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Strain Improvement of *Brevibacillus borostelensis* R1 for Optimization of α-Amylase Production by Mutagens

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Abstract

Physical and chemical mutagens are promising and are used for screening of high yielding strains. The exponential increase in the application of amylases in various fields has placed stress and demand in both qualitative improvement and quantitative enhancement through strain improvement. Ultraviolet light exerts its mutagenic effect by exciting electrons in molecules. The potent UV mutants which showed more than 20 mm zone of starch hydrolysis were screened and selected at 42% of survival time at 80minutes of exposure. The wild strain with fixed parameters yielded (3000 U/ml). The major findings of the strain improvement were out of ten mutants isolated, two (UV-3 and UV-10) showed 3000-4000 U/ml of amylase activity. The % of survival of *Brevibacillus borstelensis* R1 in Pikovskaya's medium was 25.75% at 120 minutes of exposure. Ten mutants (HNO_2-10, HNO_2-30, EMS-4, EtBr-40, EtBr-50, Acr-1, Acr-20, Acr-30, Acr-4 and 5′-FU-50) out of fifty mutants isolated showed 3000-4300U/ml of amylase activity, which was higher than the wild strain. The potent *Bacillus* species screened from marine water was *Brevibacillus borstelensis* R1. The α-amylase was found to be useful in bakery, food, fodder for poultry, automation dishwashing and laundry industries.

Keywords: *Brevibacillus borostelensis* R1; Ultra violet (UV) rays; Nitrous acid (HNO_2); Ethyl methane sulfonate (EMS); Ethidium bromide (EtBr); Acrylamide (Acr); 5′-fluoro uracil (5′-FU)

Introduction

Strain improvement

Physical and chemical mutagens are promising and are used for screening of high yielding strains [1]. Microorganisms usually produce commercially important metabolites in very low concentrations by their inherent control system. Although the yield may be increased by optimizing the cultural conditions, ultimately the productivity is controlled by the organism's genome [2]. In the last few decades, the exponential increase in the application of amylases in various fields has placed stress and demand in both qualitative improvement and quantitative enhancement through strain improvement. Such improved strains reduce the cost of the process. The challenge is to isolate those strains which are true mutants that carry beneficial mutations [3]. *Bacillus* spp. such as *B. subtilis* and *B. amyloliquefaciens* are the most commonly used organisms of choice for amylase production [4,5]. Highly active α-amylase is preferred for the conversion of starch into oligosaccharides. So, it is worthwhile to select a potent microbial strain for α-amylase production.

Physical mutagen

Ultraviolet (UV) light exerts its mutagenic effect by exciting electrons in molecules. The excitation of electrons in DNA molecules often results in the formation of extra bonds between adjacent pyrimidines in DNA. The UV light is the best studied mutagenic agent in prokaryotic organisms [6]. The UV mutant strains of *Bacillus* reported to show better ability to produce alpha amylase, which can be derived by mutagenesis and extensive screening [7-10].

Chemical mutagens are stronger mutagenic agents and cause permanent changes in DNA sequence. They bring about transitions from G: C→ A: T [11] and have preferential effect on DNA replication [12-16].

Nitrous acid (HNO_2, Hydroxidooxidonitrogen, O=NO) causes interstrand cross-linking of DNA. Nitrous acid acts as a mutagen by deamination of the NH_2 group of adenine and cytosine to an ether group, thus altering their base pairing. It causes oxidative deamination of particular bases. Conversion of the amino groups to keto groups changes the hydrogen bonding potential of the bases. Nitrous acid was reported to be a suitable mutagen for the improvement of α-amylase production in *Bacillus* spp. [17-20].

Ethyl Methane Sulfonate (EMS) is an alkylating agent which carries one, two or more alkyl groups in reactive form. The transfer of methyl or ethyl groups to the bases such that their base-pairing potentials are altered and transitions result. Thus it induces all types of mutations (transitions, transversions, frame shifts and even chromosome aberrations) with various relative frequencies. Ethyl methane sulfonate was reported as potent mutagen in amylase production in *Bacillus* spp. [20-22].

