International Journal of Microbiological Research 5 (2): 130-139, 2014
ISSN 2079-2093

DOI: 10.5829/idosi.ijmr.2014.5.2.84114

Enhancement of Alkaline Protease Production by *Bacillus* Species Through Random Mutagenesis

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Abstract: *Bacillus species* is a potent strain which produced the alkaline protease and it is further mutated to yield more amount of enzyme. An alkaline protease producing strain of *Bacillus* species was isolated from soil. The isolates were purified and screened for the production of protease enzyme. The conditions for enzyme production were optimized under solid substrate fermentation (SSF) and submerged fermentation (SF). Maximum enzyme production occurred at 50°C, pH 8. 0 at 72 hours of incubation under SF using Reese medium. Among various combinations of natural substrates used for enzyme production by SSF, mixture of paddy husk, ground nut shell 3:2 ratio served as a suitable and best for alkaline protease production. It was thermo stable and retained full activity after 1hour of incubation at 50°C. Enzyme was purified by gel filtration, Iron exchange chromatography and molecular weight of 31 KDa was confirmed by SDS-PAGE. The enzyme was retained with a maximum and minimum activity of 84. 9% at 10 minutes and 1% at 40 minutes and completely lost at 50 minutes in the presence of detergents such as Surf, RIN, Ariel, Wheel, indicating its suitability for application in detergent industry. The enzyme activity was extended with the help of immobilization technique using sodium alginate and polyacrylamide gel.

Key words: Alkaline Protease · *Bacillus* Species · Mutation · Optimization · Thermostability · Characterization · Detergent Compatibility

INTRODUCTION

Proteases, the most important group of industrial enzymes constitute nearly 60% of the total worldwide enzyme sales [1-3]. Proteases are highly exploited enzyme in food, leather, detergent and pharmaceutical industries [4, 5]. Alkaline proteases were in fact the first enzyme to be produced in bulk and current estimated value of the total worldwide sales of industrial enzymes is $1 billion. Alkaline proteases have traditionally detained the predominant share of the industrial enzyme market accounting for about 60-65 % of total global sale of enzymes [6]. Proteases with their high temperature resistant quality, high specific activities and their superior chemical and physical stability characteristics would seem to be good candidates for current and future biotechnological applications [7].

They are found in wide diversity of sources such as plant, animals and microorganisms. Microbial cells are the usual sources of proteases for industrial use. Microbial proteases account for approximately 40% of the total worldwide enzyme sale [8]. The ability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases.

In nature, *Bacillus* spp., *Aspergillus* families are the chief producers of alkaline proteases. *Bacillus* spp. high thermo and pH stabilities keep it a very good choice for alkaline protease production [9]. About 35% of total microbial enzymes used in detergent industry are derived from bacterial sources and most of them produced by *Bacillus* spp. [10]. Many species of Bacillus have been reported to produce extracellular proteases which are used in food, brewing and laundry industry. These include *Bacillus amyloliquefaciens* [11], *B. megaterium* [12], *B. subtilitis* [13], *B. stearothermophilus* and *B. themoproteolyticus* [14]. Alkaline proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures [5].

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Mutant stains of *Bacillus* produced more alkaline protease compared to the wild one. Strain exhibiting such a changed characteristic is termed a mutant and the process giving rise to it is mutation [15]. The level of production of this enzyme is usually enhanced by random mutagenesis, protoplast fusion and genetic manipulation by introducing the best gene sequence.

Huge production of enzyme in a qualitative and quantitative manner needs a specific substrate. Most plant wastes are potentially good raw materials for the production of ethanol, single cell protein and other microbial enzymes. For the production of enzymes for industrial use, isolation and characterization of new promising strain is a continuous process. They are generally produced by using submerged fermentation due to its apparent advantages in downstream in spite of the cost intensiveness for medium components.

Purification of proteases is carried out using different techniques which include precipitation methods [16, 17], chromatographic methods [18, 19] and electrophoretic methods [20]. Purification by precipitation can be carried out by inorganic salts, organic solvents and high molecular weight polymers. The most commonly used salt is ammonium sulphate [21]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an excellent method which is used to identify and monitor proteins during purification, to obtain the homogeneity of purified fractions and to determine their molecular weights [22]. Single band in such a gel is a criterion of purity [23]. The enzyme once isolated and purified, is characterized to determine the effect of pH, temperature, additives, inhibitors and chelators on its activity and stability.

The present work has been undertaken to throw light on the production of alkaline proteases by mutated and wild strain of *Bacillus* using low cost substrates, ground nut shell and paddy husk in both submerged and solid substrate fermentation. The organism which produced the enzyme, do better at which pH and temperature was selected. Partial purification and characterization of alkaline protease produced by stain and the enzymes compatibility with detergents were studied for the commercial exploitation. The enzyme activity was extended with a help of immobilization.

