http://www.zju.edu.cn/jzus; E-mail: jzus@zju.edu.cn
ISSN 1009-3095 *Journal of Zhejiang University SCIENCE* V.4, No.6, P.719-726, Nov.-Dec., 2003

Optimization of cultural conditions for thermostable β-1,3-1,4-glucanase production by *Bacillus subtilis* ZJF-1A5*

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Received Sept.2, 2002; revision accepted Nov.20, 2002

Abstract: The optimization of cultural conditions for β-glucanase production by *Bacillus subtilis* ZJF-1A5 was investigated in flask trials. Temperature had great effect on β-glucanase production which maximized at optimal temperature of 37℃ and decreased significantly when temperature was over 37℃. Charge quantity affected β-glucanase production significantly. Adding oxygen vector N-dodecane or acetic ether benefited β-glucanase production, but it depended on the concentration and charge quantity. The results of fractional factorial design showed that age and size of inoculum and shaking speed were the key factors affecting β-glucanase production and the cultivation time span to reach the highest β-glucanase activity. The optimal cultural conditions for β-glucanase production obtained with CCD were as follows: inoculum age and size (16 h, 3.82%(v/v)), shaking speed 210 r/min, charge quantity of 30 mL in 250 mL flask and initial pH 7.0, cultured at 37℃ for 50 h. Repeated experimental results accorded with those predicted by a second-order polynomial model. The amount of β-glucanase, α-amylase and neutral protease produced by *B subtilis* ZJF-1A5 was associated partially with cell growth. Those three enzymes' activities increased following the cell growth and increased significantly when cells entered the stationary phase.

Key words: β-glucanase, *Bacillus subtilis*, Optimization, Response surface methodology, Cultivation conditions
Document code: A **CLC number:** Q936

INTRODUCTION

β-1,3-1,4-glucan is the major cell wall component of barley and other cereal endosperm, and consists of polymers of glucose monomers joined by β-1,3- and β-1,4 glycosidic bonds in a non-regular fashion (Woodward *et al.*, 1983; Edney *et al.*, 1991). High-molecular-mass β-glucan is soluble owing to its unique molecular structure and can cause severe filtration problems that may lead to gelatinous precipitates. Barley β-glucan can decrease digestibility of feedstuff for broiler chicks and lead to "sticky dropping" problems. These biotechnological applications make the enzyme a focus of interest. Adding heterogeneous β-glucanase one of efficient ways to reduce or eliminate the negative effects caused by cereal β-glucan. Endo-β-glucanase produced by *Bacillus* has similar substrate specificity to endogenous malt, more stable and very useful in industry, but lower production of *Bacillus* β-glucanase limits its industrial application.

Many factors such as temperature, aeration and inoculation course affect the growth of microorganisms and their production. It is difficult to determine the key factors and to optimize the cultural conditions with traditional methods (single-dimensional) since it is laborious, time-consuming and incapable of reaching the true optimal point due to the interactions between variables. Response Surface Methodology (RSM) is a better experimental strategy for seeking optimal conditions for multi-variable system, have been successfully employed for optimizing the medium composition and operating conditions in many bioprocesses (Lee and Chen, 1997; Bazaraa and Hassan, 1996; Lee *et al.*, 1999; El-Helow and El-Ahawany, 1999; Watier *et al.*, 1996). The

* Project(No.B0608) supported by the National Natural Science Foundation of China and a grant (2001121B25) from Hangzhou Science and Technology Development Project of Zhejiang Province, China

aim of this work is to determine optimal cultural conditions using response surface methodology for improving β-glucanase production by *Bacillus subtilis* ZJF-1A5.

MATERIAL AND METHOD

1. Microorganism and media

The bacterium *Bacillus subtilis* ZJF-1A5 was isolated from soil and mutated by ultraviolet radiation and diethyl sulfate. The culture was maintained at 4℃ and sub-cultured every four weeks.

