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Bioresource Technology 78 (2001) 123–126

BIORESOURCE TECHNOLOGY

Production of glucose oxidase using *Aspergillus niger* and corn steep liquor

R.P. Kona, N. Qureshi *, J.S. Pai

Department of Chemical Technology, Food and Fermentation Technology Section, University of Bombay, Matunga, Bombay 400 019, India

Received 28 June 1999; received in revised form 27 December 2000; accepted 28 December 2000

Abstract

Glucose oxidase production was optimized using an isolated strain of *Aspergillus niger* and an economical nutrient source, corn steep liquor (CSL). The culture produced 580 ± 30 units/ml of the enzyme using 70 g/l sucrose as the carbon source. Using CSL as the sole nutrient source enzyme synthesis was increased to 640 ± 36 units/ml. None of the nitrogen sources (nitrates of calcium, sodium, ammonium, potassium and yeast extract, malt extract, and peptone) was beneficial to the enzyme synthesis. Aeration and agitation enhanced enzyme synthesis to 850 ± 45 units/ml. Glucose oxidase has numerous applications in food industry and clinical fields. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Glucose oxidase; Corn steep liquor; *Aspergillus niger*; Sucrose; Enzyme activity

1. Introduction

Glucose oxidase (β-D-glucose; 1-oxygen oxido-reductase) is an important enzyme, which has numerous applications in the food industry and clinical fields (Buck, 1983; Richter, 1983). It catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide using molecular oxygen as an electron acceptor. A number of additional applications have been proposed (Field et al., 1986; Jiang and Ooraikul, 1989; Tiina and Sandholm, 1989; Petruccioli and Federici, 1993). Glucose oxidase is produced using *Aspergillus niger*, and *Penicillium amagasakiense*. For the production of the enzyme complex a basal medium is used which results in increased production cost. It is suggested that for commercial production of biochemical(s), economical and commercially available media be investigated to reduce production costs (Qureshi and Blaschek, 2000). These commercial substrates or media include cheese whey permeate, corn steep liquor (CSL) and molasses. The chemicals, which have successfully been produced by fermentation using these more economical nutrient sources and substrates, are butanol (acetone–butanol–ethanol, ABE) (Qureshi and Maddox, 1987; Parekh et al., 1998) and calcium magnesium acetate (CMA) (Witjitra et al., 1996). A breakdown of the medium cost suggests that CSL is a cost effective medium for fermentation (Parekh et al., 1999). CSL is a rich source of nutrients and contains vitamins, minerals and carbohydrates (Formanek, 1998). The objective of the present research was to produce glucose oxidase using CSL as a cost effective nutrient source.

2. Methods

2.1. Organism and inoculum development

A. niger was isolated from soil and maintained on malt extract agar slopes containing 10 ml/l CSL. The inoculum was developed in liquid medium containing sucrose 25 g/l, KH_2PO_4 0.25 g/l, $NaNO_3$ 4 g/l, $MgSO_4.7H_2O$ 0.25 g/l, and CSL 10 ml/l. Fifty ml liquid medium (in 250 ml conical flask) was autoclaved at 121°C for 15 min and cooled to 29 ± 1°C before inoculating with a 5 ml spore suspension (in 0.5 g/l Tween 80) of the culture (7×10^5 spores/ml) from the agar slope. The flask was kept on a rotary shaker (2.54 cm stroke and 150 rpm) at 30°C for cell growth for 24 h.

* Corresponding author. Present address: Food Science Department, Biotechnology and Bioengineering Group, University of Illinois, Urbana-Champaign, 1207 W. Gregory Drive, Urbana, IL 61801, USA. Tel.: +1-217-244-6354; fax: +1-217-244-2517.
E-mail address: nqureshi@uiuc.edu (N. Qureshi).

0960-8524/01/$ - see front matter
PII: S0960-8524(01)00014-1

2.2. Production of glucose oxidase

Studies on glucose oxidase production were conducted in 100 ml liquid medium (in 500 ml conical flask) containing 60 g/l substrate and 10 ml/l CSL, unless stated otherwise. The pH of the medium was adjusted to 7.0 with 1 M NaOH prior to autoclaving at 121°C for 15 min. Upon cooling to 29 ± 1°C the medium was inoculated with 10 ml of the inoculum. The flask was placed on a shaker at 150 rpm for 48–72 h for glucose oxidase production at 30°C. At the end of the fermentation, the cell mass was centrifuged and disrupted to release glucose oxidase. Experiments were run in triplicate and three sub-samples were taken for each sample.

For glucose oxidase production in 2 l air-lift and 30 l fermentors the medium contained 20 ml/l CSL. The fermentors were sterilized at 121°C for 15–17 min. The ratio of medium to inoculum was the same as in the conical flask. The fermentors were aerated with sterilized air (passing through a glass wool filter; 25.4 × 100 mm). The glass wool filter was sterilized at 200°C for 2 h in an oven. Two fermentors [2 l glass air-lift fermentor (height 300 mm, diameter 92 mm, inner tube height 220 mm, inner tube diameter 46 mm, actual working volume 1975 ml), and 30 l stainless steel fermentor (height 620 mm, inside diameter 248 mm, Agitator six bladed impeller, impeller blade dimensions 27 × 27 mm, impeller diameter 130 mm, number of impeller sets 4)] were used to study the effect of aeration.

