Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Economical glucoamylase production by alginate-immobilized *Thermomucor indicae-seudaticae* in cane molasses medium

P. Kumar and T. Satyanarayana

Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi, India

Keywords
Ca^{2+}-alginate, cane molasses, glucoamylase, response surface methodology, *Thermomucor indicae-seudaticae*.

Correspondence
T. Satyanarayana, Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi-110 021, India.
E-mail: tsnarayana@gmail.com

2007/0210: received 12 February 2007, revised 17 May 2007 and accepted 21 May 2007

doi:10.1111/j.1472-765X.2007.02201.x

Abstract

Aims: The present investigation is aimed at assessing the suitability of cane molasses as a cheaper carbon and energy source for glucoamylase production using alginate-immobilized *Thermomucor indicae-seudaticae*.

Methods and Results: The culture variables for glucoamylase production were optimized by 'one-variable-at-a-time' strategy and response surface methodology (RSM). A high glucoamylase titre was attained when 40 alginate beads (*c.* 5×10^6 immobilized spores) were used to inoculate 50 ml of cane molasses (8%) medium in 250-ml Erlenmeyer flasks. Response surface optimization of fermentation parameters (cane molasses 7%, inoculum level 44 alginate beads per 50 ml of medium and ammonium nitrate 0·25%) resulted in 1·8-fold higher glucoamylase production (27 U ml^{-1}) than that in the unoptimized medium (15 U ml^{-1}). Enzyme production was also sustainable in 22 l of laboratory air-lift bioreactor.

Conclusions: Cane molasses served as an excellent carbon and energy source for the economical production of glucoamylase, which was almost comparable with that in sucrose yeast-extract broth. The statistical model developed using RSM allowed determination of optimum levels of the variables for improving glucoamylase production.

Significance and Impact of the Study: The cost of glucoamylase produced in cane molasses supplemented with ammonium nitrate was considerably lower (€1.43 per million U) than in synthetic medium containing sucrose and yeast-extract (€35.66 per million U). The reduction in fermentation time in air-lift bioreactor with sustainable glucoamylase titres suggested the feasibility of scale up of the process.

Introduction

Glucoamylase is one of the most important enzymes in starch industry involved primarily in the production of glucose (Ford 1999; Reilly 1999). Starting from the non-reducing end of the starch molecule, it hydrolyses all α-1,4 glycosidic bonds consecutively while cleaving α-1,6 bonds at a slow rate to produce β-D-glucose (Meagher and Reilly 1989). Glucose being an essential substrate for numerous fermentation processes, glucoamylase extends its applications to a number of other food and beverage industries (Crabb and Shetty 1999; Polakovic and Bryjak 2004). Although glucoamylases have been reported from a wide variety of micro-organisms, the huge industrial demand is fulfilled by the filamentous fungi.

Glucoamylase for industrial applications is produced from *Aspergillus* and *Rhizopus* spp. in submerged fermentation at 30–35°C, using corn and corn-steep liquor in the production medium (Nigam and Singh 1995). A number of different glucoamylase production media have been formulated (Haasum *et al.* 1991; Pedersen

et al. 2000). The use of industrial byproducts in fermentation medium not only provides a cheaper alternative but also reduces the handling problems (Jin *et al.* 1998). The optimization of cultivation variables can further improve the efficiency of fermentation. The classical 'one-variable-at-a-time' approach is an effective technique, when only a few variables are to be optimized (Pham *et al.* 1998). It, however, fails to depict the combined effect of the variables. Response surface methodology (RSM), on the other hand, has become an important statistical tool for process optimization and/or media designing, as it is not only rapid but also allows to understand the interactions among variables and their effects on product yield (Kumar and Satyanarayana 2007b).