Three different media were employed in this work and had the following compositions (g/L): (peptone, 10; beef extract, 3; NaCl, 5; agar, 20; initial pH 7.0 – 7.2 [Basal medium for slant]. Dextrin, 20; yeast extract, 20; KH_2PO_4, 1; $MgSO_4$(7H_2O, 0.2; $CaCl_2$, 0.1; initial pH 7.0; [Inoculation media]. Barley flour, 63.5; corn flour, 44.8; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2$, 0.1; initial pH 7.0; [Fermentation medium]). In all cases, media were autoclaved for 20 min at 121℃.

Barley β-glucan (Sigma company) was used as substrate for measuring β-glucanase activity. Other chemicals were analytical reagent grade and barley and corn flour were procured from local suppliers and passed through 80-mesh sieve.

2. Cultural condition

Aliquots (30 mL) of each liquid medium were put into 250 mL Erlenmeyer flasks, inoculated and incubated at 37℃ under shaking conditions. RSM was used to assess the effects of cultural conditions on β-glucanase production.

3. Analyses

β-Glucanase assay For the determination of β-glucanase activity the culture broth was centrifuged at 3000g for 10 min and the supernatant was used as the enzyme source. Culture supernatant (100 μL) diluted with pH 6.0 phosphate buffer was incubated at 50℃ for 10 min with 0.9 mL of 0.2 % barley β-glucan in the same buffer. The reducing sugar was estimated using DNS method (Miller, 1959). One unit of β-glucanase (U) was defined as the amount of enzyme required for forming 1μmol/min reducing sugar (glucose as standard).

α-Amylase assay 2% phosphate buffered starch solution was used as the substrate for the assay of α-amylase activity. One unit of α-amylase activity was defined as the amount of enzyme required to hydrolyze 1 mg starch in 30 min at 40℃ and pH 6.0.

Neutral protease assay 0.6% Tris-HCl (pH 7.5) buffered casein solution was used as the substrate for assaying neutral protease activity. One unit of protease activity was defined as the absorbance of the soluble mass in CH_3COOCl_3 equivalent to that of 1 μg tyrosine per min at 40℃. Residual reducing sugar content in the broth was determined by the DNS method.

Total sugar content in the broth was determined by the method of hydrolysis using 6 mol/L hydrochloric acid while glucose was determined by the DNS method.

4. Experimental design

To find the optimal cultural conditions the key factors affecting β-glucanase productivity had to be determined. Fractional Factorial Design (FFD) was used to pick the most important factors from a long list of candidate factors. The influences of the five variables on β-glucanase productivity were investigated using the methodology of HFFD (Table 1) in the form of both coded and natural values. Once the relevant variables were selected by screening, experiments were planned to obtain a quadratic model consisting of trials plus a star configuration with a central point with β-glucanase productivity as response. SAS package (SAS Institute, Cary, NC, USA) was used to analyze the results. For the four factors, the model obtained can be expressed as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2$$

Where Y is the response, b_0 is the intercept term, b_i are linear coefficients, b_{ij} ($i \neq j$) is the interactive coefficient, b_{ii} are quadratic coefficients, and X_i are coded independent variables.

RESULT AND DISCUSSION

1. The effect of temperature on the β-glucanase production

Temperature was found to be one of the key

factors that influence the growth of microorganism and formation of enzyme products. The effect of temperature on β-glucanase productivity is shown in Fig. 1. The optimal temperature was 37℃ (272.9 U/mL, 48h), followed by 35℃ (261.04 U/mL, 48h). The β-glucanase activity decreased greatly at 39℃ reached (167.64 U/mL, 48h). For α-amylase and protease in broth at 39℃ activities were lower than at 35℃ and 37℃ (data not shown). It indicated that temperature had significant effects on β-glucanase productivity. Comparison of the plots of β-glucanase activity at 35℃ and 37℃ showed that at early stage after 18h there was a little difference in β-glucanase productivity. β-glucanase productivity at 35℃ was lower than that 37℃. Thus, the optimal temperature for *B. subtilis* ZJF-1A5 β-glucanase production was 37℃.