Acrylamide (acrylic amide) decomposes non-thermally to form ammonia and thermal decomposition produces carbon monoxide, carbon dioxide and oxides of nitrogen. It is effective in causing bacterial mutation. It shows direct and indirect modes of interaction with DNA results in alkylating DNA adducts [23].

Base analog mutagen (5′-fluoro uracil) is a thymine analog. It

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has a higher likelihood for tautomerization to the enol form (FU*). Methyl group in 5th position of thymine and fluorine at 5'-position in 5′-FU have similar effects. After tautomeric shift to its enol form, 5'-fluorouracil pairs with guanine and thus causes A. T to G. C transitions. Base analogs cause transitions in the bidirectional mode.

Ethidium bromide (EtBr, Intercalating agent) acts as a mutagen because it intercalates double stranded DNA and deforms the DNA [24]. Ethidium bromide is a large, flat basic molecule that resembles a DNA base pair. This insertion causes "stretching" of the DNA duplex and the DNA polymerase is "fooled" into inserting an extra base opposite an intercalated molecule. This results in frame shift mutations. This affects DNA biological processes, like DNA replication and transcription.

Materials and Methods

Primary screening of α-amylase producing bacteria

The collected marine water samples were diluted by serial dilution technique. The diluted samples of 10^{-4} to 10^{-6} (0.1 ml) were spread with L-shaped glass rod by spread plate technique on the starch agar plates. After incubation at 37°C for 24 hours, the plates were flooded with Lugol solution (1% iodine in 2% potassium iodide w/v) [25].

Estimation of amylase by DNS method

Maltose produced by the hydrolytic activity of α-amylase on α-1, 4 linkages present in polysaccharides, reduce 3, 5 dinitro salicylate to an orange red colored 5-nitro 3-amino salicylate which can be measured at 520 nm. The starch substrate [0.5 ml of 0.5% in 0.1 M phosphate buffer (pH 6.8)] was mixed with 1% (0.2 ml) NaCl in a test tube and pre incubated at 37°C for 10 minutes. The supernatant collected from the centrifugation of the production media was used as enzyme source, 0.5 ml of this was added to the reaction mixture. The reaction was terminated by the addition of 1.0 ml of 3, 5-dinitrosalicylic acid reagent [1.0 gm DNS in 0.8% NaOH, 60% Na K tartrate] after incubation at 37°C for 15 minutes. The contents were mixed well and kept in boiling water bath for 10 minutes. Then they were cooled and diluted with 10 ml of distilled H_2O. The color developed was read at 520 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1.0 mmol of reducing sugar (maltose) per minute under the assay conditions [26].

UV survival curve of *Brevibacillus borstelensis* R1

Inoculate the *Brevibacillus borstelensis* R1 in nutrient broth and incubate in rotary shaking incubator at 120 rpm at 37°C for 24 hrs. Hundred ml of the culture was taken in a petridish and exposed to UV irradiation by placing at a distance of 30 cm from the UV radiation source (emitting the energy of 1.6×10^2 J/m^2//s). After every 10 minutes of exposure 5.0 ml of the sample was drawn. One ml of the sample was serially diluted, 10^{-4}-10^{-7} dilutions were used for the viable count method. The control was the culture sample without UV treatment. A graph was plotted with time in minutes on the X-axis and log cfu per ml on Y-axis in order to obtain survival curve.

Ultraviolet treatment to isolate mutants

Twenty four hours old bacterial culture prepared in nutrient broth was aseptically centrifuged at 5000 rpm for 15 minutes. The bacterial sediment was made suspension with sterile saline water. Ten ml of diluted suspension was transferred to sterilized petriplates placed under the UV lamp (emitting the energy of 1.6×10^2 J/m^2//s) for 80 minutes (42% survival time) to 120 minutes. The bacterial suspension (0.5 ml) was collected after every 10 minutes interval and transferred to solidified starch agar petriplates (180×20 mm BOROSIL) by spread plate technique. The plates were invertedly incubated in an incubator at 37°C for 24 hrs.