Glucoamylase of thermophilic fungus *Thermomucor indicae-seudaticae*, being thermostable and optimally active at neutral pH, has been found to be useful in developing an ideal starch saccharification process (Satyanarayana *et al.* 2004). Alginate-immobilized sporangiospores of *T. indicae-seudaticae* have recently been shown to produce higher glucoamylase than freely growing mycelia, and the bead inoculum was successfully reused for eight batches of repeated fermentation with sustainable enzyme production (Kumar and Satyanarayana 2007a). Optimization of cultivation conditions further improved enzyme secretion by the immobilized cells (Kumar and Satyanarayana 2007b). The present investigation was, therefore, carried out for optimizing glucoamylase production in a cheaper medium using alginate-immobilized sporangiospores of *T. indicae-seudaticae*. Cane molasses, being a rich source of carbohydrates and other nutrients (Crueger and Crueger 2000), was chosen as the substrate for glucoamylase production. The fermentation variables were optimized using a combination of conventional 'one-variable-at-a-time' and statistical approaches.

Materials and Methods

Micro-organism

The thermophilic fungus *T. indicae-seudaticae* (CBS 104·75) is a soil isolate from Pune, India (Subrahmanyam *et al.* 1977). The mould was routinely grown on Emerson's yeast-starch agar (YpSs) (Emerson 1941) at 40°C, and preserved in glycerol at –20°C.

Immobilization of sporangiospores

The sporangiospores of *T. indicae-seudaticae* were immobilized in calcium alginate according to Kumar and Satyanarayana (2007a).

Production medium and cultivation conditions

Glucoamylase production was carried out in 250-ml Erlenmeyer flasks containing 50 ml of cane molasses medium inoculated with alginate-immobilized *T. indicae-seudaticae*, and incubated at 40°C and 250 rev min^{-1} in an incubator shaker. After 48 h, the mould biomass was separated from the fermented medium by filtration through Whatman No. 1 filter paper. The filtrate was centrifuged at 10 000 ***g*** for 10 min to eliminate any muck from molasses, and the supernatant was used in glucoamylase assay.

Analytical methods

Glucoamylase was assayed by determining the amount of glucose released by its action on soluble starch (Merck, Mumbai, India) using dinitrosalicylic acid (DNS) reagent (Miller 1959). One unit of glucoamylase is defined as the amount of enzyme that releases 1 μmol of reducing sugar as glucose ml^{-1} min^{-1} under the assay conditions.

The average number of spores entrapped per alginate bead was determined by dissolving the beads in 0·1 mol l^{-1} of phosphate buffer followed by counting under compound microscope in a haemocytometer. The residual sugars in the fermented broth were quantitated spectrophotometrically using anthrone reagent (Brink *et al.* 1960).

Optimization of cultivation conditions

'One-variable-at-a-time' strategy

Production media containing various concentrations of cane molasses, i.e. 2%, 4%, 6%, 8% and 10% were used to find out the optimum concentration that supports a high glucoamylase production by immobilized *T. indicae-seudaticae*. The inoculum size was optimized by inoculating varying number of alginate-immobilized beads (20, 30, 40, 50 and 60) per 50 ml of medium (8% cane molasses) in 250-ml Erlenmeyer flasks. The effect of inorganic nitrogen supplementation was assessed by incorporating different nitrogen sources [$(NH_4)_2$ HPO_4, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3 and $CO(NH_2)_2$] at a concentration of 0·2% into the production medium, and the optimum level of the most effective nitrogen source was found out by incorporating it in different concentrations (0·05%, 0·1%, 0·2%, 0·3%, 0·4% and 0.5%). While optimizing a single factor, remaining variables were used at the levels found optimum in previous experiments. Other parameters such as pH of the production medium, temperature and agitation rate were the same as optimized earlier for glucoamylase production in sucrose yeast-extract medium (Kumar and Satyanarayana 2007b).