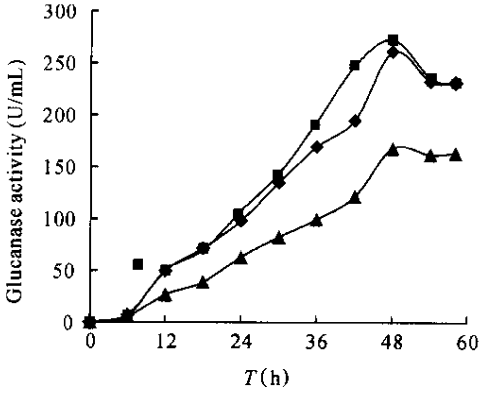

Fig. 1 Effects of temperature on β-glucanase production

Symbols(℃): ◆35.0±0.5; ■37.0±0.5; ▲39.0±0.5

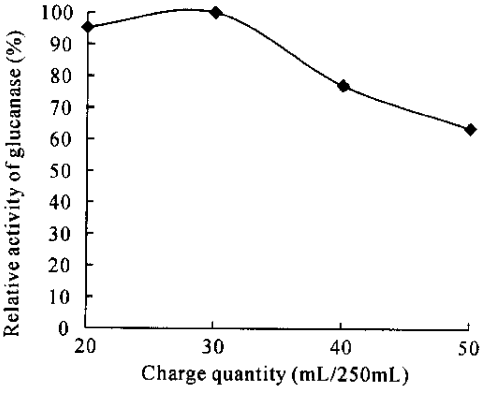

Fig. 2 The effect of charge quantity on β-glucanase production

2. The effect of the charge quantity on the β-glucanase production

Most species of *Bacillus* are obligate aerobic bacteria and the result of this work indicated that the charge quantity influence the content of the dissolved oxygen in the broth and affected enzyme formation (Fig. 2). β-Glucanase activity in the broth maximized when 30 mL liquid medium was dispersed in 250 mL flask and the β-glucanase activity decreased rapidly as the charge quantity increased. Thus, the charge quantity of 30 mL in 250 mL flask was chosen for downstream experiments.

3. The effect of oxygen vectors on the β-glucanase production

In general, increasing the aeration or/and stir rate and designing rational reactors could eliminate or reduce the oxygen limitation and as shown above, the charge quantity had effect on enzyme production. Adding oxygen vectors was another way for improving the oxygen supply. Oxygen vectors increased the level of dissolved oxygen through decreasing the oxygen transfer coefficient of the broth. The results (Fig. 3) showed that β-glucanase production depended on the type and concentration of the oxygen vectors. Oleic acid had negative effect on the β-glucanase production when added into broth. N-dodecane and acetic ether had different effects on the β-glucanase productivity depending on the concentration. Lower concentration of N-dodecane and acetic ether benefited β-glucanase production. β-glucanase activity decreased when the concentration was over 1% and 0.5% for *N*-dodecane and acetic ether respectively. Adding appropriate oxygen vector can improve the level of dissolved oxygen in the broth and promotes production, but excessive dissolved oxygen had negative effect on β-glucanase productivity.

The effect of *N*-dodecane concentration on β-glucanase production in different charge quantity was investigated (Fig. 4). The β-glucanase production decreased as the charge quantity increased. Adding *N*-dodecane into the broth could increase the β-glucanase production to some extent. Gain in yield depended on the N-dodecane concentration and charge quantity.

Thus, the type and amount of oxygen vectors to improve dissolved oxygen levels must be determined prior to addition into the broth. Economically, adding oxygen vectors increases the cost of the fermentation and influences the down-stream process of enzyme purification. Considering this, we adjusted the shaking speed to improve the oxygen supply in the next experiments.

