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ORIGINAL ARTICLES

Mutation induction and protoplast fusion of *Streptomyces* spp. for enhanced alkaline protease production

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ABSTRACT

Five isolates of *Streptomyces* sp. were evaluated for alkaline protease productivity. Results showed that *Streptomyces* sp. C_1 is the most effective strain for alkaline protease productivity (giving 223.08 U/ml). So, *Streptomyces* sp. C_1 was selected and mutagenized using UV as a physical mutagen in order to induce genetic variations. Results indicated that, the majority of mutants exhibited alkaline protease productivity higher than the wild type strain and positive correlation between alkaline protease productivity and specific activity of the most mutants. The highest record of the alkaline protease production was 337.97 U/ml which was obtained from the mutant No.12. Protoplast fusion technique was carried out in order to obtain fusants with high alkaline protease production. Results showed that, 18 out of the 22 fusants were higher alkaline protease producers than the higher parent (P1). The highest fusants F2 and F18 produced 111.85 and 102.76 percents more alkaline protease than its higher producer parent, respectively, and at the same time represents 58.17 and 51.38 percents more alkaline protease than its summation of two parents.

Key words: *Streptomyces,* identification, alkaline protease, UV-mutagenesis, protoplast fusion.

Introduction

Proteases are among the most important industrial enzymes, accounting for nearly 60% of the industrial market in the world (Mitra *et al.*, 1996; Layman, 1986). Alkaline proteases are of great interest because of their high proteolytic activity and stability under alkaline conditions (Maurer, 2004; Saeki *et al*., 2007). These enzymes find applications in detergents, feather processes, food processing, silk gumming, pharmaceuticals, bioremediation, biosynthesis and biotransformation (Bhaskar *et al*., 2007; Jellouli *et al*., 2009). Furthermore, fibrous proteins such as horn, feather, nail and hair are abundantly available in nature as waste. This waste can be converted into useful biomass, protein concentrate or amino acids using proteases from certain micro-organisms. The majority of commercial alkaline proteases are produced by bacteria, especially *Bacillus* sp. and *Streptomycse* (Jellouli *et al*., 2009).

Strain improvement plays a key role in the commercial development of microbial fermentation processes. As a rule, the wild strains usually produce limited quantities of the desired enzyme to be useful for commercial application (Glazer and Nikaido, 1995). However, in most cases, by adopting simple selection methods, such as spreading of the culture on specific media, it is possible to pick colonies that show a substantial increase in yield (Aunstrup, 1974). The yield can be further improved by the use of mutagens and the adoption of special techniques or procedures for detecting useful mutants. Shah *et al.*, (1986) developed a cysteine auxotrophic mutant of *B. licheniformis* with improved protease production. Further, increased yields of alkaline proteases have been achieved by *Bacillus* mutants that were resistant to some antibiotics (Ito *et al*., 1991). Moreover, the mutant of different species of *Streptomyces* led to produce highly enzyme activity (Catherine *et al*., 1992; Michael *et al*., 2006 and Bushell *et al*., 2006). Moreover, the genetic manipulation via protoplast fusion protocol is considered as a more recent method to promote recombinations. Furthermore, protoplast fusion led to produce superior enzyme production fusants from *Streptomyces* (Teeradakorn *et al*., 1998 and Sivakumar *et al*., 2004). In the present work we demonstrated that by induction of mutation using ultraviolet rays and protoplast fusion we can continuously construct superior strains for alkaline protease productivity.

Materials and methods

Isolation of actinomycetes:

The sediment sample was collected from the Egyptian agriculture soil, Mansoura governate, by inserting a sterilized polyvinyl corer (10 cm) into the sediments. The centre portion of the 2 cm sediment sample was taken out with the help of a sterile spatula. The collected sample was transferred to a sterile polythene bag and taken

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immediately to the laboratory. After arrival at the laboratory, the sample was air-dried aseptically for one week. An air-dried sediment sample was incubated at 30°C for 5 min (Balagurunathan, 1992). Then, 10-fold serial dilutions of the sediment samples were prepared, using filtered and sterilized 0.85% sterile saline solution (NaCl). Serially diluted samples were plated in the Actinomycetes isolation agar medium in duplicate Petri plates (Starch nitrate agar plates). To minimize bacterial and fungal contamination, all agar plates were supplemented with 20 mg/l of nystatin and cycloheximide (100 mg/l), respectively (Kathiresan *et al.*, 2005). The actinomycete colonies that appeared on the Petri plates were counted from 5th day onwards, up to 21th day. All the colonies that were growing on the Petri plates were separately streaked in Petri plates, subcultured, ensured for their phenotypes and maintained in starch nitrate slants.

