Journal of Industrial Microbiology, 12 (1993) 413–416

Published by The Macmillan Press Ltd

Starch conversion by amylases from *Aureobasidium pullulans*

Badal C. Saha[a] and Rodney J. Bothast

Fermentation Biochemistry Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, US Department of Agriculture, Peoria, IL, USA

(Received 12 April 1993; revision received 10 July 1993; accepted 30 September 1993)

Key words: Aureobasidium pullulans; Amylase; Starch conversion

SUMMARY

A color variant strain (NRRL Y-12974) of *Aureobasidium pullulans* produced a saccharifying α-amylase and two forms of glucoamylase extracellularly when grown on starch at 28 °C for 4 days. A sugar syrup containing DP1 (degree of polymerization) and DP2 (3 : 1) was made from maltodextrin DE (dextrose equivalent) 10 (35%, w/w) at 55 °C and pH 4.5 using the amylase preparation (40 U g^{-1} DS (dry substance)). The syrup composition was highly dependent upon substrate concentration but nearly independent of enzyme dose. Glucose syrup containing 93% glucose was made from maltodextrin DE 10 (35%, w/w) at 65 °C and pH 4.5 using the same enzyme preparation at 100 U g^{-1} DS. The enzyme preparation (100 U g^{-1} DS) produced 98–100% glucose from raw corn starch at pH 4.5 and 50 °C.

INTRODUCTION

Interest in amylolytic yeasts has increased in recent years because more economical methods for conversion of starchy biomass into fuel ethanol are needed. *Aureobasidium pullulans* is a yeast-like fungus that has been used for industrial production of pullulan from starch substrates [8]. Also a wide variety of industrial enzymes and products have been characterized from this organism [3]. Past studies at our Center have shown that color variant strains of *A. pullulans* overproduce xylanase with extremely high specific activity [5]. Recently, we initiated a detailed study to identify and characterize the starch-degrading enzymes from a color variant strain of *A. pullulans* [7]. We found that the organism produces an extracellular amylolytic enzyme system containing a saccharifying α-amylase (α-1,4-D-glucan glucanohydrolase, EC 3.2.1.1, endoamylase) and two forms of glucoamylase (A and B, 1,4-α-D-glucan glucanohydrolase, EC 3.2.1.3). In addition, it produces an extracellular α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20). The pH optima of α-amylase, glucoamylase A and glucoamylase B were 5.0, 4.5 and 4.0–4.5, respectively. The temperature optima of these enzymes were 55, 50–60 and 65 °C, respectively. All amylases were adsorbed onto and degraded raw corn starch. Glucoamylase B readily cleaved pullulan. None of the enzymes required a metal ion for activity.

The objective of the present study is to explore the use of these starch degrading enzymes from *A. pullulans* for industrial starch processing and alcohol fermentation.

MATERIALS AND METHODS

Enzyme preparation

A color variant strain of *A. pullulans* (NRRL Y-12794 obtained from the ARS Culture Collection, Peoria, IL) was grown at 28 °C for 4 days in a liquid medium with soluble starch as the carbon source [7]. The cell-free culture broth contained amylase (0.47 U ml^{-1}, specific activity, 1.40 U mg^{-1} protein) and α-glucosidase (0.61 U ml^{-1}). About 30 g of Q-Sepharose was added to the culture supernatant solution (3400 ml) adjusted to pH 5.0 with 1 M acetic acid and stirred for 2 h. The α-glucosidase was adsorbed by the Q-Sepharose. The matrix was then removed by centrifugation at 20000 *g* for 15 min. The supernatant solution containing the amylase was treated with $(NH_4)_2SO_4$ (0.8 saturation) and left overnight. The precipitate was collected by centrifugation at 48000 *g* for 30 min, dissolved in 50 mM acetate buffer, pH 6.0, and dialyzed overnight against the same buffer. This amylase preparation (specific activity, 10.54 U mg^{-1} protein) was then used for subsequent studies [7].