Table 1 Range of variables used for response surface optimization

Factor code	Factor	Levels				
		$-\alpha$	−1	0	+1	$+\alpha$
A	Cane molasses (%)	2·0	4·0	6·0	8·0	10·0
B	Inoculum size (number of beads)	10	25	40	55	70
C	Ammonium nitrate (%)	0·05	0·10	0·20	0·30	0·35

Response surface optimization

The effect of various interactions among variables [cane molasses (*A*), inoculum size (*B*), and ammonium nitrate (*C*)] on glucoamylase production was studied by the central composite design (CCD) of RSM. Each variable in the design was studied at five different levels, with all variables taken at a central coded value of zero (Table 1). A 2^3 factorial design with six axial points and six replicates at the centre point with a total of 20 experiments was followed (Table 2). Glucoamylase production was taken as response (Y) and a multiple regression analysis of the data was carried out to obtain an empirical model that relates the response measured to the independent variables. The following quadratic equation was used to explain the behaviour of the system,

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC,$$

where Y is the predicted response, β_0 is the intercept, β_1, β_2 and β_3 are the linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$ and $\beta_{3,3}$ are the squared coefficients, $\beta_{1,2}$, $\beta_{1,3}$, and $\beta_{2,3}$ are the interaction coefficients and A, B, C, A^2, B^2, C^2, AB, AC and BC are the independent variables.

All optimization studies were carried out in triplicate using 50 ml of the production medium in 250-ml Erlenmeyer flasks.

Table 2 Experimental design and results of central composite design (CCD) of response surface methodology for glucoamylase production

Run no.	Cane molasses (%)	Number of beads	Ammonium nitrate (%)	Glucoamylase production (U ml^{-1})*	
				Experimental	Predicted
1	$-\alpha$	0	0	18·282 ± 0·96	18·683
2	0	0	0	27·046 ± 1·24	27·084
3	0	0	0	27·014 ± 1·33	27·084
4	$+\alpha$	0	0	21·583 ± 1·32	21·199
5	0	0	$-\alpha$	22·017 ± 1·21	21·756
6	0	$-\alpha$	0	22·585 ± 1·43	22·123
7	0	0	0	27·009 ± 0·97	27·084
8	+1	−1	−1	18·157 ± 0·75	18·590
9	+1	+1	+1	27·652 ± 0·91	27·407
10	0	0	0	27·114 ± 0·93	27·084
11	−1	−1	+1	23·680 ± 1·43	23·513
12	+1	+1	−1	23·201 ± 1·21	23·350
13	0	0	$+\alpha$	24·151 ± 1·26	24·443
14	+1	−1	+1	22·276 ± 1·60	22·689
15	−1	+1	−1	20·441 ± 1·32	20·011
16	0	$+\alpha$	0	22·385 ± 1·14	22·864
17	−1	+1	+1	19·944 ± 0·93	19·494
18	−1	−1	−1	23·760 ± 1·24	23·987
19	0	0	0	27·104 ± 1·43	27·084
20	0	0	0	27·214 ± 1·33	27·084

*Mean of three values (SD within 10%).

Software and data analysis

Experimental designs were generated and analysed by using the statistical software package 'Design-Expert® 6·0' Stat-Ease Inc., Minneapolis, MN, USA.

Validation of the experimental model and large-scale glucoamylase production

Validation of the statistical model and regression equation was performed by carrying out glucoamylase production using all the three factors, *A* (cane molasses), *B* (inoculum size) and *C* (ammonium nitrate) at their respective optimized levels. Glucoamylase production was also carried out in shake flasks of varied volume (0·25, 0·50, 1·0 and 2·0 l) containing one-fifth volume of the optimized cane molasses medium. Large-scale glucoamylase production was carried out in a 22 l air-lift fermentor (B. Braun Biotech International, Germany) (H : D ratio of 5·4 : 1) containing 10 l of optimized cane molasses medium at constant aeration (1 vvm) and pH (7·0). The samples were drawn at the desired intervals and assayed for glucoamylase.