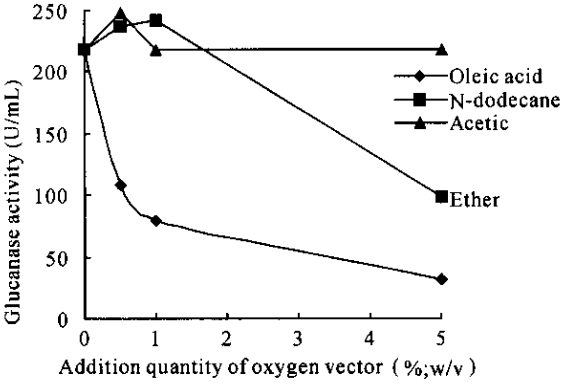

Fig.3 Effects of oxygen vector on the β-glucanase activity

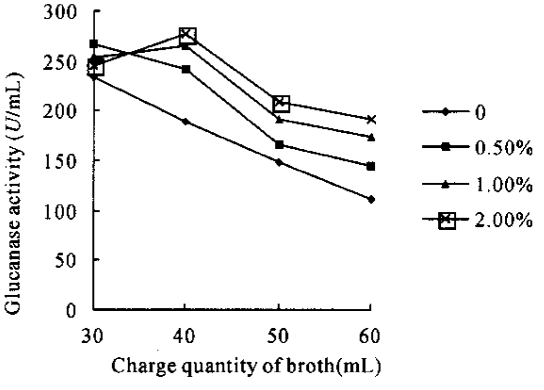

Fig.4 Effect of *N*-dodecane concentration on the β-glucanase activity

4. The optimization of cultural conditions for β-glucanase production

(1) Half Fractional Factorial Design (HFFD)

Usually the quality of inoculation (age and dosage), shaking speed and pH had different influences on the product formation and cultivation time. Different charge quantity could cause big error because of evaporation. Thus, the charge quantity was fixed (30 ml) and the shaking speed was changed to study the effect of aeration on β-glucanase production by *B. subtilis* ZJF-1A5. HFFD was chosen to study the effects of the five factors and their interactions on β-glucanase production. The design was sufficient to assess all the main effects and two factors interactions. Coded values of factors and results of experiment are shown in Tables 1 and 2, respectively.

Table 1 Definitions and levels of independent variables in HFFD

Independent variables	Symbol	Coded levels		
		−1	0	+1
Inoculum age (h)	x_1	12	16	20
Inoculum size (%, v/v)	x_2	2	3	4
Shaking speed (r/min)	x_3	160	200	240
Cultivation time (h)	x_4	44	48	52
Initial pH	x_5	6.5	7.0	7.5

The factorial analysis of variance indicated that the inoculum age, size (X_1, X_2) and shaking speed (X_3) were significant factors affecting β-glucanase production (Table 3). The interactions between inoculum age and cultivation time, inoculum size and cultivation time, shaking speed and cultivation time on β-glucanase production were significant. These results indicated that inocula quantity and shaking speed influenced the cultivation time needed to reach the highest production level of β-glucanase. The negative coefficients of interactions between inoculum size and cultivation time, shaking speed and cultivation time, means that increasing the inoculum size and shaking speed could reduce the fermentation time.

The inocula at primary stage of log phase (12 h), and at late stage of log phase or early

Table 2 Experimental design and results of HFFD

Trial	Coded levels of independent variables					Enzyme production (U/mL)
	X_1	X_2	X_3	X_4	X_5	
1	−1	−1	+1	−1	−1	170.65
2	+1	−1	+1	−1	+1	165.56
3	+1	−1	+1	+1	−1	213.54
4	+1	+1	−1	+1	−1	279.50
5	−1	+1	−1	+1	+1	185.36
6	+1	−1	−1	+1	+1	269.37
7	−1	+1	+1	+1	−1	135.03
8	−1	+1	+1	−1	+1	234.89
9	−1	+1	−1	−1	−1	206.61
10	+1	+1	+1	+1	+1	241.21
11	−1	−1	+1	+1	+1	156.46
12	−1	−1	−1	−1	+1	176.28
13	+1	−1	−1	−1	−1	150.20
14	+1	+1	−1	−1	+1	173.03
15	−1	−1	−1	+1	−1	197.87
16	+1	+1	+1	−1	−1	170.31
17	0	0	0	0	0	266.61
18	0	0	0	0	0	274.75
19	0	0	0	0	0	264.50
20	0	0	0	0	0	285.45

