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REFERENCES

[1] S.H.Yoo, E.J.Yoon, J.Cha, H.G.Lee; Biological Macromolecules, **34**, 37-41 **(2004)**.
[2] H.W.Doelle, L.Kirk, R.Crittenden, T.Hsien; Critical Reviews in Biotechnol, **13**, 57-98 **(1993)**.
[3] Y.W.Han, M.A.Clarke; J.Agric.Food Chem., **38**, 393-396 **(1990)**.
[4] T.Tanaka, S.Oi, T.Yamamoto; J.Biochem., **87**, 297-303 **(1980)**.
[5] Y.W.Han; Advances in Applied Microbiol., **35**, 171-194 **(1990)**.
[6] C.H.Shu, M.Y.Lung; Proc.Biochem., **39**, 931-937 **(2004)**.
[7] M.Bekers, D.Upite, E.Kaminska, J.Laukevics, M.Gurbe, A.Vigants, R.Linde; Proc.Biochem., **40**, 1535-1539 **(2005)**.
[8] G.M.T.Calazans, C.E.Lopes, R.M.O.C.Lima, F.P.França; Biotechnol.Letters, **19**, 19-21 **(1997)**.
[9] G.M.T.Calazans, R.C.Lima, F.P.França, C.E.Lopes; Biological Macromolecules, **27**, 245-247 **(2000)**.
[10] I.W.Sutherland; Microbiol.Today, **29**, 70-71 **(2002)**.
[11] I.W.Sutherland; Int.Dairy J., **11**, 663-674 **(2001)**.
[12] D.Kim, J.F.Robyt, S.Y.Lee, J.H.Lee, Y.M.Kim; Carbohydr.Res., **338**, 1183-1189 **(2003)**.
[13] P.L.Pham, I.Dupont, D.Roy, G.Lapoint, J.Cerning; Appl.Environ.Microbiol., **66**, 2302-2310 **(2000)**.
[14] B.Degeest, L.Vuyst; Appl.Environ.Microbiol., **65**, 2863-2870 **(1999)**.
[15] H.P.Seo, C.H.Son, C.H.Chung, D.I.Jung, S.Y.Kim, R.A.Gross, D.L.Kaplan, J.W.Lee; Bioresource Technol., **95**, 293-299 **(2004)**.
[16] R.Chambert, M.F.P.Glatron; Carbohydr.Res., **191**, 117-123 **(1989)**.
[17] M.L.Cazzeta, M.A.P.C.Celligoi, J.B.Buzato, I.S.Scarmino, R.S.F.Silva; Proc.Biochem., **40**, 747-751 **(2005)**.
[18] Y.W.Han, M.A.Watson; J.Ind.Microbiol., **9**, 257-260 **(1992)**.
[19] M.Bekers, R.Linde, D.Upite, E.Kaminska, U.Viesturs; Acta Biotechnol, **21**, 89-96 **(2001)**.
[20] O.Sreekumar, N.Chand, S.C.Basappa; J.Biosci. Bioeng., **88**, 334-338 **(1999)**.
[21] D.R.Hamsaveni, S.G.Prapulla, S.Divakar; Proc. Biochem., **36**, 1103-1109 **(2001)**.
[22] M.Somogy; Journal Biology Chemistry, **160**, 61-68 **(1952)**.
[23] N.Nelson; Biochem., **84**, 375-380 **(1944)**.
[24] M.Dubois, K.A.Gilles, P.A.Roberts, F.Smith; Anal. Chem., **9**, 77-103 **(1956)**.
[25] V.K.Ananthalakshmy, P.Gunasekaran; Brazilian Archives of Biol.Technol., **42**, 291-297 **(1999)**.
[26] J.H.Lee, J.H.Kim, I.H.Zhu, X.B.Zhan, J.W.Lee, D.H.Shin, S.K.Kim; Biotechnol.Letters, **23**, 817-820 **(2001)**.
[27] G.E.D.Box, N.R.Draper; 'Empirical Model Building and Response Surface'. John-Wiley & Sons, New York, **(1987)**.