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Isolation of a transglucosidase-nonproducing mutant of *Aspergillus awamori* yielding improved quality and production of amyloglucosidase preparations

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SUMMARY

The fermentative production of amyloglucosidase (AMG) by different *Aspergillus* species simultaneously yields transglucosidase (TG), which is undesirable in the conversion of starch to dextrose, as it catalyses the reversion of dextrose and maltose to maltosaccharides, in turn providing disproportionately lower yields of dextrose (DX). To overcome this problem, using UV-irradiation, a novel mutant (ND-1-283) has been isolated from *Aspergillus awamori*, which has lost the ability to produce TG and which secretes 45% more AMG than its parent strain, giving the mutant a dual operational advantage. The inability of this mutant to produce TG was demonstrated by thin layer chromatography (TLC) of starch hydrolysate; this was substantiated by obtaining 96.0% DX (w/w) at the end of saccharification.

INTRODUCTION

Amyloglucosidase (AMG) or glucoamylase (α-D-(1 → 4)-glucan glucanohydrolase, EC 3.2.1.3) is widely used in the saccharification of liquefied starch into dextrose, and any improvement in the quality of its preparation has significant industrial importance. It is produced by many *Aspergillus* species in the presence of starch and its hydrolysis products [3,11,14] with transglucosidase (TG, also called transglucosylase or glucosyltransferase, EC 2.4.1.24) as a contaminating enzyme [4,7,14]. The presence of TG is undesirable in the conversion of starch to dextrose, as it catalyses transglucosylation between dextrose and maltose to yield higher maltosaccharides [2,7,17]. This phenomenon, called reversion, ultimately yielded a hydrolysate with only 90–92% (w/w) DX, affording lower yields of dextrose, thereby imparting a higher cost of production, more wastage and a lower margin of profitability. Thus, it is desirable that AMG preparations be free of TG or, if this cannot be achieved, that they contain minimal TG contamination. In the present study, UV rays were used to obtain a mutant of *A. awamori*, which yields TG-free AMG preparations at higher levels than those produced by the parent strain. The AMG preparation ob-

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tained from this mutant was more suitable than that obtained from its parent strain for use in starch hydrolysis to achieve almost quantitative conversion of starch to dextrose, confirming the absence of TG.

MATERIALS AND METHODS

Strain. Aspergillus awamori, hereinafter referred to as strain ND-1, isolated from the industrial effluent of a starch processing unit, was the parent strain used in the present study.

Chemicals. Corn flour (Maize Products, Ahmedabad), corn steep liquor (Anil Starch, Ahmedabad), bacto-agar (Difco), tapioca starch (Synbiotics Ltd., Baroda), maltose, maltotriose and maltotetrose (Sigma), bacterial alpha amylase, and silica gel with binder (Sarabhai M. Chemicals, Baroda), AMG 200-L (Novo Industri A/S, Denmark), Tween-80 (BDH, U.K.), soluble starch (E. Merck), analar grade chemicals and metal distilled (DM) water were used.

Media. Strain ND-1 and mutants were maintained on PDA agar slants as described in Ref. 6. PDA was also used as the complete medium (CM) in plating the mutant colonies. The minimal medium used was that of MacDonald et al. [9]. The inoculum medium (IM) adjusted to pH 6.0 contained 2% corn flour, 0.5% tapioca starch and 3% corn steep liquor (CSL). The production or screening medium (PM) comprising 15% (w/v) corn flour, 5% (w/v) starch and 3% CSL (w/v) was liquefied (pH 7.2, 76°C, 10 min) with 0.2 units of alpha amylase per g of starch, further liquefaction was arrested (96°C, 5 min), and after the medium had cooled the pH was adjusted to 6.4.

UV mutagenesis. A PDA slant inoculated with the spores of strain ND-1 was incubated (28°C, 7 days) to allow good sporulation, and spores were collected in sterile 0.01% (v/v) aqueous Tween-80. The suspension of spores was aseptically transferred to a sterile petri dish and exposed to UV-radiation for 6 min as described by Shah et al. [13]. During irradiation, samples were withdrawn every minute and kept in the dark for 2 h before diluting suitably and plating on PDA plates. After incubating the plates at 28°C for 48 h, mutants were randomly picked and examined for ability to secrete TG-free AMG.

Protocol for the study of AMG secretion

Inoculum preparation. About 1×10^8 spores of each mutant were incubated (28 ± 1°C, 24 h) in 100 ml IM in a 500-ml Erlenmeyer flask on a rotary shaker operated at 220 rpm. A 10-ml suspension from this inoculum was transferred to 100 ml PM in a 500-ml Erlenmeyer flask.

