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Improvement of Glucoamylase Production by *Aspergillus awamori* Using Microbial Biotechnology Techniques

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Abstract: This study was initiated to take advantages of mutagenesis in conjunction with protoplast fusion technique to obtain hyper-glucoamylase strains of *Aspergillus awamori*. The resulted data can successfully, be applied in the production of many superior mutants which produced at least 80% of enzyme more compared with the parental strain. Through antifungal resistance study of the highest producer mutants, only five of them were selected according to different response to antifungal agents and intraspecific protoplast fusion between them was carried to obtain hyper-glucoamylase fusants. All obtained fusants after all carried crosses (four crosses) showed higher activities than the original strain. Moreover, among 20 isolated fusants, 13 showed higher activities than their corresponding higher parent strain.

Key words: *Aspergillus awamori*, glucoamylase, improvement, UV, mutants, fusants

INTRODUCTION

Glucoamylase (1,4-α-D-glucan glucohydrase, EC 3.2.1.3) catalyzes the release of glucose progressively from the non-reducing ends of starch and related oligo- and polysaccharides by cleaving α--I,4 and α--l,6 glycosidic bonds. Glucoamylase (GA) find potential application in a number of industrial processes such as in the food, fermentation, textiles and paper industries (Quang *et al*., 2002). Microbial Glucoamylase (GA) have successfully replaced the chemical hydrolysis of starch in starch-processing industries. Glucoamylases are used mainly in the production glucose syrup, high fructose corn syrup and in whole grain and starch hydrolysis for alcohol production. They could be potentially useful in the pharmaceutical and fine chemicals industries provided that enzymes with suitable properties can be prepared.

Glucoamylase play an important role in degradation of starch and produced commercially in bulk from microorganisms (*Bacillus* and *Aspergillus* sp.) and represent about 25-33% of the world enzyme market And ranks second after proteases (Quang *et al*., 2002).

The filamentous fungus *Aspergillus* is an organism of considerable importance for a variety of biotechnological industries. Several *Aspergillus* species, such as *A. niger*, *A. awamori*, *A. awamori* var. *kawachi* and *A. shirousami* were used for GA production.

Aspergillus awamori var. *kawachi* produces three types of GA, namely, GAI, I` and II. These GA, showed different properties and hydrolysis patterns on various substrates and can be were obtained selectively under the specific submerged culture conditions (Shinsaku *et al*., 1982, 1988).

Mutagenesis and protoplast fusion have been used widely by several workers as a tool of protein engineering to achieve strains with higher enzyme productivity or desired characters. Svensson and Sogaard (1993) reviewed the effects of mutation on the structure and function of GA and related enzymes. Mutagenesis has mainly been employed to obtain GA hyper-producing strains. It can be enhanced by the use of chemicals such as N-methyl-N-nitro-N nitrosoguanidine (NTG), or by radiation, such as UV radiation. Suntornsuk and Hang (1994) reported strain improvement of *R. oryzae* for GA production using NTG as well as UV radiation. One mutant, 3N4, produced more GA compared with the parent strain. The mutagenesis of a GA-producing strain of *A. awamori* resulted in a mutant that exhibited 80% increase in enzyme productivity (Witkowska-Gwiazdowska *et al*., 1991).

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Flory *et al.* (1994) carried out random mutagenesis of *A. awamori* to generate thermosensitive mutants, that were expressed in *S. cerevisiae*. Huelseweh *et al.* (1997) carried out site-directed mutagenesis to define the active site of the *Sch. occidentalis* GA. The mutated *GAM*1 genes were expressed in *S. cerevisiae* and transformants were evaluated. Mutants were transcribed and translated similarly to wild-type GA. The aim of this study was to improve the GA productivity of *Aspergillus awamori* NRRL 3112 through UV-mutagenesis and protoplast fusion between the selected hyperproducing mutants.

MATERIALS AND METHODS

Microorganism and culture conditions: The present study was conducted between 2006-2007. The strain of *Aspergillus awamori* NRRL 3112 was obtained from the Northern Regional Research Laboratories (NRRL), Peoria, Illinois, USA and was used as glucoamylase producer and was stored on potato dextrose agar, PDA, (Difco) slants. Complete medium for glucoamylase production was prepared according to Helena and Palva (1979) and contained (g L^{-1}): molasses, 8.5; wheat flour, 37.5; $(NH_4)_2HPO_4$, 1.14; $(NH_4)_2SO_4$, 5.6; $MgSO_4.7H_2O$, 0.14 and $CaCO_3$, 0.14. The pH was adjusted to 4.8 with HCl.

