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Improvement of Xylanase Production from *Streptomyces Pseudogriseolus* via UV Mutagenesis

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Abstract: *Streptomyces pseudogriseolus* isolated from terrestrial environment was subjected to UV irradiation for different periods producing 139 mutants. Twenty-nine mutants were considered as morphological mutants as they showed abnormality in growth, color and/or shape. Qualitative screening has been done to those mutants using agar plate medium stained with Congo red after growth for 3 days at 30°C. Ninety-four mutant exhibited clear zones more than the wild strain, 48 mutants were selected to be tested quantitatively using submerged culture medium containing xylan as sole carbon source. Quantitatively, 16 mutants showed xylanase activity more than the wild strain. Mutant no. 121 was the promizing potent xylanase-producing mutant. It had an activity of 161% as compared to the wild strain.

Key words: Xylanase, Production, UV irradiation, Streptomyces spp.

INTRODUCTION

Hemicellulose is the second most abundant renewable biomass and accounts for 25–35% of lignocellulosic biomass which is considered to be a promising biomass feedstock for the production of biofuels. β-1,4-xylan is the major hemicellulose component (Kumar *et al*., 2008). Several enzymes are required to complete hydrolysis and assimilation of xylan, including β-1,4-xylanase (1,4-β-d-xylanohydrolase; E.C. 3.2.1.8) that cleaves glycosidic bonds to produce xylooligosaccharides and β-xylosidase responsible for the final breakdown of small xylooligosaccharides into xylose (Sharma and Bajai, 2005). Xylanases are hydrolytic enzymes, which catalyze the endohydrolysis of β-1,4-xylosidic linkages in xylan. The xylan hydrolysis end product has considerable industrial applications in biofuel, artificial sweetener, animal feed production, baking, textile, clarification of fruit juices and coffee extraction. Besides, xylanases showed increasing interest applications as eco-friendly bleaching agent of pulp in paper industries (Viikari *et al*., 1994 and Li *et al*., 2010). Xylanase in synergism with numerous enzymes can be used for the generation of biofuel, such as ethanol and xylitol from lignocellulosic biomass (Girio *et al*., 2010). Actinomycetes are gram positive filamentous bacteria widely considered as producers of important industrial enzymes involved in lignocellulose degradation (Holtz *et a*l., 1991 and Flores *et al.*, 1997). Xylanases observed in mesophilic actinomycetes are mainly of endotype (β-1,4-xylan xylanohydrolase). Several species of *Streptomyces* such as *Streptomyces olivaceoviridi*s E-86, *Streptomyces viridosporus* T7A, *Streptomyces lividans* 66 and *Streptomyces cyaneus* SN32 were investigated as endotype xylanase producers (Kluefel et al.,1990; Timothyet al., 1997; Ding et al., 2004 and Suchita et al., 2008).

Ultraviolet light has been widely used in inducing mutations in the field of microbial breeding (Gadgil *et al*., 1995 and Adsul *et al*., 2007). Kuek and Kidby (1984) improved the glucoamylase production over 140% after UV mutagenesis in *Aspergillus pheonesis*. Similar improvement related to cellulolytic enzymes was achieved by Szczodrak (1989) after mutagenesis of *Trichoderma reesii*.

The present work aimed to obtain a xylanase overproducing mutant strain after UV irradiation for different periods by qualitative and quantitative screening of the resulting mutants.

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MATRIALS AND METHODS

Microorganism:

A locally isolated *Streptomyces pseudogriseolus*, identified by Mansour *et al.* (2010), had been subjected to UV irradiation for different periods.

UV mutagenesis:

A Philips T UV-30 W, WL 254 nm lamp type number 57413, as the source of irradiation treatments was used. Spore suspension from 10 days old slants of the wild type strain (*Streptomyces pseudogriseolus*) had been prepared in saline solution (0.85% NaCl). The spores were irradiated for 0, 30, 60, 90, 120 and 180 sec. Treated spores were kept in dark for one hour before their dilution. Serial dilution up to 10^{-5} for treated samples and 10^{-6} for control had been constructed and spread onto complete medium (CM) having the following composition (g/l): glucose, 10; peptone, 2; hydrolyzed casein, 2; $MgSO_4.7H_2O$, 0.5; yeast extract, 2; agar, 15-20; tap water, 1000 ml and the pH was adjusted to 7.0. Plates were incubated for three days at 29°C, then single colonies were transplanted onto minimal medium (MM) plates having the following composition (g/l): glucose, 10; asparagines, 0.5; K_2HPO_4, 0.5, $FeSO_4$, 0.01; KOH, 0.3; agar, 20, tap water, 1000 ml and the pH was adjusted to 7.0.