Production. These flasks, in duplicate for each mutant, were then incubated (28 ± 1°C on a rotary shaker, 220 rpm). One flask was terminated at 72 h and the other at 120 h. From each, the broth was filtered to remove mycelium and the filtrate was used to assay AMG, pH, percent residual reducing sugars and TG activity. On a larger scale, inoculum was initiated in a shake flask, transferred to germinator (300 litres IM) and subsequently transferred to the fermentor (3000 litres PM).

AMG concentration. The filtrate from a large batch was subjected to ultrafiltration (Permionics Ltd., Baroda) to obtain a 20–25-fold concentrated AMG preparation which was subsequently used in starch hydrolysis. Concentration facilitated the detection of TG, even if present in traces, and also reduced the volume of AMG to be added during saccharification.

Starch hydrolysis. A 35% (w/w) tapioca starch slurry in DM water fortified with 0.2% calcium chloride and pH adjusted to 7.2 was liquefied using alpha amylase (0.2 units/g starch, 10 min). The liquefaction was arrested (95°C, 5 min) and saccharification was initiated (pH 4.5, 56°C) by adding 2.5 units of AMG/g starch and continued till optimum saccharification was obtained, which was judged by withdrawing aliquots of hydrolysate at intervals to examine percent DX (w/w).

Analytical methods. Reducing sugars were estimated according to the method described by Bernfeld [5]. Alpha amylase was assayed as described by Redfern [12]. One unit of alpha amylase is defined as the amount of enzyme giving red colour equivalent to 20 mg standard dextrin in 30 s at 40°C at

pH 7.0 as a result of starch liquefaction. AMG was estimated by incubating it with 2% (w/v) soluble starch (60°C, pH 4.3, 10 min) and determining the reducing sugars formed [5]. One unit of AMG is defined as the amount of enzyme releasing 1 mg dextrose per min under the assay conditions. Dextrose was estimated using a Beckman Glucose Analyser-2. Dissolved solids (DS) in the starch hydrolysate were estimated using Abbe's refractometer and the standard chart of refractive index vs. dextrose concentration [16]. The ratio of dextrose/DS expressed in percentage indicated percent DX (w/w) produced from tapioca starch.

Chromatography. The presence of TG in AMG was detected by TLC, essentially according to the method described by Aunstrup [2]. A 1.0-ml aliquot of concentrated AMG was incubated (37°C, 60 min) with 1 ml of 6% (w/v) maltose, and the reaction was terminated by placing it in a boiling water bath for 10 min. A 2-μl aliquot of this reaction mixture was spotted on a silica gel plate (0.2-mm thickness). Authentic dextrose, maltose, maltotriose and maltotetrose were spotted simultaneously for identification and comparative evaluation of the resolved constituents of starch hydrolysate prepared from AMG preparations derived from strain ND-1 and its mutants. The plates were developed in a solvent phase [15] comprised of ethyl acetate/acetic acid/methanol/water, 12:3:3:2 (v/v) for 75 min, dried, sprayed with a solution [2] containing diphenylamine/aniline/phosphoric acid/ethanol, 0.16: 0.16:0.85:100 (w/v/v/v), and resolved sugar spots were made visible by keeping the plates at 85°C for 10 min. When TG was present in AMG, isomaltose and maltosaccharides synthesized by it appeared in the chromatogram between the spotted aliquot and maltose spot. An absence of these spots below the maltose spot was considered to be a definite indication of the absence of TG in AMG. The TLC afforded 97% efficiency as judged from quantitative and internal recovery runs.

RESULTS AND DISCUSSION

UV-irradiation of ND-1

Fig. 1A shows percent survival of the mutants isolated after UV-irradiation as a function of time and their AMG secretion profiles. Out of about 100 colonies screened for each exposure duration, plus and minus mutants (showing higher and lower activity than strain ND-1, respectively) were obtained. After an exposure of 1, 2 or 3 min, the largest group of mutants exhibited AMG activity equivalent to strain ND-1 (100%) even though a few plus mutants were obtained. Between 4 and 6 min, this peak shifted beyond 100%, indicating an increased probability of obtaining plus mutants, even though some minus mutants were also obtained. Thus, UV-exposure for a longer time (or lower percent survival) was necessary to obtain an increased number of plus mutants from strain ND-1. The percent of plus mutants increased dramatically at 4 min (Fig. 1B) and remained almost constant thereafter. Table 1 summarizes the AMG yield and TG secretion of representative mutants in