Induction and isolation of mutant strains: Philips T-UV-30 W lamp type No. 57413 P/40 was used as a source of UV-Light to induce genetic variabilities. Conidia from ten days old slants were suspended in 0.9% (w/v) NaCl solution containing 0.01% (v/v) Tween 80 for UV-treatments. Spore suspension in saline solution was transferred into sterile Petri-dish containing a sterile magnetic rod and irradiated using UV- lamp with steering at 20 cm distance for different period (0, 2, 4, 6, 8 and 10 min). The treated spore was kept in dark for 2 h to avoid the photo-reactivation.

The potato dextrose agar was inoculated with diluted treated suspensions and incubated at 30°C. The growing colonies were counted against the control of the same dilution and transferred onto slants for further studies.

Isolation of antifungal resistant mutants for protoplasting: Mutants with high GA were streaked on the surface of PDA agar plates, each supplemented with specific antifungal agent (benomyl, cycloheximide, griseofulvin, miconazole and nystatin) and incubated for 5 days at 30°C. Mutants exhibited resistance to specific antifungal agents were retested for its stability.

Protoplast formation: For each antifungal resistant mutant, 50 mL of the PDA were inoculated with spores suspension (2%, v/v) and incubated overnight (30°C) on rotary shaker (180 rpm). The formed mycelium was recovered by centrifugation (6000 rpm, 4°C), washed twice with sterile distilled water, then resuspended in 5 mL citrate phosphate buffer (0.1 M, pH 5.8) containing 0.7 M NaCl, 0.2 M $CaCl_2$ and Novozyme 234 (10 mg mL^{-1}) and incubated (30°C) with gentle shaking up to 3 h, this treatment resulted in release of protoplasts.

Intraspecific protoplast fusion and fusants isolation: Equal volumes of crude protoplast suspension from 2 parents were mixed and centrifuged at 2500 rpm for 10 min at 4°C. The formed protoplasts pellet was resuspended in 2 mL glycine-NaOH buffer (0.05 M, pH 7.5) containing 30% (w/v) polyethylene glycol 6000 (PEG), 50 mM $CaCl_2$ and 0.7 M NaCl and incubated for 20 min at 30°C. PEG treated protoplast suspensions were plated onto the surface of PDA agar containing 0.7 M NaCl and supplemented with the antifungal agents and incubated for 5-7 days at 30°C. Colonies grown on the surface of the plates were considered as fusants.

Assay of glucoamylase activities: Conical flasks (250 mL), each containing 50 mL of complete medium were inoculated with 5 mL of the spore suspensions from ten days old slants with water containing a drop of Tween 80. Flasks were incubated with shaking (200 rpm) at 30°C for 5 days.

Glucoamylase activity was assayed in the cultured supernatant obtained by centrifugation for 5 min at 5000 rpm under cooling in an expender centrifuge. A half milliliter of the clear supernatant was diluted in 4.5 mL of 100 mM acetate buffer pH 5.5 containing 200 mM $CaCl_2$. The dilution was used to assay the enzyme activity.

A half milliliter of 0.15% soluble starch in 100 mM acetate buffer, pH 5.5 containing 200 mM $CaCl_2$ and 0.5 mL of diluted enzyme were mixed and incubated at 65°C for 10 min. The reaction was stopped with 3 mL dinitrosalisylic acid and the color can be obtained after heating for 10 min. The reducing sugar was measured at wavelength 570 nm (Shimadzu UV-VIS Spectrophotometer model UV-VIS Spectrophotometer model UV-240). One unit of enzyme liberate 1 μmol reducing sugar in one min (Miller, 1959; Nahas and Waldemarin, 2002).

RESULTS AND DISCUSSION

Induction of genetic variabilities was carried out using ultraviolet light (UV) to produce many superior mutants for glucoamylase production by

Table 1: No. of the counted colonies, survival percentages and induced morphological variants (No. and %) following different exposure periods of *Aspergillus awamori* to UV-light

			Morphological variants	
Treatment (min)	No. of counted colonies	Survival (%)	No.	%
0	2174	100.00	0	0.00
2	1073	49.40	0	0.00
4	469	21.60	5	1.10
6	138	6.30	11	8.00
8	78	3.60	14	17.90
10	33	1.50	7	21.20

Table 2: Glucoamylase productivity of different isolates obtained following exposure of *Aspergillus awamori* to UV- light for 2 min