Enzyme assays

Amylase activity was assayed by measuring the reducing sugar released from soluble starch. The reaction mixture

Correspondence to: B.C. Saha, Fermentation Biochemistry Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, US Department of Agriculture, Peoria, IL 61604, USA.

[a] Visiting Scientist from the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, USA.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other firms or similar products not mentioned.

Abbreviations: DE, dextrose equivalent (an indication of total reducing sugars as percentage glucose); DP, degree of polymerization; DP1, glucose; DP2, disaccharide; DP3, trisaccharide; DP4, tetrasaccharide; DS, dry substance.

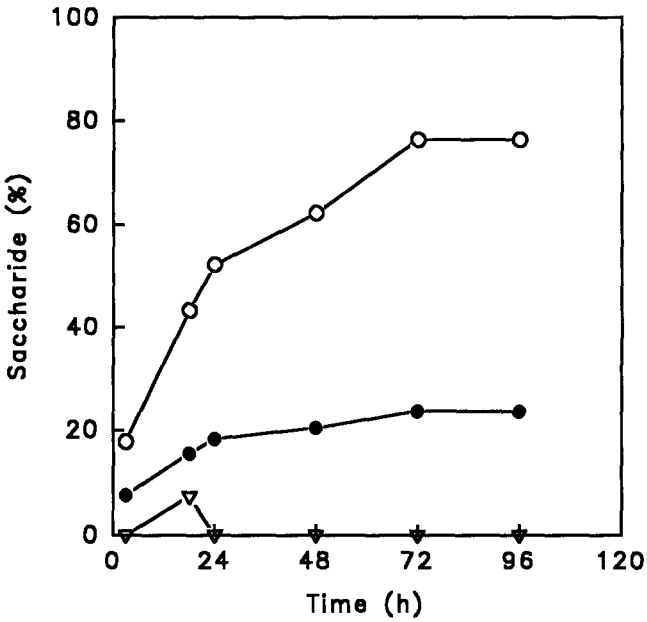

Fig. 1. Time course of saccharide formation by *A. pullulans* amylase preparation (40 U g^{-1} DS) from maltodextrin DE 10 (35%, w/w) at pH 4.5 and 55 °C. ○, DP1; ●, DP2; ▽, DP3.

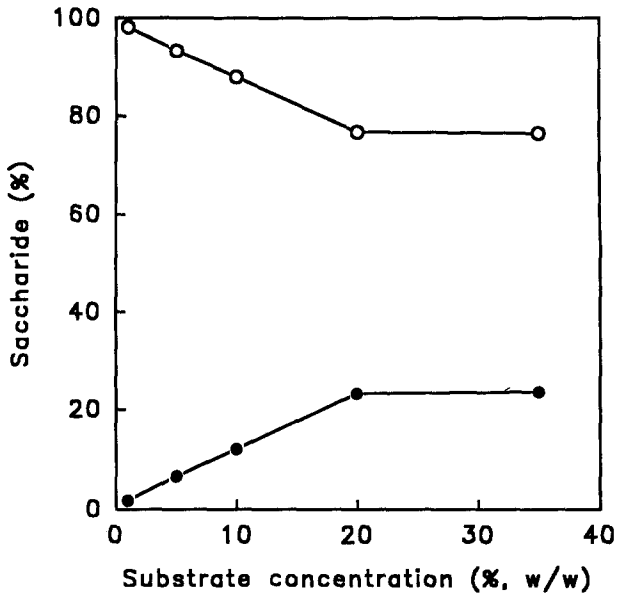

Fig. 3. Effect of substrate concentration on DP1 and DP2 production from maltodextrin DE 10 by *A. pullulans* amylase preparation (40 U g^{-1} DS) at pH 4.5 and 55 °C after 72 h. ○, DP1; ●, DP2.