Table 3 Regression results of HFFD

Parameter	Estimate	$Pr > \|T\|$	Std error of estimate	Parameter	Estimate	$Pr > \|T\|$	Std error of estimate
Intercept	195.37	0.0001	3.405	X_5	4.90	0.1931	3.405
X_1	12.47	0.0080	3.405	$X_1 \times X_4$	28.64	0.0001	3.405
X_2	7.88	0.054	3.405	$X_2 \times X_4$	−7.39	0.0665	3.405
X_3	−9.41	0.028	3.405	$X_3 \times X_4$	−13.82	0.0048	3.405
X_4	14.43	0.0039	3.405				

$F = 17.29$; $P = 0.0006$; $R^2 = 0.952$

stage of stationary phase (20 h), activities of α-amylase, β-glucanase, neutral protease and biomass of inucula at 12 h were lower than those at 20 h (data not shown). The inocula quantity resulted in the difference of β-glucanase productivity. Shaking speed affected β-glucanase production and cultivation time, indicating that dissolved oxygen in the broth affected enzyme production. The initial pH of the culture medium ranged from 6.0 to 7.0 and had insignificant effect on β-glucanase production, so it was fixed at 7.0 in this study.

A regression equation could be obtained from the regression results of HFFD as follows:

$$Y = 195.37 + 12.47X_1 + 7.88X_2 - 9.41X_3 14.43X_4 28.64X_1X_4 - 7.39X_2X_4 - 13.82X_3X_4 \quad (1)$$

The coefficient and determination coefficient (R^2) for the regression model of β-glucanase production are presented in Table 3. The regression model for β-glucanase production was highly significant ($P < 0.01$) and had a satisfactory determination coefficient ($R^2 = 0.952$).

The center point data allowed assessing the gross curvature in the enzyme production response. A t-test comparing the mean of the center points (272.83 U/mL) to the mean of the fractional factorial point (195.37 U/mL) yielded

a P value of 0.03, providing strong evidence of gross curvature. Since the mean of the center points exceeds the mean of the factorial points, it indicated that the optimum is near or within the design space. Thus values of center point of X_1, X_2, X_3 and X_4 were chosen to optimize the cultural conditions for β-glucanase production with CCD.

(2) Central composite design (CCD)

The CCD strategy was an efficient way to find the optimal cultural conditions for bioprocess. A Box-Wilson central composite design with

Table 4 Definitions and levels of independent variables in CCD

Independent variables	Symbol	Coded levels				
		−2	−1	0	+1	+2
Inoculum age (h)	X_1	8	12	16	20	24
Inoculum size (%, v/v)	X_2	1	2	3	4	5
Shaking speed (r/min)	X_3	120	160	200	240	280
Cultivation time (h)	X_4	40	44	48	52	56