Isolate No.	Glucoamylase activity (U mL^{-1})	% to WT	Isolate No.	Glucoamylase activity (U mL^{-1})	% to WT
Original strain	215	100.00	1-20	126	58.60
1-1	237	110.23	1-21	231	107.44
1-2	182	84.65	1-22	62	28.84
1-3	160	74.42	1-23	220	102.33
1-4	170	79.07	1-24	316	146.98
1-5	261	121.40	1-25	143	66.51
1-6	124	57.67	1-26	230	106.98
1-7	190	88.37	1-27	184	85.58
1-8	283	131.63	1-28	218	101.40
1-9	172	80.00	1-29	130	60.47
1-10	296	137.67	1-30	259	120.47
1-11	180	83.72	1-31	175	81.40
1-12	138	64.19	1-32	208	96.74
1-13	190	88.37	1-33	197	91.63
1-14	187	86.98	1-34	309	143.72
1-15	266	123.72	1-35	188	87.44
1-16	160	74.42	1-36	103	47.91
1-17	269	125.12	1-37	233	108.37
1-18	43	20.00			
1-19	140	65.12			

Aspergillus awamori. In the present study, UV-irradiation was applied in different exposure periods, i.e., 0, 2, 4, 6, 8 and 10 min.

Data in Table 1 showed clearly that the survival percentage decreased gradually by increasing exposure time. After the application of UV- doses, data showed that morphological variants percentages is directly proportional to exposure time. The highest percentage of the obtained morphological variants was recorded following 10 min and decreased gradually by decreasing exposure periods. After every mutagenic treatment, some of the grown colonies were transplanted separately and tested for their glucoamylase activity.

The obtained results showed that the majority of the tested isolates (23 out 37) produced glucoamylase lower than their original strain. Meanwhile, six isolates, i.e., 1-1, 1-21, 1-23, 1-26, 1-28 and 1-37 proved to have slightly more efficiency glucoamylase productivity compared with the original strain. The glucoamylase productivity percentages of these isolates ranged from 101.40 to 110.23% (Table 2).

On the other hand, eight isolates (1-5, 1-8, 1-10, 1-15, 1-17, 1-24, 1-30 and 1-34) out of the tested isolates proved to be higher glucoamylase producers compared with the original strain. The glucoamylase productivity percentages of these isolates ranged from 121.40 to 147%.

Data in Table 3 showed that the majority of the tested isolates (24) produced less glucoamylase than their parental strain. Two isolates (2-17 and 2-22) gave the same productivity of the parental stain. The highest record of glucoamylase production was 275 U mL^{-1} (with 27.91% more than the original untreated strain which was obtained from the isolate No. 2-34 Meanwhile, 12 isolates showed glucoamylase activities higher than original strain but lower than the activities in the superior stain (No. 2-34).

Moreover, data in Table 4 showed that, 13 isolates out of 37 proved to be higher glucoamylase producers than the untreated original stain. Meanwhile, the increasing percents ranged from 6.98 (isolates No. 3-13, 3-20 and 3-33) to 58.14 (isolate No. 3-16). Five isolates No. 3-14, 3-18, 3-26, 3-28 and 3-37 gave the same productivity of the parental strain. The remaining isolates exhibited glucoamylase productivity lower than the wild type strain with the exception of the isolates No. 3-10 and 3-31 which lost completely its glucoamylase productivity activity.

Results in Table 5 proved to be higher glucoamylase producers than the original strain. Four of the isolates, i.e., 4-9, 4-17, 4-22 and 4-31 showed the following percent increase than the original strains (155.81, 173.02, 160.47 and 183.72%, respectively. On the other hand, 22 isolates

Table 3: Glucoamylase productivity of different isolates obtained following exposure of *Aspergillus awamori* to UV- light for 4 min

Isolate No.	Glucoamylase activity (U mL^{-1})	% to WT
Original strain	215	100.00
2-1	235	109.30
2-2	225	104.65
2-3	185	86.05
2-4	160	74.42
2-5	185	86.05
2-6	190	88.37
2-7	230	106.98
2-8	218	101.40
2-9	170	79.07
2-10	145	67.44
2-11	200	93.02
2-12	240	111.63
2-13	190	88.37
2-14	175	81.40
2-15	210	97.67
2-16	225	104.65
2-17	215	100.00
2-18	185	86.05
2-19	196	91.16
2-20	228	106.05
2-21	176	81.86
2-22	215	100.00
2-23	265	123.26
2-24	200	93.02
2-25	105	48.84
2-26	190	88.37
2-27	145	67.44
2-28	183	85.12
2-29	217	100.93
2-30	195	90.70
2-31	142	66.05
2-32	175	81.40
2-33	163	75.81
2-34	275	127.91
2-35	204	94.88
2-36	190	88.37
2-37	257	119.53
2-38	228	106.05