2.3. Crude extract preparation

A crude extract of the enzyme was prepared by removing the cell mass from the culture samples, washing with distilled water 2–3 times to remove medium ingredients and disrupting the cells in cold buffer (0.02 M citric acid and 0.058 M Na_2HPO_4, pH 5.6). The homogenate was centrifuged at 27,000 *g* at 4°C for 15 min. The volume of the supernatant was made equivalent to the sample volume and was used as the crude extract for the assay.

2.4. Analyses

The sugars were measured by the dinitro-salicylic acid (DNSA) method using glucose as the standard (Miller, 1959). The same method was used for measuring enzyme activity viz., measuring residual reducing sugars. Sucrose was measured after hydrolysis with 1 N HCl to reducing sugars. The reducing sugars were treated with 3,5 dinitro-salicylic acid (DNSA) which is reduced to 3-amino-5-nitro-salicylic acid. The latter was quantified by measuring absorbance at 540 nm using a spectrophotometer (Spectronic 2000: Milton Roy, USA). The DNSA reagent consisted of 1 g DNSA dissolved in 20 ml 2 N NaOH and 50 ml distilled water. Thirty grams of Rochelle salt (potassium sodium tartrate tetrahydrate: $KO_2CCH(OH)CH(OH)CO_2Na \cdot 4H_2O$; Aldrich, Milwaukee, WI, USA) was added and the volume was brought up to 100 ml with distilled water. The reducing sugars were measured as follows: 0.2 ml reducing sugar solution (containing 1–3.5 g/l reducing sugar), 1.8 ml distilled water and 2 ml DNSA reagent were boiled for 5 min followed by cooling to room temperature and diluting to 24 ml. A standard curve was prepared using known concentrations of glucose (0.5–5.0 g/l: linear range). From the standard curve the concentration of reducing sugar was determined.

The enzyme assay mixture consisted of 0.2 ml reducing sugar solution (2.5–5.0 g/l), 0.2 ml crude enzyme preparation, 1 ml citrate phosphate buffer (pH 5.6) and 0.6 ml distilled water. The citrate phosphate buffer contained 0.02 g/l sodium nitrate to inhibit catalase activity without affecting glucose oxidase activity. The reaction mixture was incubated at 30°C for 30 min. The reaction was stopped by keeping the tube in boiling water. To measure residual sugar, 2 ml DNSA reagent was added to the above tube and the mixture boiled for 5 min followed by cooling to room temperature and diluting to 24 ml. The absorbance was read and glucose concentration was determined from the standard curve as described above. The blank (containing de-ionized water instead of crude enzyme) was immediately boiled after adding the enzyme preparation.

The cell mass was measured as dry weight. The filtered cell mass was washed with distilled water 2–3 times to remove medium ingredients and dried at 60°C to a constant weight. One unit of glucose oxidase activity was expressed as that amount of enzyme which converts 1.0 μg of glucose per 30 min at 30°C. Since the method of measuring glucose/reducing sugar by DNSA method is accurate, it was possible to measure as low as 5 units of enzyme activity.

2.5. Statistical analysis

The results were analyzed by SAS (a statistical computer program; Version 8) Analysis of Variance method. The probability values (*P*-values) are given in Section 3. Two activity values are significantly different for *P*-values <0.05.

3. Results and discussion

Cell concentration in the fermentation broth and glucose oxidase activity using various carbon sources are shown in Table 1. The initial concentration of the substrate was 60 g/l and fermentation was allowed to continue for 48 h. The results showed that sucrose resulted in the highest enzyme activity followed by glucose. Statistical analysis showed that there was not a signifi-

Table 1
Effect of various carbon sources and corn steep liquor on the production of glucose oxidase using *A. niger*

Carbon source	Dry cell weight	Enzyme activity (units/ml)
Effect of various carbon sources		
Glucose	3.60 ± 0.18	450 ± 20
Sucrose	3.90 ± 0.20	500 ± 35
Lactose	–	–
Maltose	3.30 ± 0.17	125 ± 10
Fructose	3.60 ± 0.21	150 ± 9
Starch	2.70 ± 0.15	100 ± 8
Molasses	2.85 ± 0.14	100 ± 8
Potato extract	3.00 ± 0.17	50 ± 8
Effect of corn steep liquor[a]		
Volume of CSL (ml/l)	Glucose oxidase activity (units/ml)	
15	580 ± 28	
20	640 ± 36	
25	490 ± 20	
30	450 ± 22	
60	380 ± 20	

[a] Sucrose as substrate at 70 g/l.