Table 5 Experimental design and results of CCD

Trials	X_1	X_2	X_3	X_4	Y(U/mL)
1	+1	−1	+1	+1	202.84
2	−1	−1	−1	+1	205.11
3	−1	−1	+1	+1	208.96
4	−1	+1	+1	−1	221.34
5	+1	+1	−1	+1	259.01
6	+1	−1	−1	+1	217.40
7	−1	−1	−1	−1	168.09
8	−1	−1	+1	−1	197.46
9	+1	−1	+1	−1	214.40
10	+1	+1	−1	−1	170.89
11	0	0	−2	0	149.32
12	+1	−1	−1	−1	190.63
13	0	0	0	2	248.65
14	−1	+1	−1	+1	219.84
15	0	0	0	0	256.49
16	0	0	0	0	264.57
17	−2	0	0	0	178.49
18	−1	+1	+1	+1	257.15
19	0	0	0	0	275.83
20	0	0	0	0	269.97
21	−1	+1	−1	−1	146.57
22	0	+2	0	0	250.95
23	+2	0	0	0	233.08
24	+1	+1	+1	+1	209.67
25	0	0	+2	0	243.75
26	0	0	0	0	265.65
27	0	0	0	0	264.15
28	0	0	0	0	259.71
29	+1	+1	+1	−1	212.12
30	0	0	0	−2	174.67
31	0	−2	0	0	227.47

five settings for each of four factors was run to optimize the process for β-glucanase production. The coded values of factors, experimental design and results are shown in Tables 4 and 5, respectively.

This regression model for β-glucanase production was highly significant ($P < 0.01$) and had a satisfactory value of determination coefficient ($R^2 = 0.917$). This means that 91.7% of the variability in β-glucanase production in the flask trials can be accounted for by the second-order polynomial prediction equation (2) given below.

$$Y = 265.20 + 6.74X_1 + 5.78X_2 + 13.97X_3 + 16.93X_1^2 - 2.42X_1X_2 - 7.65X_2^2 - 9.02X_1X_3 + 3.84X_2X_3 - 18.32X_3^2 - 3.55X_1X_4 + 8.18X_2X_4 - 11.99X_3X_4 - 14.54X_4^2 \quad (2)$$

Canonical analysis was a mathematical approach employed to examine the over-all shape of the curve, to locate the stationary point of the response surface and to decide whether it described a maximum, minimum or saddle point. According to the results obtained by canonical analysis the response surface for cultural conditions showed a maximum point. The optimal culture conditions for β-glucanase production by B subtilis ZJF-1A5 were calculated to be: inoculum age and size 16 h and 3.82 %(v/v); shaking speed 210 rev/min; cultivation time 50 h and initial pH 7.0. The maximum response predicted from the model was 275.25 U/mL. Repeated experiments were performed for the production of β-glucanase cultivated under the optimal cultural conditions. The results from three replications (i.e.277.33 U/mL, 269.78 U/mL and 276.33 U/mL) accorded with the estimated values so the model was proven to be adequate.

5. The time course of β-glucanase production

A primary enzyme associated with *B. subtilis* ZJF-1A5 is β-glucanase and other useful hydrolysis enzymes (α-amylase and protease) were included in the broth. *Bacillus subtilis* amylase is particularly useful due to its activity on a broad range of starches. Protease is also useful in brewing industry for hydrolyzing protein to amino acid. The time course of enzymes production under the optimized cultural conditions was investigated and the results are shown in Fig. 5. After the short lag phase, *B. subtilis* ZJF-1A5 entered the log phase and total sugar content in the broth decreased rapidly and residual reducing sugar content increased. β-glucanase, α-amylase and neutral protease started to form and accumulated in this period. After 30 h, cells growth entered the stationary phase and the consuming rate of sugar decreased and the β-glucanase, α-amylase and neutral protease activities increased rapidly. The β-glucanase and neutral protease production maximized at 48 h and α-amylase production at 54 h. The three enzymes produced by *B subtilis* ZJF-1A5 were partially associated with cell growth.