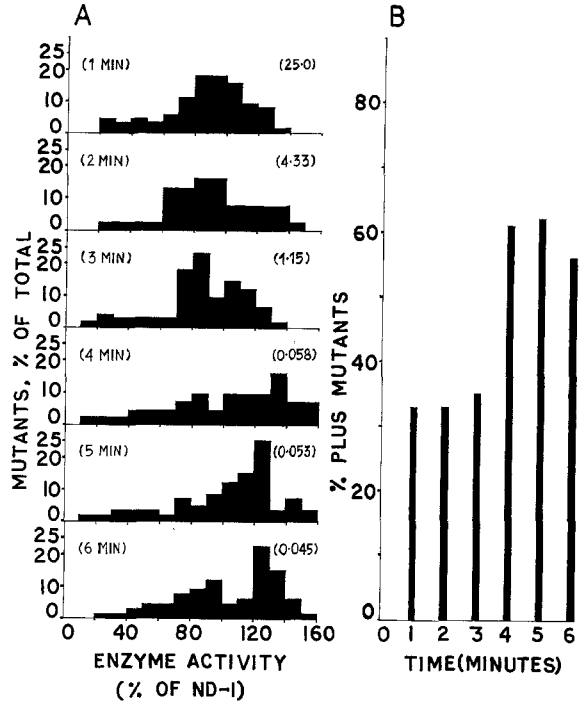

Fig. 1. (A) Pattern of distribution of AMG secreting ability of the mutants, isolated after UV exposure of differing duration. Figures in left parentheses indicate duration of UV exposure in min, and in right parentheses percent survival. The data are based on the figures given in Table 1, column 3. (B) Percent of plus mutants obtained, after UV exposure of differing duration.

Table 1

AMG and TG secretion profiles by representative mutants as a function of UV exposure duration

The presence of TG was ascertained by TLC. When the chromatogram showed a spot of high molecular weight saccharide(s) corresponding to maltotriose and maltotetrose between the spotted aliquot and an authentic maltose spot, reverse synthesis by TG was assumed. Such preparations are marked positive (+) in the last column; the absence of these spots indicated the absence of TG and such strains were marked negative (−). The level of significance in the differences in AMG yield in TG-positive mutants were not calculated, since our objective was to isolate TG-negative mutants.

Strain No.	UV exposure (min)	No. of mutants screened in each UV exposure	AMG yield (percent of ND-1)	Presence of TG
ND-1-101	1	98	136	−
-393	2	99	149	+
-001	3	102	136	+
-204	4	104	154	+
-283	5	103	145	−
-288	5	103	160	+
-365	6	107	156	+
ND-1 (control)	−	−	100	+

each group producing AMG in higher quantum than strain ND-1, obtained as a function of the UV exposure period. It shows that: (a) in each group more AMG-producing mutants were obtained, (b) maximum AMG was secreted in the groups exposed for 4, 5 or 6 min, and (c) AMG secretion never exceeded 160% of the value for strain ND-1. Another round of mutagenesis might increase the yield of AMG further [1].

Since no method existed for the selective isolation of a mutant which lacked an ability to produce TG and yet produced more AMG, all the mutants screened for AMG secretion ability were also examined by TLC for TG production. Out of about 600 mutants thus screened, only two (ND-1-101 and ND-1-283) produced AMG in quantities higher than strain ND-1 with no detectable TG activity as judged from the TLC pattern (Table 1). Of the two, the higher AMG producer, strain ND-1-283, was selected for the production of AMG on a larger scale. The AMG preparation derived from it was used to saccharify starch. Although different mutagens have been used [8,10] to eliminate the co-production of TG with AMG by different strains of *Aspergillus,* only UV mutants of *A. niger* exhibited the ability to produce TG-free AMG [7,14]. Thus the mutants of *A. awamori* obtained in the present study appear to be the first to produce TG-free AMG, and at a 45% higher yield than the parent *A. awamori* strain.

Time course studies of AMG secretion by strains ND-1 and ND-1-283

Fig. 2 shows time course studies of AMG secretion by strain ND-1 and strain ND-1-283 under identical conditions. The rate of secretion was different, although maximum yield in both of the strains was obtained at 120 h. After the initial 24 h, the rate of AMG secretion in strain ND-1 was, on average, 25 units/ml/24 h compared to 39 units/ml/24 h secretion in strain ND-1-283. In both strains, continued fermentation beyond 120 h gave slightly reduced AMG yields even though reducing sugars were present in the broth (Fig. 2). The percent reducing sugars liberated reached a maximum during the initial 24 h and tapered subsequently in both cases, at the rate of 1.9 and 2.1% per 24 h in strains ND-1 and ND-1-283, respectively. Thus, the maximum AMG yield of 175 U/ml by strain ND-1-283 as compared to 120 U/ml by strain ND-1 indicated an increase of 45%.