Qualitative screening of alkaliphilc isolates for alkaline protease production:

Skim milk agar medium described by (Ventosa *et al*., 1982) was used to detect alkaline protease-producing isolates. This medium had the following constituents (g/l): skim milk, 50.0; yeast extract, 5.0; $Na_2CO_{3,}$ 10.0; Agar, 15-20 and distilled water, 1000ml. Skim milk, Na_2CO_3 and the other constituents were sterilized separately and mixed just before solidification. The medium showed a final pH of 9.5.

Alkaline protease production medium:

The medium composed of (%): casein (5), glucose (10), peptone (5), yeast extract (5) and 50ml salt solution {KH_2PO_4 (0.25); $MgSO_4$ (0.1);K_2HPO_4 (0.25); $FeSO_4$ (0.1)} in 1000 distilled water. The pH was adjusted to be 9.5 and was sterilized in an autoclave for 15 min at 121^{o}C. After cooling the medium was inoculated with a loopfull of *Streptomyces* strains and then incubated at 30^{o}C in an orbital shaker set at 150 rpm for 4 days. At the end of fermentation period, the culture was centrifuged and the supernatant was assayed for protease activity according the method of Kathiresan and Manivannan (2007).

Alkaline protease assay and protein measurement:

The activity of alkaline protease in the cell-free supernatant was measured by modified method of Takami *et al.*, (1989) using casein as a substrate at a concentration of 1% w/v in 50 mM Glycine-NaOH buffer (pH 10). The assay was carried out routinely in a mixture containing 0.5 ml of a suitably diluted enzyme solution and 2.5 ml casein solution. After incubation for 1 hour at 50^{o}C, the reaction was terminated by the addition of 2.5ml of 0.44 M TCA (trichloroacetic acid) solution. After 10min the mixture was centrifuged at 8000 rpm for 10min. An aliquot of 0.5 ml of supernatant was mixed with 2.5ml of 0.5M Na_2CO_3 and 0.5ml of Folin-Ciocalteu's phenol solution and kept for 30 min at room temperature. The developed color of the solutions were determined with respect to sample blanks at 660 nm. Protein was tested according to Lowry *et al.* (1951).

Identification of the genus Streptomyces:

The identification was carried out according to Lechevalier and Lechevalier (1970) by cultivating the tested microorganisms on tryptone yeast extract broth at 30^{o}C for 5-10 days. Cells were then harvested by filtration, washed with water and ethyl alcohol and then dried in an open air at room temperature. Cells were subjected to hydrolysis and tested for both diaminopimelic (DAP) acid and whole cell sugar pattern (Hasegawa *et al*, 1983).

UV-mutagenesis:

Streptomyces spores from old slants (5 days) were suspended in sterile distilled water and exposed to UV-light (Philips T – UV – 30 W Lamp type number 57413 p /40) for 0 , 3 , 6 and 9 minutes at a distance of 20 cm. After irradiation, the treated spore suspensions were protected from light for two hours. Appropriate dilutions were spread on starch- nitrate medium and incubated at 28 ºC for five days. The growing colonies were transplanted on slants for the forward assay of alkaline protease productivities.