Isolation and identification of irradiated auxotrophic mutants were performed as follows:

Single isolates, which appeared on CM plates after each mutagenic treatment, were replica plated on CM and MM plates and incubated at 29°C for three days. The isolates, which failed to grow on MM plates, were considered as auxotrophs. In addition, all colonies, which showed abnormality in growth, color and/or shape, were considered as morphological variants. The stable mutants were chosen for testing their xylanase activity.

Qualitative and quantitative screening for xylanase production:

Mutants were screened qualitatively using Congo red and /or absolute ethyl alcohol (Ninawe *et al.*, 2006 and Saadoun *et al.*, 2007), after cultivating on agar plate medium (described by Wang *et al.*, 2003) had the following ingredients (%): xylan (oat spelt), 0.25; $NaNO_3$, 0.1; K_2HPO_4, 0.1; $MgSO_4.7H_2O$, 0.05; yeast extract, 0.05 and agar, 2.0. the pH was adjusted to be 7.0. The cultivated plates were incubated at 30°C for 3 days. The mutants which showed best results (clears zones) were screened quantitatively by cultivating on submerged culture medium (Nascimento *et al.*, 2002) having the following composition (%): xylan, 1.0, $NaNO_3$, 0.15; KH_2PO_4, 0.3; K_2HPO_4, 0.6; $MgSO_4.7H_2O$, 0.02; $CaCl_2$, 0.005; $MnSO_4.7H_2O$, 0.001 and $ZnSO_4.7H_2O$. The pH was adjusted to be 7.0 using diluted NaOH and HCl. After inoculation with 5% of spore suspension (1.7×10^6 cell/ml),the cultures were incubated at 30°C for 6 days on a rotary shaker at150rpm. At the end of incubation period, the cultures had been centrifuged at 5000 rpm for 20min. using a cooling centrifuge (Jouan E956). Xylanase activity was assayed in the culture supernatant.

Xylanase assay and protein measurement:

Enzyme activity was assayed as described by Xiong *et al.* (2003) as follows: 1% xylan (from oat spelts) dissolved in 0.05M acetate buffer (pH 5.0) was used as a substrate. One ml of diluted culture supernatant was added to 1.0 ml of substrate in test tube (16x160mm). The mixture was incubated for 15 min in water bath adjusted at 50°C. The released reducing sugars were determined by the method of Miller (1959). Xylanase unit is the amount of enzyme that liberates 1µ mole of reducing sugar per min. Protein was measured according to the method of Lowry *et al.* (1951).

Results:

Exposing *Streptomyces pseudogriseolus* spores to UV irradiation for different incubation periods and cultivating the resulting colonies from CM on the MM. The colonies which failed to grow on MM medium as well as the colonies that showed abnormities in growth, color and shape were considered as mutants. 139 colonies were considered as mutant strains. Results in Table 1 revealed that, by increasing the UV irradiation time the number of mutants was increased, while the survival ratio decreased gradually with increment of UV exposure time. The survival ratio reached 20% after 30 sec., then decreased drastically after 60, 90, 120 and 180 sec to be 11.67, 2.72, 1.56 and 0.39%, respectively.

Twenty-nine mutants were considered as morphological mutants as they showed abnormalities in growth, colour and shape in comparison to the wild strain (Photo. 1). On the other hand, the other 110 mutants were considered as auxotrophs as they couldn't grow on MM.

Most stable mutants were tested for xylanase production using the agar plate techniques (qualitative test). The results in Table 2 summarize the different xylanase-producing mutants and clear zones. It is clear that the wild type (WT) strain had a clear zone of 3.7cm, 104 mutants exhibited clear zones less than the WT, 22 mutants showed a clear zones more than the WT strain while 13 mutants had a clear zone similar to the WT. Photo. 2 explains the clear zone formation by some produced mutants in relation to the wild type strain of *Streptomyces pseudogriseolus*.