cant difference between glucose and sucrose (P-value 0.2483). The difference between fructose and maltose was not significant either but very close with a P-value of 0.057. The P-values for other carbon sources were <0.05 (with most P-values <0.0001) meaning that they were significantly different. Lower enzyme activities were obtained with substrates other than sucrose and glucose. No growth was observed in lactose suggesting that the culture lacked the β-galactosidase enzyme to hydrolyze lactose. Fructose resulted in a cell concentration of 3.6 ± 0.21 g/l, equal to that of glucose, however, the enzyme activity was three times lower. Starch and potato extract resulted in cell concentrations of 2.7 ± 0.15 and 3.0 ± 0.17 g/l and enzyme activities were 100 ± 8 and 50 ± 8 units/ml, respectively. Surprisingly the enzyme activity with molasses was 100 ± 8 units/ml compared to sucrose of 500 ± 35 units/ml. Possibly high concentration of minerals in the molasses may have inhibited enzyme synthesis (Paturau, 1989). Next, the sucrose level was optimized (by conducting experiments at various initial sucrose concentrations) and it was found that 70 g/l sucrose resulted in 580 ± 30 units/ml. At above 80 g/l sucrose enzyme synthesis was inhibited.

Optimization studies of glucose oxidase production using CSL were conducted. CSL was added to the fermentation medium at 15, 20, 25, 30, and 60 ml/l and fermentation was allowed without supplementing with any other nutrients. At 20 ml/l CSL glucose oxidase activity of 640 ± 36 units/ml was observed (Table 1). Above 20 ml/l CSL enzyme synthesis was inhibited. The P-values of <0.05 (with most P-values <0.0001) showed that the enzyme activity was significantly different at all levels of CSL. Possibly higher concentrations of lactic acid and γ-amino-butyric acid may have been inhibitory to the culture at higher levels of CSL. Lactic acid, as sodium lactate has been reported as a potent inhibitor in cultures of *Klebsiella oxytoca* (Qureshi and Cheryan, 1989). CSL is a rich source of nutrients (Formanek, 1998) and has been successfully used for other fermentations including ABE (Parekh et al., 1999) and CMA (Witjitra et al., 1996). In addition to being rich in nutrients, it is an economical fermentation nutrient source (Parekh et al., 1998).

In order to determine whether CSL was deficient in nutrients, various inorganic and organic nitrogen sources were supplemented to a level of 4 g/l in addition to 20 ml/l CSL (sucrose 70 g/l). The nitrogen sources included nitrates of calcium, sodium, ammonium and potassium, while organic sources included yeast extract, malt extract and peptone. The results indicated that supplementation of the nitrogen sources was not beneficial to the production of glucose oxidase (data not shown). With sodium nitrate, which resulted in the maximum activity among all the nitrogen sources used, enzyme activity was 595 ± 30 units/ml while with CSL it was 640 ± 36 units/ml.

Bioreactors of 2 and 30 l capacity were used to produce glucose oxidase (Table 2). Initially, a 2 l air-lift fermentor was used. The rate of aeration in this fermentor was 0.5–1.5 vvm (volume of air per volume of medium). At an aeration rate of 1.0 vvm the glucose oxidase activity was 670 ± 34 units/ml. Statistical analysis showed that the enzymatic activity was significantly higher at 1.0 vvm than the other two levels of aeration (P-values <0.002). At 0.5 and 1.5 vvm the enzyme activities were 550 ± 27 and 450 ± 23 units/ml, respectively. This showed that in shake flask cultures, aeration was limiting the enzyme production. The medium contained CSL at 20 ml/l and no other nutrients were added. In addition it was investigated whether agitation would improve production of glucose oxidase. This time a larger reactor (30 l) was chosen. The aeration was kept constant at 1.0 vvm and agitation was varied. At 125 rpm enzyme activity was 700 ± 37 units/ml and at

Table 2
Effect of aeration and agitation on the production of glucose oxidase by *A. niger*[a]

Fermentor type and aeration level (vvm)	Agitation (rpm)[b]	Enzyme activity (units/ml)
2 l air-lift fermentor		
0.5	–	550 ± 27
1.0	–	670 ± 34
1.5	–	450 ± 23
30 l fermentor		
1.0	125	700 ± 37
1.0	250	850 ± 45

[a] Sucrose level 70 g/l. Working volume 70% of the total volume.
[b] Air-lift fermentor does not require mechanical agitation as air creates circulation of fermentation broth.

250 rpm it was 850 ± 45 units/ml suggesting that aeration and agitation improved enzyme synthesis significantly (P-value <0.009).

4. Conclusions

A. niger was isolated and it produced 500 ± 35 units/ml glucose oxidase using 60 g/l sucrose. Use of CSL resulted in the production of 640 ± 36 units/ml of glucose oxidase. Supplementation by nitrogen sources did not result in the improvement of enzyme synthesis. Aeration and agitation resulted in an increased production of the enzyme (850 ± 45 units/ml).

Acknowledgements

The authors would like to thank Dr. Susanne Aref (Manager of Statistical Consulting Services) and Mr. Nan Lin (Graduate Student) of Statistics Department of University of Illinois at Urbana-Champaign, IL, USA, for analyzing these results using SAS (statistical program).

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