Protoplast preparation, fusion and regeneration:

Different superior isolates and original strains were tested against six antibacterial agents and their responses were recorded and used as markers for fusants detection after protoplast fusion. The cells were grown in 50 ml of screening medium containing 0.5% glycine in a 100-ml Erlenmeyer flask at 30°C for 24 h. Then the cells were harvested by centrifugation at 8000 rpm for 10 min and transferred to a protoplasting medium(PM) consisting of (g/liter):sucrose, 300.0; $MgCI_2.6H_20$, 2.0; $CaCI_2.2H_2O$, 3.5; 0.25 M TES- HCl(buffer, pH 7.2), containing 10 mg /ml of lysozyme (grade 1, Sigma Chemical Co.). After observation of protoplast formation

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with a phase contrast microscope, protoplasts were fused by suspension in 10 ml of PM containing 30% PEG 4000, 15% dimethyl sulfoxide (DMSO), and 10 mM $CaCl_2$. After gentle shaking for 30 min at 0°C, the suspension was diluted 10-fold with PM buffer. Protoplasts were harvested by centrifugation at 2500 rpm for 5 min at 4°C and then resuspended in 10 ml of regeneration medium. The protoplast suspension was diluted and plated and the plates were incubated for 5 to 8 days at 30°C.The growing recombinant colonies (fusants) were transplanted on slants for the forward assay of alkaline protease productivities.

Results and discussion

Protease was considered as industrially important enzyme with a wide range of applications in pharmaceutical, leather, laundry, food, waste processing as well as in textile industry. Alkaline protease was the most preferable type of proteases as it is used as detergent additive. In our search for enhancement of alkaline protease, five *Streptomyces* strains isolated from soil were selected as they were able to produce alkaline protease on skim milk agar plate screening medium as shown in Figure(1). Several investigators used different screening plate agar media in their search for alkaline proteases (Aftab *et al.,* 2006; Kasana and Yadav, 2007; Rao and Narasu, 2007). Isolates that showed positive results were screened quantitatively using broth medium containing casein, peptone and yeast extract as nitrogen source and the enzyme was assayed at different pHs. Isolate C1 overproduce alkaline protease than the others at pH 9 as shown in Table(1).

Identification of selected isolates:

The whole cell hydrolysates of the tested isolates were examined to determine the isomers of diaminopimelic acid (DAP) and sugar pattern of isolates following the methods of Becker *et al.* (1965) and Lechevalier and Lechevalier (1970). The chromatographic analysis of the cell wall hydrolysates revealed that the all tested isolates contain LL-DAP and no characteristic sugars and were, therefore, classified belong to the genus *Streptomyces.*

Alkaline protease productivity after UV-treatments:

The physical mutagen ultraviolet light (UV) was used for the induction of genetic variabilities. Spores suspension of *Streptomyces* sp $C_1$was exposed to UV light as mentioned previously in the adopted methods. Results presented in Table (2) clearly showed that, out of selected 24 mutants which was exposed to UV-light for different time periods, only 7 mutants produced alkaline protease lower than their wild type strain. Meanwhile, four mutants, i.e., 9, 27, 43 and 48 proved to have a little bit more efficiency alkaline protease productivity than the original strain. On the other hand, 13 mutants of the tested 24 ones produced alkaline protease higher than their original parental strain. The highest record of the alkaline protease production was 337.97 U/ml which was obtained from the mutant No.12. The following superior mutant for alkaline protease

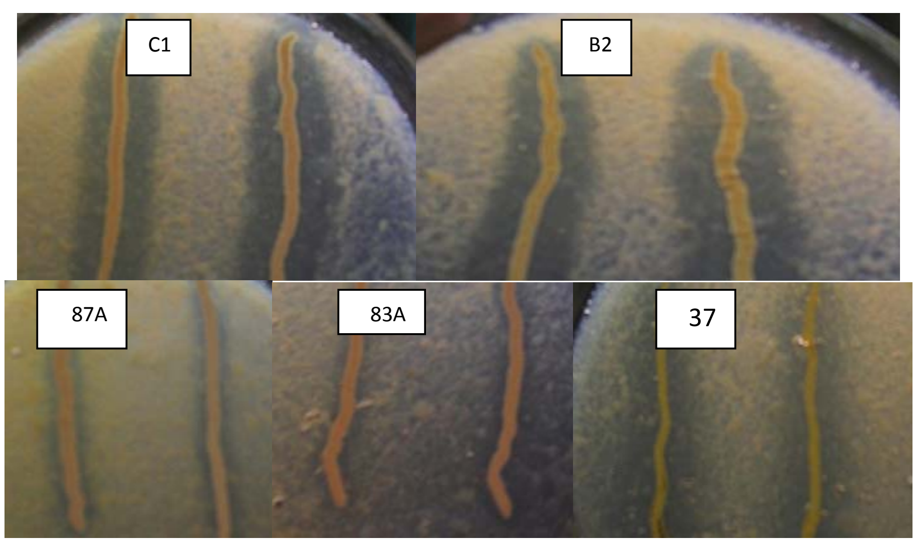

Fig. 1: Photographs of wild types strains of *Streptomyces* produced alkaline protease as a clear area around the growth area on the skim milk agar medium.