(0.5 ml) containing boiled soluble starch solution (1%, w/v), acetate buffer (50 mM, pH 5.0), and appropriately diluted enzyme solution was incubated at 50 °C for 30 min. The reducing sugar was measured by the dinitrosalicylic acid (DNS) method [6]. One unit (U) of amylase activity is defined as the amount of enzyme which produces 1 μmol reducing sugar as glucose min^{-1} under these assay conditions.

α-Glucosidase activity was assayed in a reaction mixture (1.0 ml) containing 5 mM *p*-nitrophenyl α-D-glucoside, 50 mM acetate buffer, pH 5.0 and enzyme solution. After 30 min incubation at 50 °C, the reaction was stopped by adding 1 ml of ice-cold 0.5 M Na_2CO_3, and the change in color was measured at 405 nm. One unit (U) of α-glucosidase activity is defined as the amount of enzyme that liberates 1 μmol *p*-nitrophenol min^{-1} under these assay conditions.

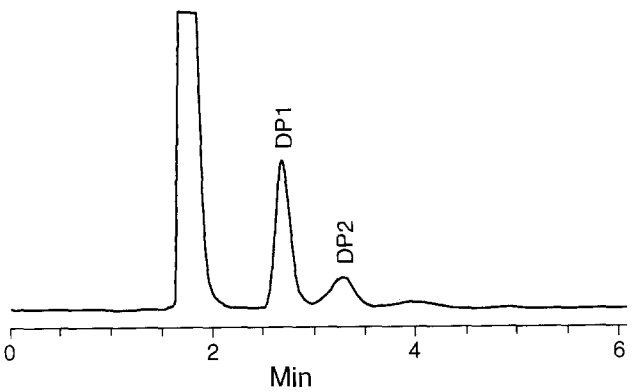

Fig. 2. HPLC analysis of maltodextrin DE 10 (35%, w/w) hydrolyzate of *A. pullulans* amylase preparation (40 U g^{-1} DS) at pH 4.5 and 55 °C after 72 h. A Regis reversible amino column and acetonitrile–water (70 : 30, v/v) solvent system were used.

Maltodextrin DE 10 hydrolysis

Maltodextrin DE 10 (Maltrin M105, Grain Processing Corp., Muscatine, IA) was dissolved in water and then the *A. pullulans* amylase preparation (40 U g^{-1} DS) was added. The pH was adjusted to 4.5 with acetate buffer (final concentration, 50 mM) and the mixture was incubated at 55 °C. At suitable time intervals, aliquots were removed and heated at 100 °C for 15 min to inactivate the enzymes.

Raw corn starch hydrolysis

Raw corn starch (United States Biochemical Corporation, Cleveland, OH) was slurried in water and the amylase preparation from *A. pullulans* (100 U g^{-1} DS) was added. The pH was adjusted to 4.5 with acetate buffer (final concentration, 50 mM) and the mixture was agitated at 50 °C. At suitable time intervals, aliquots were removed, centrifuged to remove the residual starch, and heated at 100 °C for 15 min to inactivate the amylase preparation.

Analysis of sugars

Sugar analysis was performed by high-pressure liquid chromatography (HPLC) on a Regis reversible amino column (Regis Chemical Co., Morton Grove, IL; 250 × 4.6 mm), with acetonitrile : water (70 : 30, v/v) as the eluent (flow rate, 2 ml min^{-1}) at room temperature [2]. Peaks were detected by refractive index, and identified and quantitated by comparison to retention times by authentic saccharide standards (glucose to maltoheptaose, Sigma Chemical Co., St Louis, MO).