Results

Glucoamylase production increased gradually with increase in the concentration of cane molasses. The enzyme titres at 8% and 10% were comparable, and hence, 8% cane molasses was used in further experiments. Glucoamylase titre was comparatively higher at an inoculum level of 40 alginate beads per 50 ml of cane molasses

medium and decreased on either sides of this inoculum size. Among different inorganic nitrogen supplements, glucoamylase titre was marginally higher in NH_4NO_3 in comparison with others, and it was optimum at 0·1–0·2% (data not shown).

Response surface optimization

Based on the results obtained from 'one-variable-at-a-time' optimization, the aforementioned variables were considered for response surface optimization to delineate their interactive responses on glucoamylase production. The results obtained from the CCD of RSM were analysed by standard analysis of variance (ANOVA), and the mean predicted and observed glucoamylase titres are presented in Table 2. The following regression equation was obtained for predicting the glucoamylase production:

$$Y = 27084 + 0{\cdot}629 \times A + 0{\cdot}185 \times B + 0{\cdot}896 \times C - 1{\cdot}786 \times A^2 - 1{\cdot}148 \times B^2 - 1{\cdot}771 \times C^2 + 2{\cdot}184 \times A \times B + 1{\cdot}143 \times A \times C - 0{\cdot}011 \times B \times C$$

where Y indicates glucoamylase production (U ml^{-1}), and A, B and C are, concentration of cane molasses (%), inoculum size (number of beads) and concentration of ammonium nitrate (%), respectively.

The statistical model used in the present investigation had an '*F*-value' of 108·51, as indicated by ANOVA for glucoamylase production, which implied that the model is significant. Model terms A, C, A^2, B^2, C^2, AB and AC, having values of 'Prob > *F*' less than 0·05, were significant for enzyme production, while B and BC did not exhibit a significant influence on glucoamylase production ('Prob > *F*' > 0.10). The coefficient of determination (R^2) for glucoamylase production was 0·9899, which can explain upto 98·99% variability of the response. The 'predicted R^2' value of 0·9108 was in close agreement with the 'adjusted R^2' value of 0·9807. Adequate precision is a measure of the signal-to-noise ratio and a value greater than 4 is generally desirable. The 'adequate precision' value of 29·047 indicated an adequate signal and suggested that the model can be used to navigate the design space.

To find out the optimum level of each variable and to study the effect of their interactions on glucoamylase production, three-dimensional response surface curves and contour plots were generated for any two independent variables, while keeping the other variables at their respective optimum 'O' levels. For three factors, three contour plots/response surface curves were generated to study the interactions between A (cane molasses) and B (number of beads), B (number of beads) and C (ammonium nitrate), and A (cane molasses) and C (ammonium nitrate). The contour plot showing the interaction between concentrations of cane molasses and ammonium nitrate is depicted in Fig. 1. A linear increase in glucoamylase titre was observed with increasing ammonium nitrate concentration up to around 0·25% (between 'O' and '+1' level) and further increase in ammonium nitrate concentration resulted in adverse effect. Similarly, glucoamylase production gradually increased with increasing concentration of cane molasses up to 7·0% (between 'O' and '+1' level) and declined, thereafter. On the other hand, a high glucoamylase production was attained at just above the optimum 'O' level of inoculum size, when 44 alginate-immobilized beads were used for inoculation (data not shown). The response surface optimized parameters [cane molasses (7%), inoculum level (44 Ca^{2+}-alginate beads per 50 ml of medium) and ammonium nitrate (0·25%)] supported an overall 1·8-fold-enhanced glucoamylase production. Glucoamylase production by *T. indicae-seudaticae* was higher than that reported in *Humicola grisea* var. *thermoidea* (Tosi *et al.* 1993) and *Humicola lanuginosa* (Mishra and Maheshwari 1996).