Chemical treatment

Five ml of chemical mutagens [Nitrous acid (National), Ethyl methane sulfonate (HIMEIDA), Ethidium bromide (MERCK), Acrylamide (HIMEDIA) and 5′-fluoro uracil (ONCO FLUOR)] of 50-250 µg/ml were transferred to sterilized centrifuge tube separately containing 5.0 ml of bacterial suspension. The tubes were placed at room temperature for 120 minutes. Then the tubes were centrifuged at 5000 rpm for 15 minutes. The supernatant was discarded to remove the chemical mutagen from the bacterial cells. Ten ml of sterile saline water was added to each centrifuge tube. The tubes were re-centrifuged thrice for the removal of traces of chemical mutagen from bacterial cell sediment. After washing the cells, 10 ml of sterilized saline water was added to each tube to form bacterial suspension. The suspension (0.5 ml) was transferred to the petriplates (180×20 mm) containing solidified starch agar by spread plate method and incubated at 37°C for 24 hrs.

From both physical and chemical mutagen treatment the mutant colonies showing more than 20 mm zone of starch hydrolysis in starch agar medium were isolated in pure culture (streak plate technique) on nutrient agar plates. The mutants were single line streaked over starch agar plates to confirm the size of the zone of starch hydrolysis. The confirmed mutants were subjected to submerged fermentation (SmF) at 120 rpm in rotary shaking incubator in Pikovskaya's fermentation medium (100 ml) in Erlenmeyer flasks with predetermined physical and chemical parameters. The α-amylase activity was assayed by DNS method.

Results and Discussion

Isolation of mutant strains by using physical and chemical mutagens

The optimum production of α-amylase by *Brevibacillus borstelensis* R1 was observed in Pikovskay's medium at temperature-37°C, pH-7.0, salinity-1%, with 5% jaggary, 2% beef extract and 0.8% calcium chloride [27]. The activity of α-amylase produced by wild strain was found to be 3000 U/ml with zone of starch hydrolysis (Figure 1). The above optimized conditions were used for all physical and chemical mutagen experiments for strain improvement studies in our laboratory experiments.

Physical mutagen

The potent physical mutants which showed more than 20 mm zone of starch hydrolysis were screened and selected at different minutes of exposure of UV irradiation. Control is the culture unexposed to UV radiations. The potent UV mutants which showed more than 20 mm zone of starch hydrolysiswere screened and selected at 42% of survival time at 80 minutes of exposure (Figure 2). The wild strain with fixed parameters yielded (3000 U/ml). Out of ten mutants isolated, two UV-3 and UV-10 showed 1.32 and 1.29 fold increase in of amylase activity, respectively (Table 1). The % of survival of *Brevibacillus borstelensis* R1 in Pikovskaya's medium was 25.75% after 120 minutes of exposure.

Jin et al. [28] have developed a hyper producing α-amylase mutant

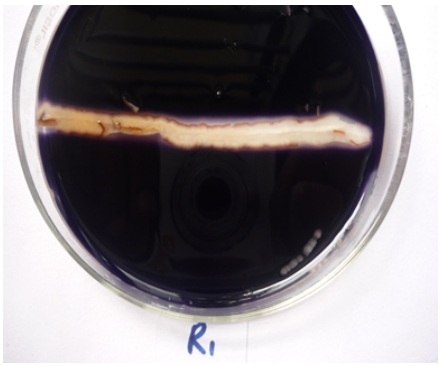
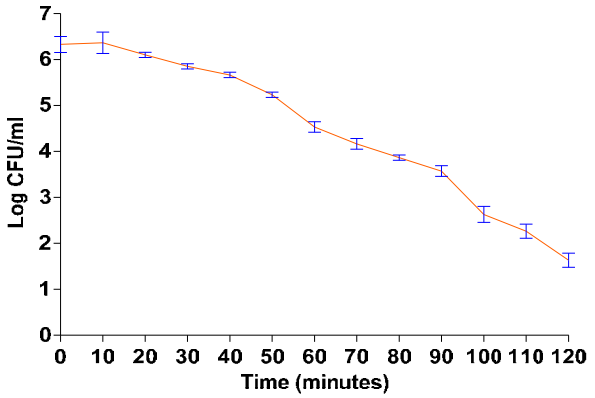


Figure 1: Zone of starch hydrolysis *Brevibacillus borstelensis* R1.