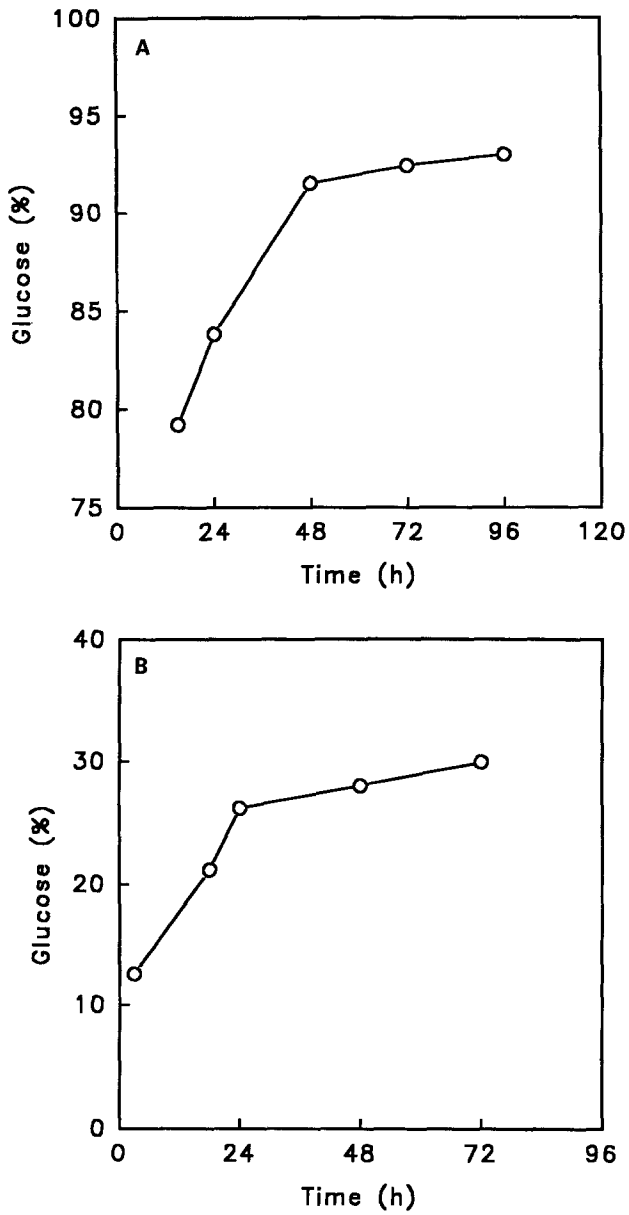

Fig. 4. Time course of glucose production by *A. pullulans* amylase preparation (100 U g^{-1} DS) from (A) maltodextrin DE 10 (35%, w/w) at pH 4.5 and 65 °C and (B) raw corn starch (35%, w/w) at pH 4.5 and 50 °C.

DE value

DE value was calculated from the saccharide analysis as reported previously [1].

RESULTS AND DISCUSSION

Maltodextrin DE 10 hydrolysis by an amylase preparation from A. pullulans

The time course of saccharide formation from maltodextrin DE 10 (35%, w/w) at pH 4.5 and 55 °C by the amylase preparation from *A. pullulans* (40 U g^{-1} DS) is shown in Fig. 1. The hydrolyzate after 72 h contained 76.3% DP1 and 23.6% DP2, and the DE value of the syrup was 89. HPLC analysis of the enzymatic hydrolysis products of maltodextrin DE 10 after 72 h is shown in Fig. 2. A corn syrup containing DP1 and DP2 (3 : 1) may give a different functionality than a glucose (92–96%) syrup [1]. This DP1–DP2 (3 : 1) syrup should also be highly fermentable by yeast for production of biofuel. Fermentability may be estimated as the sum of DP1, DP2 and DP3 saccharides on the assumption that these simple sugars are completely utilized by brewing yeast and fermentability as determined by laboratory fermentation tests is in agreement with the calculated values for a variety of starch hydrolysis products [4].