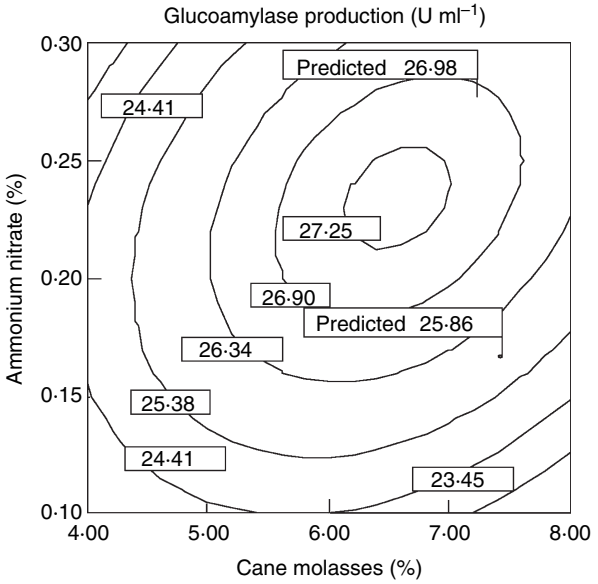

Figure 1 Contour plot showing the interactive effect of concentrations of cane molasses and ammonium nitrate on glucoamylase production.

Validation of the statistical model

The glucoamylase production under optimized conditions (27·05 U ml^{-1}) was comparable with the values predicted by the statistical model (27·32 U ml^{-1}) that proved the validity of the experimental model. A slight decline in

Table 3 Glucoamylase production in shake flasks and air-lift fermentor using optimized cane molasses medium

Flask volume (l)	Production medium (l)	Glucoamylase production (U ml^{-1})*
0·25	0·05	27·1 ± 1·2
0·50	0·10	26·1 ±1·4
1·00	0·20	24·8 ± 1·3
2·00	0·40	24·1 ± 1·8
22·00 (air-lift fermentor)	10·00	26·0 ± 1·2

*Mean of three values (SD within 10%).

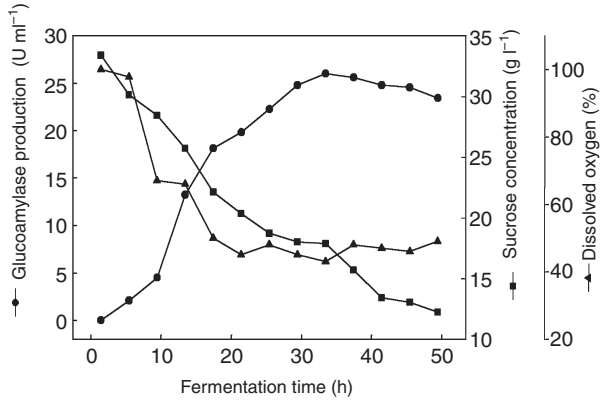

Figure 2 Glucoamylase production profile of alginate-immobilized *Thermomucor indicae-seudaticae* in air-lift bioreactor.

enzyme production was recorded when enzyme production was carried out using higher-volume flasks/medium (Table 3).

Glucoamylase production in air-lift bioreactor

Glucoamylase production profile of immobilized *T. indicae-seudaticae* using cane molasses medium in air-lift fermentor is shown in Fig. 2. A low titre of glucoamylase was detected up to the initial 6–8 h, which gradually increased with the progression of fermentation and attained a peak at 32 h, and decreased slightly afterwards. The concentration of sugars decreased gradually as the fungal growth commenced, and after 48 h, the mould utilized about 63% of the sugar in the production medium. Dissolved oxygen (DO) content (set 100% at the start of fermentation) dropped sharply to reach 50% after 16 h, and thereafter, it was maintained at around 40–50%.

Discussion

The presence of almost all essential nutrients required for sustaining normal growth and metabolism makes cane molasses an attractive alternative for its exploitation as a cheaper substrate for fermentation. For designing an efficient and productive process, it becomes essential to optimize the critical parameters. The variables for optimization, in this investigation, were selected on the basis of their effects on chemical composition of the medium and the glucoamylase production. The concentration of cane molasses, inoculum size and inorganic nitrogen supplement, were therefore, considered for optimization, while other parameters were kept at the same level as optimized earlier (Kumar and Satyanarayana 2007b).