Table 4: Glucoamylase productivity of different isolates obtained following exposure of *Aspergillus awamori* to UV- light for 6 min

Isolate No.	Glucoamylase activity (U mL^{-1})	% to WT
Original strain	215	100.00
3-1	174	80.93
3-2	190	88.37
3-3	137	63.72
3-4	290	134.88
3-5	180	83.72
3-6	169	78.60
3-7	212	98.60
3-8	185	86.05
3-9	325	151.16
3-10	0	0.00
3-11	172	80.00
3-12	156	72.56
3-13	230	106.98
3-14	215	100.00
3-15	286	133.02
3-16	340	158.14
3-17	310	144.19
3-18	215	100.00
3-19	162	75.35
3-20	273	126.98
3-21	140	65.12

Table 4: Continued

Isolate No.	Glucoamylase activity (U mL^{-1})	% to WT
3-22	185	86.05
3-23	125	58.14
3-24	173	80.47
3-25	235	109.30
3-26	215	100.00
3-27	175	81.40
3-28	215	100.00
3-29	258	120.00
3-30	183	85.12
3-31	0	0.00
3-32	210	97.67
3-33	230	106.98
3-34	315	146.51
3-35	268	124.65
3-36	145	67.44
3-37	215	100.00
3-38	260	120.93

Table 5: Glucoamylase productivity of different isolates obtained following exposure of *Aspergillus awamori* to UV-light for 8 min

Isolate No.	Glucoamylase activity (U mL^{-1})	% to WT
Original strain	215	100.00
4-1	185	86.05
4-2	173	80.47
4-3	165	76.74
4-4	280	130.23
4-5	145	67.44
4-6	160	74.42
4-7	230	106.98
4-8	247	114.88
4-9	335	155.81
4-10	0	0.00
4-11	290	134.88
4-12	138	64.19
4-13	180	83.72
4-14	0	0.00
4-15	165	76.74
4-16	190	88.37
4-17	372	173.02
4-18	210	97.67
4-19	140	65.12
4-20	95	44.19
4-21	212	98.60
4-22	345	160.47
4-23	0	0.00
4-24	215	100.00
4-25	193	89.78
4-26	205	95.35
4-27	70	32.56
4-28	168	78.14
4-29	0	0.00
4-30	200	93.02
4-31	395	183.72
4-32	182	84.65
4-33	125	58.14
4-34	0	0.00
4-35	310	144.19
4-36	175	81.40
4-37	205	95.35

from the same treatment produced glucoamylase lower than the wild type strain. Furthermore, five isolates lost completely its glucoamylase productivity.

After exposure of the parental strain to UV-light for 10 min, the majority of the tested isolates (22 out of 33),

Table 6: Glucoamylase productivity of different isolates obtained following exposure of *Aspergillus awamori* to UV-light for 10 min

Isolate No.	Glucoamylase activity (U mL^{-1})	% to WT
Original strain	215	100.00
5-1	125	58.14
5-2	180	83.72
5-3	145	67.44
5-4	260	120.93
5-5	212	98.60
5-6	200	93.02
5-7	146	67.91
5-8	135	62.79
5-9	397	184.65
5-10	218	101.40
5-11	197	91.63
5-12	143	66.51
5-13	245	113.95
5-14	210	97.67
5-15	155	72.09
5-16	230	106.98
5-17	277	128.84
5-18	170	79.07
5-19	163	75.81
5-20	105	48.84
5-21	75	34.88
5-22	250	116.28
5-23	173	80.47
5-24	165	76.74
5-25	138	64.19
5-26	70	32.56
5-27	210	97.67
5-28	385	179.07
5-29	182	84.65
5-30	90	41.86
5-31	118	54.88
5-32	195	90.70
5-33	257	119.53

produce glucoamylase activity lower than their original strain. However, this treatment gave the highest record of the glucoamylase production (184.65% more than the original untreated strain) which was obtained from the isolate No. 5-9. On the other hand, the lowest record of the glucoamylase production was (30% lower than the original strain) which was obtained from the isolate No. 5-26.