The effect of substrate concentration (1–35%, w/w) at an equal enzyme dose (40 U g^{-1} DS) was investigated on glucose production from Maltrin M105 at pH 4.5 and 55 °C. Results after 72 h are shown in Fig. 3. The DP1 production decreased significantly and DP2 increased proportionately with an increase in substrate concentration up to 20% (w/w) after which the ratio of DP1 and DP2 remained unchanged. The effect of enzyme dose (2–50 U g^{-1} DS) was also investigated at one substrate concentration (35%, w/w) at pH 4.5 and 55 °C. Higher enzyme doses increased the reaction rate but had no significant effect on the ratio of DP1 and DP2 (data not shown). A glucose solution (35%, w/w) was also incubated with the 2.5 times higher dose of the enzyme (100 U g^{-1} DS) at 55 °C for 4 days. No reversion products were detected within 24 h of incubation. After 72 h, the reversion products were 7.8%. This result indicates that enzyme catalyzed glucose condensation to maltose and isomaltose was not primarily responsible for DP2 production.

Glucoamylase B from *A. pullulans* showed an optimum activity at 65 °C whereas α-amylase and glucoamylase A showed optimum activity at 55 °C [7]. Maltrin M105 (35%, w/w) hydrolysis was performed at 65 °C and pH 4.5 using the same enzyme preparation (100 U g^{-1} DS) as in Fig. 1. Results are shown in Fig. 4 (A). Glucose syrup containing 93% glucose was made from maltodextrin DE 10 (35%, w/w) at 65 °C and pH 4.5 using the amylase preparation at 100 U g^{-1} DS. This is within the expected level of glucose (92–96%) production by a commercial glucoamylase but at a higher temperature (65 °C vs 55–60 °C). Consequently, this glucoamylase B may have potential for reducing the energy input in an industrial operation.

Raw corn hydrolysis by A. pullulans *amylase preparation*

Based on HPLC analysis, raw corn starch (35%, w/w) was hydrolyzed to 98–100% glucose by *A. pullulans* amylase preparation (100 U g^{-1} DS) at pH 4.5 and 50 °C. The time course of glucose production is shown in Fig. 4 (B). Conversion of raw corn starch into glucose was dependent upon substrate concentration. Nearly complete conversion (100%) was observed after 96 h up to 5% (w/w) substrate concentration using the same enzyme quantity (100 U g^{-1} DS). At 10, 20 and 35% (w/w) substrate concentration, 65, 47 and 32% conversions were achieved, respectively after 96 h. The residual starch was available for recycling.

Advances in the areas of enzyme and starch hydrolysis

are providing new options and greater flexibility in the development of many new corn syrups with a variety of saccharide components and a broad range of syrup compositions [4]. The functionality of a corn syrup is a reflection of its composition [1]. New enzymes from new sources and processing technology will facilitate better and more economical production of various corn syrups that may be useful in many applications [4]. The results presented in this paper indicate that the amylase preparation from *A. pullulans* may have practical use in fuel ethanol production and certain food applications such as baking and brewing.

REFERENCES

1 Anonymous. 1986. Nutritive Sweeteners from Corn, p. 28, Corn Refiners Association, Inc., Washington, D.C.

2 Cotta, M.A. 1988. Amylolytic activity of selected species of ruminal bacteria. Appl. Environ. Microbiol. 54: 772–776.

3 Deshpande, M.S., V.B. Rale and J.M. Lynch. 1992. *Aureobasidium pullulans* in applied microbiology: a status report. Enzyme Microb. Technol. 14: 514–527.

4 Hebeda, R.E. and C.R. Styrlund. 1986. Starch hydrolysis products as brewing adjuncts. Cereal Foods World 31: 685–687.

5 Leathers, T.D. 1986. Color variants of *Aureobasidium pullulans* overproduce xylanase with extremely high specific activity. Appl. Environ. Microbiol. 52: 1026–1030.

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

7 Saha, B.C., R.W. Silman and R.J. Bothast. 1993. Amylolytic enzymes produced by a color variant strain of *Aureobasidium pullulans*. Curr. Microbiol. 26: 267–273.

8 Tsujisaka, Y. and M. Mitsuhashi. 1988. Manufacture of pullulan. In: Handbook of Amylases and Related Enzymes (The Amylase Research Society of Japan, ed.), pp. 221–223, Pergamon Press, Oxford.