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Table 1: Alkaline protease activity from positive *Streptomyces* isolates at different incubation pHs.

Streptomyces isolate no.	Total protein (mg/ml)	pH 8		pH 9		pH10	
		Activity (U/ml)	Specific activity U/mg protein	Activity (U/ml)	Specific activity U/mg protein	Activity (U/ml)	Specific activity U/mg protein
37	3.99	77.63	19.46	139.92	35.07	132.92	33.31
83A	4.97	173.83	34.98	192.92	38.82	192.04	38.64
87A	4.31	113.42	26.32	114.71	26.61	109.67	25.45
B2	4.05	192.92	47.63	216.08	53.35	192.17	47.45
C1	4.04	191.42	47.38	223.08	55.22	194.50	48.14

production was No.44 which produced 303.25 U/ml. Moreover, data in Table (2) showed that positive correlation between alkaline protease productivity and specific activity of the most mutants. Out of the previous obtained results of *Streptomyces* sp C_1exposed to different UV-exposure times, it could be clearly concluded that the highest promising mutants for alkaline protease production counted as the follows: i) One mutant as a result of 12 min. exposure time, i.e., mutant No.12 which produced 51.50 percent higher than the original strain. ii) One mutant as a result of 6 min. exposure time, i.e., mutant No.44 which produced 35.94 percent higher than the original strain.

Protoplast fusion and alkaline protease productivity:

Protoplast fusion was the main method for exchanging genetic material between two cell types and subsequently obtaining the new gene recombinants towards the isolation of higher alkaline protease producing fusant(s). In order to investigate the effect of protoplast fusion on alkaline protease production, three original strains and three UV- treated mutants were selected to determine their antibiotic resistance or sensitivity as an additional selective marker for fusant(s) detection and isolation. Table (3) presents the selected strains and mutants response to six different types of antibiotics. Results showed that, the original strain and all UV-mutants were resistant to Ap and Lm. Meanwhile, they were sensitive to Nb, Km, Tc and Em. On the other hand, the other wild type strains showed different responses. The strain B2 was resistant to Ap, Nb and Lm while, it was sensitive to the others. Also, the strain 83A was resistant to Ap but it was sensitive to the others. Finally, the strain 87A was resistant to Nb but it was sensitive to the others. From these antibiotic responses differences, we can fuse different strains in the protoplast fusion experiment. To study the effect of protoplast fusion on alkaline protease productivity, cross between mutant No.12 and wild strain No. 87A which varied in both their productivity and antibiotic responses was done. Table (4) presents the alkaline protease productivity of the parents and the protoplast fusants which were obtained following the protoplast fusion cross and detection on the regeneration medium containing 50µg/ml of Lincomycin and 20 µg/ml of Novobiocin.

Table 2: Alkaline protease production using UV- mutants obtained from *Streptomyces* strain No. C_1.

Mutant No.	UV-exposure time(min.)	Productivity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Productivity (%) to W.T.
Wild type	0	223.08	4.04	55.22	100.00
1	12	208.98	3.931	53.16	93.68
9	12	235.51	3.989	59.04	105.57
10	12	291.57	4.242	68.73	130.70
11	12	193.57	3.745	51.69	86.77
12	12	337.97	4.159	81.26	151.50
18	9	164.43	4.074	40.36	73.71
20	9	165.59	4.049	40.90	74.29
23	6	273.18	3.935	69.42	122.46
27	6	227.34	3.938	57.73	101.91
28	9	193.57	3.868	50.04	86.77
29	9	248.31	3.773	65.81	111.31
30	6	208.60	4.031	51.75	93.51
31	6	259.64	4.125	62.94	116.39
36	9	267.99	4.074	65.78	120.13
40	9	266.31	4.144	64.26	119.38
42	9	248.31	4.171	59.53	111.31
43	9	236.26	3.893	60.69	105.91
44	6	303.25	3.794	79.93	135.94
45	6	283.40	3.637	77.92	127.04
46	9	185.58	4.019	46.18	83.19
47	9	290.83	4.016	72.42	130.37
48	6	238.85	4.110	58.11	107.07
49	12	263.90	4.023	65.60	118.30
50	12	272.98	4.097	66.58	122.21

Table 3: Response of the superior mutants for alkaline protease production against five antibacterial agents.