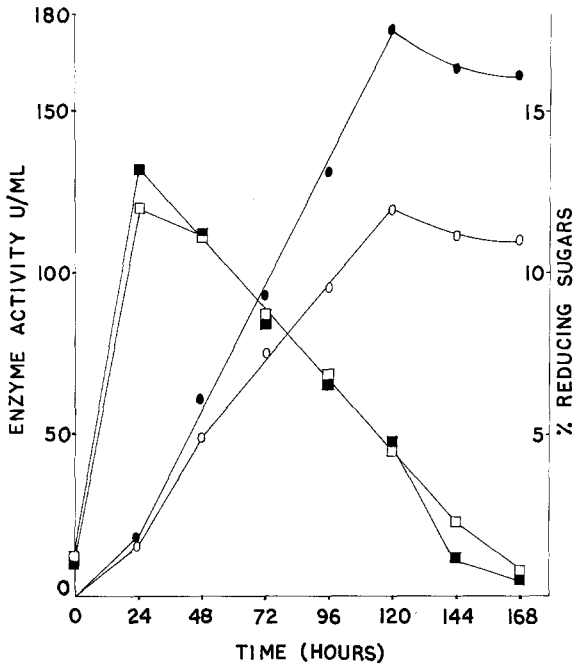

Fig. 2. Profiles of AMG secretion, (○) ND-1 and (●) ND-1-283, and profiles of residual % reducing sugars, (□) ND-1 and (■) ND-1-283, as a function of time.

Starch hydrolysate by AMG preparation from strain ND-1-283

The presence of TG in AMG is undesirable in starch hydrolysis, as it catalyses reversion, ultimately providing 90–92% DX. Therefore, besides evidence from TLC, the absence of TG would be conclusively proved by the ability of an AMG preparation to produce 96–97% DX. On this basis, it was necessary to ascertain the quality of the AMG preparation elaborated by strain ND-1-283 (ND-1-283 AMG), to determine its commercial utility in the preparation of starch hydrolysate. To explore this, ten 3000-litre AMG production batches were prepared using strain ND-1-283, and the AMG preparation from each batch was processed. AMG preparations obtained in this manner were used in starch hydrolysis, conducted individually for each ND-1-283 AMG preparation.

Table 2 shows the progress of saccharification of liquefied starch as a function of AMG preparation from strain ND-1 (ND-1 AMG), ND-1 AMG after removing TG by 0.8% calcium chloride treatment [7] (ND-1 AMG-treated), ND-1-283 (ND-1-283 AMG) and AMG 200-L (Novo Industri A/S, Denmark; Novo AMG). The ND-1 AMG preparation produced maximally 94% DX at 55 h, and further saccharification decreased the DX value to 91.0% at 68 h, indicating that it contained maltosaccharide-synthesising activity (TG). As compared to this, ND-1 AMG-treated also produced 94% DX at 55 h, but did not show reversion in DX value upon further incubation. At 68 h, it produced 95.9% DX, which was similar to the amount that

Table 2

Percent DX produced by different AMG preparations during the saccharification of liquefied starch

The presence or absence of TG in the above AMG preparations was ascertained by TLC; only ND-1 AMG showed the presence of TG, the rest being TG-free.

Duration of saccharification (h)	Percent DX, by preparation			
	ND-1 AMG	ND-1 AMG (treated)	ND-1-283 AMG	Novo AMG
9	62.0	62.5	70.0	70.4
25	84.2	84.0	89.2	92.0
35	89.0	89.0	91.6	94.3
45	92.0	92.2	93.6	95.0
50	93.2	93.5	94.9	95.9
55	94.0	94.0	95.0	96.0
60	92.5	94.9	95.2	95.8
68	91.0	95.9	96.0	96.1

the ND-1-283 AMG preparation yielded after the same saccharification time (96% DX), without reversion. However, an AMG preparation with calcium chloride treatment for the removal of TG would be costly due to 30% inactivation of AMG during recovery, as well as the cost of an extra step in the protocol. The best saccharification results were, however, obtained by Novo AMG in that 96% DX was obtained after 55 h of incubation. These results show that AMG from strain ND-1-283 was free from TG, recommending its commercial use.

Percent DX values as a function of AMG preparation from strain ND-1, ND-1-treated, and ND-1-283 (Table 2) suggest that TG activity is not associated with AMG activity, as TG was removed by calcium chloride treatment. Removal of TG from ND-1-treated AMG preparation and its absence from ND-1-283 AMG preparation further suggest the latter to be a mutation effect. However, over-production of AMG could be due to either another mutation or modification at the active site of AMG.

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