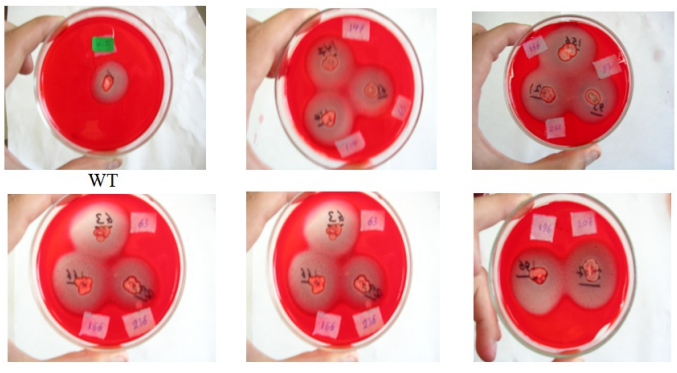
Table 1: Survival ratio and mutation percentages of *Streptomyces pseudogriseolus* after UV irradiation treatments

Treatment time (sec.)	Survival		No. of tested colonies	Mutants	Mutation (%)
	(No.)	(%)			
0	514	100	-	-	-
30	103	20.04	19	7	36.84
60	60	11.67	61	32	52.46
90	14	2.72	63	39	61.09
120	8	1.56	48	27	56.25
180	2	0.39	49	34	69.39

Photo. 1: (a, b and c): Some morphological variants in *Streptomyces pseudogriseolus* obtained after UV irradiation

Table 2: Different xylanase-producing mutants from qualitative test

Clear zone (cm.)	Number of mutants	Type of mutant	
		Auxotrophs	Morphology
<1	1	1	0
1.2-2.0	8	7	1
2.1-3.0	50	39	11
3.1-4.0	76	59	7
4.1-5.0	7	4	0
Total	139	110	29



WT

Photo. 2: Some hyper-xylanase-producing mutants and the wild type of *Streptomyces pseudogriseolu*

Mutants that exhibited clear zones over 3.5cm, including the wild type, were selected to be screened quantitatively using submerged culture medium. It has been found that 48 mutants had clear zones over 3.5cm including 10 morphological variants. Results in Table 3 showed that 16 mutants overproduced xylanase activity

compared with the wild type. On the other hand, 32 mutants had xylanase activity less than or approximately equal to the wild type strain. Mutant No. 121 was found to have the highest xylanase activity and productivity than other tested mutants, having activity of 35.2U/ml with a percent of 161.62 compared to WT strain and it had specific activity of 100U/mg protein resemble 156.56% relation to WT strain. *Streptomyces psedogriseolus* mutant showed xylanase activity more than the wild type strain by 61% when exposed to UV irradiation for 120sec.

Table 3: Xylanase production by the produced mutants from *Streptomyces psedogriseolus*

Serial No.	Mutant no.	Mutation type	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Activity (%) to W.T W.T.	Productivity (%) to w W.T
Wild	-	None	21.78	0.31	70.26	100	100
1	2	Auxotroph	28.13	0.32	87.91	129.16	125.12
2	6	Auxotroph	22.72	0.36	63.11	104.32	89.82
3	14	Auxotroph	16.07	0.32	50.22	73.78	71.48
4	19	Auxotroph	20.39	0.36	56.64	93.62	80.61
5	23	Auxotroph	28.13	0.36	78.14	129.16	111.22
6	28	Auxotroph	22.15	0.32	69.22	101.7	98.52
7	31	Morphology	21.58	0.35	61.66	99.08	87.76
8	37	Morphology	19.31	0.21	91.95	88.66	130.87
9	53	Auxotroph	20.17	0.36	56.03	92.61	79.75
10	56	Auxotroph	19.31	0.32	60.34	88.66	85.88
11	58	Auxotroph	20.45	0.31	65.97	93.89	93.39
12	61	Morphology	17.61	0.32	55.03	80.85	78.32
13	62	Morphology	22.55	0.33	68.33	103.54	97.25
14	63	Auxotroph	22.72	0.33	68.85	104.32	97.99
15	67	Auxotroph	17.61	0.32	55.03	80.85	78.32
16	68	Auxotroph	27.71	0.33	83.7	127.33	119.13
17	69	Morphology	21.87	0.33	66.27	100.4	94.32
18	70	Auxotroph	26.07	0.32	81.47	119.7	115.96
19	67	Auxotroph	23.14	0.33	70.12	106.24	99.8
20	77	Auxotroph	29.81	0.35	85.17	136.87	121.22
21	97	Auxotroph	24.65	0.35	70.43	113.18	100.24
22	88	Auxotroph	19.31	0.31	62.29	88.66	88.66
23	90	Auxotroph	27.06	0.33	82	124.24	116.71
24	92	Auxotroph	23.62	0.33	71.58	108.45	101.88
25	95	Auxotroph	20.45	0.32	63.91	93.89	90.96
26	97	Auxotroph	33.33	0.34	98.03	153.03	132.52
27	98	Auxotroph	22.46	0.43	52.23	103.12	74.34
28	100	Auxotroph	24.97	0.33	75.12	118.77	106.92
29	102	Auxotroph	30.8	0.34	90.85	141.41	129.31
30	104	Auxotroph	28.82	0.37	77.89	132.32	110.86
31	105	Auxotroph	20.73	0.32	64.78	95.18	92.2
32	106	Auxotroph	29.47	0.37	79.65	135.31	113.36
33	107	Auxotroph	20.76	0.4	51.9	95.32	73.78
34	108	Auxotroph	21.02	0.32	65.69	96.51	93.5
35	109	Auxotroph	19.31	0.33	58.52	88.66	83.29
36	110	Auxotroph	19.6	0.28	70	89.99	99.63
37	114	Auxotroph	20.34	0.38	53.53	93.39	76.19
38	115	Morphology	20.45	0.32	63.91	93.89	90.96
39	116	Morphology	28.82	0.32	90.06	132.32	128.18
40	118	Auxotroph	20.39	0.34	59.97	93.62	85.35
41	119	Auxotroph	27.68	0.4	69.2	127.09	98.49
42	121	Auxotroph	35.2	0.32	110	161.62	156.56
43	122	Auxotroph	17.33	0.34	50.97	79.57	72.54
44	124	Auxotroph	17.9	0.33	54.24	82.19	77.2
45	127	Auxotroph	21.9	0.32	68.43	100.55	97.4
46	128	Morphology	23.51	0.31	75.84	107.94	107.94
47	131	Morphology	25.79	0.32	80.59	118.41	114.7
48	133	Morphology	22.43	0.35	64.09	102.98	91.22