Wild types* &Mutant strains	Antibacterial agents					
	Ap	Nb	Km	Tc	Lm	Em
Original strain*	+	-	-	-	+	-
12	+	-	-	-	+	-
44	+	-	-	-	+	-
45	+	-	-	-	+	-
B_2*	+	+	-	-	+	-
83A*	+	-	-	-	-	-
87A*	-	+	-	-	-	-

Ap(Ampicillin): 125µg/ ml; Km(Kanamycin): 50µg/ml; Nb(Novobiocin): 20 µg/ml; Em(Erythromycin):100 µg/ml; Lm(Lincomycin): 50µg/ml; Tc(tetracycline): 50µg/ml.

Results in this table showed that, 18 out of the 22 fusants were higher alkaline protease producers than the higher parent (P_1). For instance, the highest fusants F2 and F18 produced 111.85 and 102.76 percents more alkaline protease than its higher producer parent, respectively, and at the same time represents 58.17 and 51.38 percents more alkaline protease than its summation of two parents. Meanwhile, only four fusants, i.e., F2.4, F2.7, F3 and F17 showed less alkaline protease production than the higher parent (P1) but these fusants exhibited higher alkaline protease production than the lower parent (P2). Moreover, 13 fusants of the tested 22 ones produced alkaline protease higher than its summation of two parents, while, 9 fusants of the tested 22 ones produced alkaline protease lower than its summation of two parents.

Table 4: Alkaline protease production using fusants obtained after protoplast fusion crosses.

Parents &Fusant No.	Protein (mg/ml)	Productivity (U/ml)	Specific activity (U/mg protein)	Productivity (%) to parent(P_1)	Productivity (%) to (P_1+ P_2)
P_1(12)	4.13	337.97	81.83	100.00	74.66
P_2(87A)	4.00	114.71	28.68	33.94	25.34
F1	4.56	674.84	147.99	199.67	149.08
F2	4.74	715.99	151.05	211.85	158.17
F2.1	4.66	370.42	97.49	109.60	81.83
F2.2	5.47	679.23	124.17	200.97	150.05
F2.3	4.85	468.00	96.49	138.47	103.38
F2.4	5.28	327.00	61.93	96.75	72.24
F2.5	5.68	368.15	64.77	108.93	81.33
F2.6	5.29	599.68	113.36	177.44	132.47
F2.7	5.01	293.52	58.59	86.86	64.84
F3	4.76	299.02	62.82	88.47	66.06
F4	4.49	546.46	121.71	161.69	120.72
F8	4.03	586.51	145.54	173.54	129.56
F3.8	4.13	393.38	95.25	116.40	86.90
F3.9	5.13	544.26	106.09	161.04	120.23
F3.10	5.89	486.65	82.45	143.99	107.50
F3.11	4.30	496.53	115.47	146.92	109.69
F3.12	4.53	603.52	133.23	178.57	133.32
F3.13	4.43	402.71	90.91	119.16	89.62
F3.15	3.74	542.62	145.08	160.55	119.87
F16	4.61	377.47	81.88	111.69	83.39
F17	3.30	182.15	55.20	53.90	40.24
F18	5.27	685.27	130.04	202.76	151.38

Discussion:

The importance of the isolation of new strains of *Streptomyces* from the nature become a global programs to produce new type of alkaline protease with high activity. In our search for alkaline protease five *Streptomyces* strains isolated from soil were selected as they were able to produce alkaline protease, on skim milk agar plate screening medium. Isolates that showed positive results were screened at different pHs to detect the best isolate for enzyme production.