Y bars indicate the standard deviation of mean value
****P< 0.0001Values differ significantly at p<0.05 with high degree of negative correlation

Figure 2: Ultraviolet survival curve of *Brevibacillus borstelensis* R1.

S.no.	Time of exposure in minutes	Isolate code	Zone of starch hydrolysis (mm)	Alpha-amylase activity (U/ml) in Pikovskay's medium
1	80	UV-1	21 ± 1	903 ± 6
2		UV-2	24 ± 4	2633 ± 58
3	90	UV-3	43 ± 6	3967 ± 58
4		UV-4	21 ± 4	1830 ± 52
5	100	UV-5	27 ± 4	2433 ± 58
6		UV-6	22 ± 2	1417 ± 29
7	110	UV-7	20 ± 2	1633 ± 58
8		UV-8	27 ± 4	2533 ± 58
9	120	UV-9	18 ± 4	823 ± 4
10		UV-10	33 ± 5	3867 ± 58

Values presented in the table are means of triplicates ± SD

Table 1: Ultra violet treated mutant isolates showing α-amylase potency in starch media and Pikovskaya's Medium.

of *Bacillus licheneniformis* which yielded 50 times higher enzyme activity than the parental strain. The α-amylase production was decreased in 48 mutant strains out of 60mutant strains isolated when compared to parent strain/wild strain. Similarly decrease in production of α-amylase was reported by Haq et al. [29] in mutant strain of *Bacillus amyloliquefaciens* UNG-16 when compared to the wild parental strain. The effect of UV irradiation on *Bacillus* spp. was most effective in the production of α-amylase [30-33].

Chemical mutagens

The wild strain with fixed physical parameters and chemical parameters yielded 2100 U/ml and 3000 U/ml enzyme activity

S.no.	Conc. of chemical mutagen (µg/ml)	Isolate code	Zone of starch hydrolysis (mm)	Alpha-amylase activity (U/ml) in Pikovskaya's medium
1	50	HNO_2-1	21 ± 2	910 ± 17
2		HNO_2-10	35 ± 4	3967 ± 58
3	100	HNO_2-2	29 ± 3	2433 ± 58
4		HNO_2-20	25 ± 4	2833 ± 58
5	150	HNO_2-3	22 ± 4	1213 ± 23
6		HNO_2-30	33 ± 6	3833 ± 116
7	200	HNO_2-4	33 ± 5	2617 ± 29
8		HNO_2-40	24 ± 4	2033 ± 58
9	250	HNO_2-5	21 ± 3	2233 ± 58
10		HNO_2-50	25 ± 4	2017 ± 29
11	50	EMS-1	21 ± 2	2027 ± 46
12		EMS-10	33 ± 5	2367 ± 58
13	100	EMS-2	25 ± 4	2230 ± 52
14		EMS-20	21 ± 2	1567 ± 58
15	150	EMS-3	23 ± 5	1310 ± 17
16		EMS-30	28 ± 4	2825 ± 43
17	200	EMS-4	36 ± 2	4167 ± 58
18		EMS-40	24 ± 3	1313 ± 23
19	250	EMS-5	21 ± 5	1827 ± 46
20		EMS-50	27 ± 3	2167 ± 58
21	50	EtBr -1	26 ± 3	2233 ± 58
22		EtBr -10	21 ± 4	1023 ± 25
23	100	EtBr -2	28 ± 2	2367 ± 58
24		EtBr -20	27 ± 4	2433 ± 58
25	150	EtBr -3	29 ± 2	2767 ± 58
26		EtBr -30	21 ± 5	813 ± 23
27	200	EtBr -4	23 ± 2	1133 ± 58
28		EtBr -40	37 ± 3	4166 ± 58
29	250	EtBr -5	21 ± 5	1833 ± 58
30		EtBr -50	33 ± 5	4017 ± 76
31	50	Acr-1	33 ± 5	3950 ± 87
32		Acr-10	26 ± 2	2433 ± 58
33	100	Acr-2	25 ± 5	2833 ± 58
34		Acr-20	33 ± 5	4042 ± 72
35	150	Acr-3	22 ± 5	1333 ± 58
36		Acr-30	35 ± 5	3883 ± 29
37	200	Acr-4	33 ± 6	4237 ± 64
38		Acr-40	29 ± 2	2833 ± 58
39	250	Acr-5	39 ± 6	2833 ± 58
40		Acr-50	22 ± 4	1140 ± 53
41	50	5′-FU-1	30 ± 4	2783 ± 29
42		5′-FU-10	20 ± 5	1830 ± 52
43	100	5′-FU-2	21 ± 5	813 ± 23
44		5′-FU-20	27 ± 4	2577 ± 40
45	150	5′-FU-3	29 ± 2	2827 ± 46
46		5′-FU-30	30 ± 5	2810 ± 17
47	200	5′-FU-4	21 ± 5	717 ± 29
48		5′-FU-40	32 ± 5	2817 ± 58
49	250	5′-FU-5	20 ± 1	1227 ± 46
50		5′-FU-50	33 ± 5	3650 ± 50