Discussion:

Development and implementation of a mutation and selection strategy for improving production of extracellular xylan-degrading enzymes was considered. Irradiation with UV has been applied to intensify xylanase activity via mutagensis. Results obtained after exposure of spore suspension from *Streptomyces pseudogriseolus* UV irradiation presented in Table (1), indicated that survival rate had decreased by increasing UV exposure time. Survival percentage reached 20% after 30 seconds, and then decreased drastically after 60, 90, 120 and 180 seconds to be 11.67, 2.72, 1.56 and 0.39%, respectively. Decrease in survival rate might be

attributed to damage in nucleic acid and of defects in other cell components caused by UV irradiation. Szczodrak *et al*. (1994) observed that survival rate in *Penicillium notatum* 1 after exposure to UV irradiation from 2 to 4 minutes reached 4.6% and 27.5%, respectively. However, Talkhan (2000) found that survival rate percent in *Aspergillus foetidus* decreased gradually with the gradual increase in exposure time of fungal conidia to UV light. These observations declare that these kinds of fungal cells have great effect on survival rate during exposure to UV irradiation. Mutation percent was also affected by exposure time to UV irradiation. As increased with increasing the exposure time. It was 11.71% after 4 min and reached to 30.48 after 15min.

Some of the obtained mutants that differed morphologically from the original strain in color, shape or growth vigor were chosen (Photo.1) and the others were selected as auxotrophs. Similar results had been obtained when *Penicillium funiculosum* was exposed to UV-irradiation (Hoffman and Wood, 1985). Mutants quite different in the morphology of the mycelia were isolated from *Penicillium pinophillum* by Brown *et al*. (1987). Moreover, other authors had reported that mutants of *Penicillium* sp. isolated after exposure to UV irradiation could be used as a tool for strain improvement (Agrawal *et al.*, 1999). The mutation technique was used to enhance indigenous lipase production from *Rizopus* sp. BTS24 using either UV or NTG (N-methyl-N'-nitro-N- nitroso guanidine) mutagens as shown by Bapiraju *et al.,* (2004), who obtained a mutant with 1645 lipase production compared with the parent strain by UV treatment. Also, Steiner *et al. (*1998) mutated *Penicillium purpurogenum* with UV light to increase xylanase production, a mutant showed an increase in xylanase activity by 181% as compared with the wild type strain. Xylanase from the fungus *Thermomyces lanuginosus* was also increased to 1.5 fold by UV mutation (Kumar *et al.,* 2009). Mutagenesis had also been used for increasing other microbial enzymes e.g., cellulase production by *Penicillium pinophillum*, dextranase production by constitutive mutant of *Lipomyces starkeyi* and amylase production by Aspergillus *foetidus* (Talkhan, 2000).

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