Before the advent of protoplast fusion technology, random mutagenesis with UV-rays and screening was the main technique applied for strain improvement. Mutation is the ultimate source of all genetic variation. The efficiency of induced mutation depends on the type of damage (base pair substitution, insertion, deletion etc.) a given mutagen causes on DNA and the cellular mechanism(s) involved in the repair of that damage. Using UV-light as a mutation-inducer has been recommended as the method of choice for increasing the enzyme productivity from *Streptomyces* (Catherine *et al.*, 1992; Michael *et al*., 2006). For many reasons, it appeared that UV-induced mutants were more stable through long term of generation and subculturing (Thoma, 1971). In addition, UV-light also induced tolerance to different environmental stresses and changes in protein synthesis (Hartke *et al.*, 1995). Furthermore, UV-light is safety usage and causing no pollution. The results obtained after UV-mutagenesis were in agreement with those obtained by Bushell *et al.*, (2006) who obtained a *zwf* mutant which has superior glucose-6-phosphate dehydrogenase activity properties than *Streptomyces coelicolor* original strain. Moreover, Bapiraju (2004) isolated mutant from the parent after its mutagenesis by UV and NTG mutagens. They found that, UV and NTG were effective mutagenic agents for strain improvement of *Rhizopus* sp. BTS-24 for enhanced lipase productivity. Caob and Zhanga (2000) reported an increase in lipase using a *Pseudomonas* mutant of UV and NTG. Also, a 200% increase in lipase yield by *Aspergillus niger* mutant of UV and NTG was reported by Ellaiah *et al*. (2002).

To study the effect of protoplast fusion between different genomes on alkaline protease productivity, cross between mutant No.12 and wild strain No. 87A which varied in both their productivity and antibiotic responses was done. Results showed that, 18 out of the 22 fusants were higher alkaline protease producers than the higher parent (P_1). For instance, the highest fusants F2 and F18 produced 111.85 and 102.76 percents more alkaline protease than its higher producer parent, respectively, and at the same time represents 58.17 and 51.38 percents more alkaline protease than its summation of two parents. These results were in agreement with those obtained by Teeradakorn *et al*. (1998) who isolated new fusants, which appeared to involve a rearrangement in genetic material and produced high levels of xylose isomerase when grown on hemicellulosic materials such as xylan as the carbon source, after protoplast fusion between *Streptomyces cyaneus* 190-1 and *Streptomyces griseoruber* 42-9. Moreover, Kanatani *et al*., (1990) isolated fusants which exhibited recombination between a range of chromosomal marker after intraspecific protoplast fusion between isogenic *Lactobacillus plantarum* strains. Furthermore, Chassy (1987) and Ward *et al*., (1993) reported that protoplast fusion could be used for the genetic manipulation and improvement of *Lactobacilli*. In addition, Ward *et al.* (1993) studied the molecular analysis of the obtained fusants after protoplast fusion between *Lactococcus lactis* sub sp. *cremoris* strains and showed that, out of the four protoplast fusion examined, three appeared to involve a rearrangement in genetic material while in the fourth, the fusants appeared similar to one of the parental strains. Moreover, Lin *et al*., (2007) after two rounds of genome shuffling, isolated several fusants. The lipase activity of these fusant strains was increased 317% over the starting strain. Intra-specific fusants increased extracellular glucose oxidase activity in *Aspergillus niger* (Khattab and Bazaraa, 2005). *Bacillus* strain was engineered for improvement of fibrinolytic enzyme yield through the rounds of protoplast fusion (genome shuffling) (Liang and Guo, 2007). The enzyme production of shuffled strain (fusant) was increased by 4–5 fold compared with the starting mutants.

Conclusion:

The isolation of new S*treptomyces* strains is an important way and good starting point to obtain the new genotypes which produce alkaline protease with highly activity. And also, genetic improvement protocols such as mutagenesis and protoplast fusion are an elementary part of process development, generally aiming at reduction of production costs. The results obtained confirmed that UV-mutagenesis technique is an important tool in *Streptomyces* improvement for increasing production of alkaline protease enzyme. Furthermore, protoplast fusion is used to combine genes from different isolates for creating strains with high activity and productivity of alkaline protease.

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