The gene encoding β-1,3-1,4-glucanase was expressed when cell growth entered the stationary stage while the contents of GTP in cells

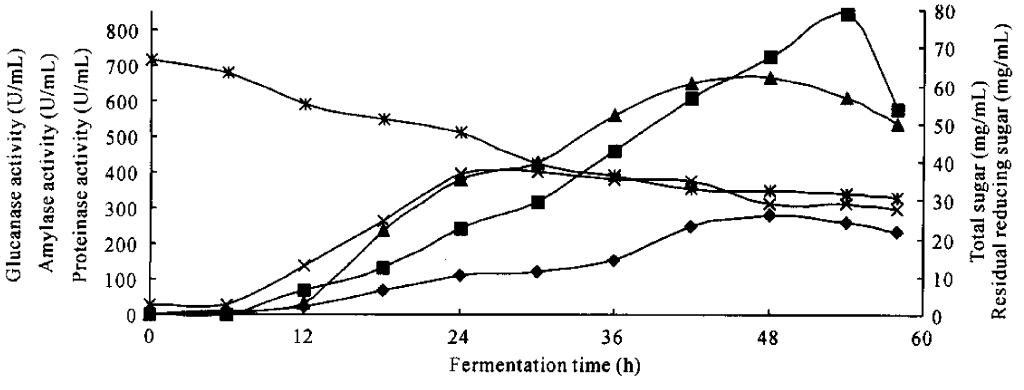

Fig.5 Effect of the time course on the enzyme production

♦ β-glucanase; ■ α-amylase; ▲ neutral protease; ✱ total sugar; × residual reducing sugar

decreased (Stulke *et al*., 1993). The production of β-glucanase by *Bacillus* was also associated with the biomass and was repressed by carbon catabolism such as that of glucose (Kruger *et al*., 1993; Tobisch *et al*., 1997). The key to obtain high β-glucanase production was to get high amount of biomass and avoid accumulation of glucose in the broth through controlling the culture conditions or adopting appropriate feed strategy.

References

Bazaraa, W.A. and Hassan, E.E., 1996. Response surface optimization for the continuous glucose isomerization process. *J Ind Microbiol*, **17**:100 – 103.

Edney, M.J., Marchylo, B.A. and Macgregor, A.W., 1991. Structure of total barley-glucan. *J. Inst Brew*, **97**:39 – 44.

El-Helow, E.R. and El-Ahawany, A.M., 1999. Lichenase production by catabolite repression-resistant *Bacillus subtilis* mutants: optimization and formulation of an agro-industrial by-product medium. *Enzyme Microb Technol*, **24**:325 – 331.

Kruger, S., Stulke, J. and Hecker, M., 1993. Catabolite repression of β-glucanase synthesis in *Bacillus subtilis*. *J Gen Microbiol*, **139**:2047 – 2054.

Lee, S. and Chen, W., 1997. Optimization of medium composition for the production of glucosyltransferase by *Aspergillus niger* with response surface methodology. *Enzyme Microbial Technol*, **21**:436 – 440.

Lee, M., Chen, W. and Chou, C., 1999. Optimization and kinetic analysis of cholesterol oxidase production by *Rhodococcus equi* no.23 in submerged cultures. *Enzyme and Microb Technol*, **25**:598 – 604.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*, **31**:426 – 427.

Stulke, J., Hanschke, R. and Hecker, M., 1993. Temporal activation of β-glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool. *J Gen Microbiol*, **139**:2041 – 2045.

Tobisch, S., Glaser, P., Kruger, S., Kruger, S. and Hecker, M., 1997. Identification and characterization of a new β-glucoside utilization system in *Bacillus subtilis*. *J Bacteriol*, **179**:496 – 506.

Watier, D., Dubourguier, R.A., Leguerinel, I. and Hornez, J.P., 1996. Response surface models to describe the effects of temperature, pH, and ethanol concentration on growth kinetics and fermentation end products of a *Pectinatus* sp. *Appl. Environ. Microbiol*, **62**:1233 – 1237.

Woodward, J.R., Phillips, D.R. and Fincher, G.B., 1983. Water-soluble (1→3), (1→4)-β-D- glucans from barley (*Hordeum vulgare*) endosperm. I. Physicochemical properties. *Carbohydrate Polymers*, **3**:143 – 156.