By 'one-variable-at-a-time' optimization, 8% cane molasses seemed to be sufficient to meet the nutritional requirements of alginate-immobilized *T. indicae-seudaticae*, as there was no significant improvement in glucoamylase at higher concentration (10% cane molasses). An inoculum density of 40 alginate beads (*c.* 5×10^6spores) per 50 ml of the production medium supported optimum glucoamylase production, and further increase in inoculum size resulted in decline in the enzyme titre. The nutrients available from 50-ml medium appeared to be inadequate for larger mould population, and therefore, led to reduction in glucoamylase production. Although, cane molasses is nutritionally very rich containing various growth factors and metal ions (Crueger and Crueger 2000), inorganic nitrogen supplementation is generally desired for attaining higher product yields. Inclusion of ammonium nitrate in cane molasses medium slightly improved the enzyme production, which was highest at 0·1–0·2%.

In sucrose yeast-extract medium, a high glucoamylase production (29·7 U ml^{-1}) was achieved when 40 alginate beads were inoculated per 50-ml medium containing 3% sucrose (Kumar and Satyanarayana 2007b). By 'one-variable-at-a-time' optimization, the same inoculum size was found to be optimum for 50-ml cane molasses (8%). However, response surface optimization indicated the requirement of a slightly higher inoculum (44 alginate beads), while the optimum concentration of cane molasses was found to be 7%. Similarly, 0·25% ammonium nitrate was required in the response surface optimized medium, while the optimum concentration by 'one-variable-at-a-time' strategy was 0·1–0·2%. The interactive analysis of variables by RSM helped in understanding the actual optimum levels of individual variables in relation to other parameters and achieving 1·8-fold increase in glucoamylase production, as reported earlier by Kumar and Satyanarayana (2007b) and Sharma *et al.* (2007).

The validity of the experimental model generated by the response surface optimization was confirmed by the closeness in the predicted and experimental glucoamylase titres. Slightly lower enzyme titres at higher flask/medium volume could be because of the inadequate mixing of nutrients and oxygen availability. On the other hand, sus-

tainable glucoamylase production was attained in air-lift bioreactor with an early peak within 32 h. A reduction in fermentation time and higher enzyme production in bioreactor are generally expected because of better control of process parameters (Humphrey 1998).

Glucoamylase production in *T. indicae-seudaticae* is constitutive, and higher enzyme titres were achieved in the production medium containing sucrose. The optimized cane molasses medium contained approximately 3·5% sugar, which was almost equivalent to the amount present in the medium (3% sucrose) optimized earlier (Kumar and Satyanarayana 2007b). However, considering the presence of yeast-extract, K_2HPO_4, asparagine and other micronutrients, the cost of the production medium optimized earlier is approximately 25 times higher than cane molasses, which reflects the economy of glucoamylase production in cane molasses. Cane molasses is a complex medium containing various growth factors and trace elements, and thus glucoamylase production could be higher in a medium containing cane molasses than that in the medium containing sucrose as the carbon source.

Acknowledgements

The financial assistance provided by University Grants Commission, Government of India, during the course of this investigation is gratefully acknowledged. P. Kumar is grateful to the Council of Scientific and Industrial Research, Government of India for awarding Junior/Senior Research Fellowship.

References

Brink, R.H., Dubach, P. and Lynch, D.L. (1960) Measurement of carbohydrate in soil hydrolases with anthrone. *Soil Sci* **89**, 157–166.

Crabb, W.D. and Shetty, J.K. (1999) Commodity scale production of sugars from starches. *Curr Opin Microbiol* **2**, 252–256.

Crueger, W. and Crueger, A. (2000) Substrates for industrial fermentation. In *Biotechnology, A Textbook of Industrial Microbiology* ed. Crueger, W. and Crueger, A. pp. 59–62 New Delhi: Panima Publishing Corporation.