Intra-specific protoplast fusion: To investigate the effect of protoplast fusion on GA production, eight superior GA-over producing mutants (3-9, 3-16, 4-9, 4-17, 4-22, 4-31, 5-9 and 5-28) were selected, then tested for their resistance to the antifungal agents used as selected markers during detection of fusants after protoplast fusion of two parents strains. As presented in Table 7, it was found that all mutants exhibited resistance to griseofulvin and with different response to the other agents. Mutants 4-9 and 4-31 were resistant to benomyl and sensitive to the other agents. Mutants 4-9 and 5-9 resistant to cyclohexamide but sensitive to the other agents. Mutants 4-17 and 4-31 was found to be resistant to micanozole and sensitive to the other agents.

Table 7: Antifungal response of superior GA mutants to various antifungal agents

Mutant No.	GA activity (U mL^{-1})	Antifungal agent (mg mL^{-1})				
		B (5)	C (100)	G (250)	M (5)	N (100)
3-9	325	-*	-	+**	-	-
3-16	340	-	-	+	-	-
4-9	325	+	+	+	-	-
4-17	372	-	-	+	+	+
4-22	345	-	-	+	-	+
4-31	395	+	-	+	+	-
5-9	397	-	+	+	-	-
5-28	385	-	-	+	-	+

B: Benomyl, C: Cycloheximide, G: Grisofulvin, M: Miconazole, N: Nystatine *-: Means sensitive **+: Means resistance

Table 8: Design of crosses using different selective antifungal markers

Cross	Parents	Selective markers
C1	4-9 and 4-17	B+M or C+N
C2	4-22 and 4-31	B+N
C3	4-31 and 5-09	B+C or C+M
C4	5-9 and 5-28	C+N

Finally, mutants 4-17, 4-22 and 5-28 were resistant to nystatin and sensitive to the other antifungal agents. Accordingly, six mutants 4-9, 4-17, 4-22, 4-31, 5-9 and 5-28 were selected for four different inter-specific protoplast fusions. Due to different antifungal responses of the superior mutants, four crosses were designed and executed (Table 8). The mycelium of each of the six mutants was induced to form protoplasts and the parental protoplasts were used for fusion as designed.

Five fusants were Randomly selected after each protoplast fusion cross to determine their GA activities (Table 9). The results revealed that all tested fusants exhibited higher GA productivity than the original strain. Moreover, two fusants (obtained after cross 1) exhibited higher GA productivity than their higher parental strain (4-17). The other fusants obtained after cross 1 exhibited higher GA productivity than their lower-parent strain (4-9). The same trend was recorded after cross 2. On the other hand, four fusants (obtained after cross 3) and five fusants (obtained after cross 4) exhibited higher GA productivity than their higher-parent strain (5-9). Only one fusants No. (3-3) gave the same productivity of their lower- parent strain (4-31).

Mutagenesis of industrial microbial strains is widely used for the improvement of microbial synthesis of enzymes. Various methods have been used to obtained high GA-producing mutants (Sierks *et al*., 1990, 1993; Witkowska-Gwiazdowska *et al*., 1991; Sierks and Svensson, 1993; Svensson and Sogaard, 1993; Flory *et al*., 1994; Frandsen *et al*., 1994, 1995; Suntornsuk and Hang, 1994; Huelseweh *et al*., 1997). Current literature didn't reveal the use of protoplast fusion technique to improve GA production by *Aspergillus awamori*. Therefore, this study was initiated

Table 9: Glucoamylase (GA) production of fusants obtained after four different interspecific protoplast fusion cross

Parents and fusants	GA activity (U mL^{-1})	%to WT
Original strain	215	100
C_1		
P1 4-9	335	155.81
P2 4-17	372	173.02
C1-1	350	162.79
C1-2	379	176.28
C1-3	365	169.77
C1-4	384	178.61
C1-5	358	166.51
C_2		
P1 4-22	345	160.47
P2 4-31	395	183.72
C2-1	370	172.09
C2-2	383	178.14
C2-3	415	193.02
C2-4	406	188.84
C2-5	382	177.67
C_3		
P1 4-31	395	183.72
P2 5-9	397	184.65
C3-1	416	193.49
C3-2	427	198.61
C3-3	395	183.72
C3-4	425	197.67
C3-5	409	190.23
C_4		
P1 5-9	397	184.65
P2 5-28	385	179.07
C4-1	415	193.02
C4-2	462	214.88
C4-3	398	185.12
C4-4	445	206.98
C4-5	417	193.95

to take advantages of mutagenesis in conjunction with protoplast fusion technique to obtain GA over producer strains of *Aspergillus awamori*.

In general, it can be concluded that UV-mutagenesis proved to an effective technique to enhance GA production. In addition, intraspecific protoplast fusion between higher GA-producing mutants proved effective in achieving superior GA-producing fusants.

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