Values presented in the table are means of triplicates ± SD

Table 2: Chemical mutant strains [Nitrous acid (HNO2), Ethyl methane sulfonate (EMS), Ethidium bromide (EtBr), Acrylamide (Acr) and 5′-fluoro uracil (5′-FU)] showing α-amylase potency in starch media and Pikovskaya's medium.

respectively. Out of fifty mutants isolated ten mutants HNO_2-10, HNO_2-30, EMS-4, EtBr-40, EtBr-50, Acr-1, Acr-20, Acr-30, Acr-4 and 5′-FU-50 showed 1.32, 1.27, 1.38, 1.38, 1.34, 1.32, 1.35, 1.29, 1.41 and 1.22 fold increase in amylase activity, respectively which was higher than the wild strain (Table 2).

Haq et al. [12] reported that nitrous acid was found to be the suitable mutagen for improvement of α-amylase production in *Bacillus* spp. Similarly, maximum production of α-amylase (285 U/ml) was reported in *Aspergillus oryzae* NA17 and also in *Aspergillus oryzae* by Szafraniec et al. [19] and Haq et al. [30].

Haq et al. [29] screened mutant strains of *Bacillus amyloliquefaciens* EMS-6 which gave 102.78 ± 2.22 U/ml/min α-amylase activities. The activity was around 1.4 fold higher than the parental strain *Bacillus amyloliquefaciens* UNG-16. Similarly, Szafraniec et al. [19] and Haq et al. [30] worked with *Aspergillus oryzae* (EMS-18) isolate which gave maximum production of α-amylase 347 U/ml.

Haq et al. [12] reported the mutagenesis for improvement of α-amylase production of bacterial strains with EMS. Similarly Sarikaya and Gürgün [21] worked on EMS mutants of *Bacillus subtilis*. Ikram-ul-haq et al. [22] reported on mutants of *Bacillus licheniformis* GCB-30UCM strain. Sarikaya and Gürgün [21] reported about the mutants of *Bacillus subtilis* obtained by ethidium bromide which increased amylase production.

Conclusions

Strain improvement of *B. borestelensis* R1 was carried out by using physical and chemical mutagens to develop a better yielding strain to improve the production of α-amylase. Various mutagenic agents such as ultraviolet rays (UV), nitrous acid (HNO_2), ethyl methyl sulfonate (EMS), ethidium bromide (EtBr), acrylamide (Acr), 5′-fluoro uracil (5′-FU) were used for strain improvement studies. Out of ten mutant strains isolated, two strains (UV-3 and UV-10) showed 3000-4000 U/ml of α-amylase activity. Out of fifty chemical mutant strains isolated, ten mutant strains (HNO2-10, HNO2-30, EMS-4, EtBr-40, EtBr-50, Acr-1, Acr-20, Acr-30, Acr-4 and 5′-FU-50) showed 3000-4300 U/ml of α-amylase activity which was higher than the wild parent strain. Chemical mutagens are more effective in strain improvement than physical mutagen.

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