Emerson, R. (1941) An experimental study of life cycle and taxonomy of *Allomyces*. *Lloydia* **4**, 77–144.

Ford, C. (1999) Improving operating performance of glucoamylase by mutagenesis. *Curr Opin Biotechnol* **10**, 352–357.

Haasum, I., Eriksen, S.H., Jensen, B. and Olsen, J. (1991) Growth and glucoamylase production by the thermophilic fungus *Thermomyces lanuginosus* in a synthetic medium. *Appl Microbiol Biotechnol* **34**, 656–660.

Humphrey, A. (1998) Shake flask to fermenter: what have we learnt? *Biotechnol Prog* **14**, 3–7.

Jin, B., van Leeuwen, H.J., Patel, B. and Yu, Q. (1998) Utilisation of starch processing wastewater for production of microbial biomass protein and fungal α-amylase by *Aspergillus oryzae*. *Bioresour Technol* **66**, 201–206.

Kumar, P. and Satyanarayana, T. (2007a) Production of thermostable and neutral glucoamylase using immobilized *Thermomucor indicae-seudaticae*. *World J Microbiol Biotechnol* **23**, 509–517.

Kumar, P. and Satyanarayana, T. (2007b) Optimization of culture variables for improving glucoamylase production by alginate entrapped *Thermomucor indicae-seudaticae* using statistical methods. *Bioresour Technol* **98**, 1252–1259.

Meagher, M.M. and Reilly, P.J. (1989) Kinetics of the hydrolysis of di- and trisaccharides by *Aspergillus niger* glucoamylses I and II. *Biotechnol Bioeng* **34**, 689–693.

Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**, 426–428.

Mishra, R.S. and Maheshwari, R. (1996) Amylases of the thermophilic fungus *Thermomyces lanuginosus*: their purification, properties, action on starch and responses to heat. *J Biosci* **21**, 653–672.

Nigam, P. and Singh, D. (1995) Enzyme and microbial systems involved in starch processing. *Enzyme Microb Technol* **17**, 770–778.

Pedersen, H., Beyer, M. and Nielsen, J. (2000) Glucoamylase production in batch, chemostat and fed-batch cultivations by an industrial strain of *Aspergillus niger*. *Appl Microbiol Biotechnol* **53**, 272–277.

Pham, P.L., Taillandier, P., Delmas, M. and Strehaiano, P. (1998) Optimization of a culture medium for xylanase production by *Bacillus* sp. using statistical experimental designs. *World J Microbiol Biotechnol* **14**, 185–190.

Polakovic, M. and Bryjak, J. (2004) Modelling of potato starch saccharification by an *Aspergillus niger* glucoamylase. *Biochem Eng J* **18**, 57–64.

Reilly, P.J. (1999) Protein engineering of glucoamylase to improve industrial performance – a review. *Starch/Starke* **51**, 269–274.

Satyanarayana, T., Noorwez, S.M., Kumar, S., Rao, J.L.U.M., Ezhilvannan, M. and Kaur, P. (2004) Development of an ideal starch saccharification process using amylolytic enzymes from thermophiles. *Biochem Soc Trans* **32**, 276–279.

Sharma, L., Singh, A.K., Panda, B. and Mallick, N. (2007) Process optimization for poly-β-hydroxybutyrate production in a nitrogen fixing cyanobacterium, *Nostoc muscorum* using response surface methodology. *Bioresour Technol* **98**, 987–993.

Subrahmanyam, A., Mehrotra, B.S. and Thirumalachar, M.J. (1977) *Thermomucor*, a new genus of Mucorales. *Georgia J Sci* **35**, 1–6.

Tosi, L.R.O., Terenzi, H.F. and Jorge, J.A. (1993) Purification and characterization of an extracellular glucoamylase from the thermophilic fungus *Humicola grisea* var. *thermoidea*. *Can J Microbiol* **39**, 846–852.