MATERIALS AND METHODS

Isolation of Bacterial Strain: *Bacillus* species were isolated from the local soil by serial dilution technique. The colonies were confirmed as *Bacillus* spp. by morphology and biochemical tests. The bacterial colonies were purified by repeated streaking on fresh medium and maintained at 4°C on slants of nutrient agar containing 1% gelatin, which act as a inducer for production of proteolytic enzymes.

Screening and Identification: The bacterial strains were screened for proteolytic activity using gelatin yeast peptone culture medium (GYP) and skim milk agar medium. The culture medium was prepared and the cultures were inoculated at the center of the plates and incubated for 3-5 days. After incubation the developing reagent (Mercuric chloride– 15g and 20mL of conc. HCl was dissolved in 80mL distilled water) was flooded with each plates of GYP medium and the indicator (5mL of trichloroacetic acid) was flooded with each plates of skim milk agar medium. The clear zone around the colonies indicates the presence of protease activity. Protease producing bacteria were tentatively identified on the basis of morphological, cultural, biochemical characteristic according to Bergey's manual of systematic Bacteriology [24].

Mutant Isolation

Exposure to UV: The isolated protease producing *Bacillus* spp. was mutated using ultraviolet radiation by germicidal lamp-66T5-6W – SANKYO-DENKI. Cultures exposed to UV then 0. 1 mL of cells were taken at 10 minutes interval for 60 minutes and inoculated onto agar media and incubated for 24 hours at 37°C. The non mutated and mutated strains were named as Wild, BM1, BM2, BM3, BM4, BM5 and BM6 respectively.

Alkaline Protease Production under Submerged Fermentation: Erlenmeyer flasks (250 mL) containing 50 mL of Reese medium [25] gL-1[K2HPO4, 2; $(NH4)_2$ SO4, 1. 5; $MgSO_4$ 7 H_2O, 0. 3; Urea, 0. 3; CaCl2, 0. 3; and trace elements solution-1mL. It was supplemented with gl-1 casein, 5; glucose, 2. 5 and yeast extract, 0. 5] were inoculated with 2% of 48 hours old inoculums (wild & mutant) of each producing isolate and incubated at 37°C for 72 hours on a rotary shaker at a speed of 150 rpm. The cultures were then used for carrying out studies on the protease activity changes of different incubation time. Each time 5 mL is drawn from the culture and protease estimation was carried out according to Ellaiah *et al.* [26]. The culture broths were centrifuged at 8000 rpm for 10 minutes to remove the debris. The supernatant was used as a crude source of alkaline protease. The estimation was read at 660 nm and a graph was plotted against tyrosine standard.

Alkaline Protease Production under Solid Substrate Fermentation: For solid substrate fermentation, paddy husk, ground nut shells (3:2) were used as substrates. Substrates moistened with 15mL of Reese broth excluding casein solution. Under solid substrate fermentation conditions, the flask containing solid substrate medium were inoculated with 1mL of culture suspension of *Bacillus* and incubated at 35°C without shaking. After 72 hours the entire quantity of substrate was homogenized with 100mL of 0. 1 M Tris - HCl buffer and centrifuged at 5000 rpm and supernatants were used as a crude source of alkaline protease. The enzyme activity was measured at the intervals of 24 hours for 3 days.

Estimation of Protease: The activity of alkaline protease was determined by the method of Ellaiah *et al.* [26]. Sample is taken in 5mL and centrifuged at 3000 rpm for 20minutes and the supernatant was collected to which 0. 5mL of casein solution (2% casein in 0. 2 M carbonate buffer pH (10. 0) was added with equal volume of suitably diluted enzymes, incubated at 40°C/10 minutes. After 10 minutes the reaction was terminated by 1mL of 10% trichloroacetic acid and the mixture was centrifuged and 5mL of 0. 44 M Na_2CO_3 and 1mL of two fold diluted folin- Ciocalteau reagent was added. After 30 min the colour was read at 660 nm. The concentration was determined using tyrosine as a standard. One unit (u) of enzyme activity represents the amount of enzyme required to liberate 1µg of tyrosine min-1 under standard assay conditions and expressed as unit mL^{-1} of enzyme. In case of solid substrate fermentation conditions, activity was expressed as unit g^{-1} dry weight of the solid substrate.

Protein Assay: Protein was quantified by the method of Lowry *et al.* [27], with bovine serum albumin as standard.

Optimization of Culture Parameters

Influence of Temperature and pH on the Enzyme Activity: Temperature and pH have a profound influence on the production of protease by microorganisms, for this Reese medium was prepared at the pH range of 6. 0-9. 0. On the other hand the Reese medium inoculated with the strain was kept at different temperatures from 30 to 60°C. Then enzyme activity was measured in the ample at one hour interval.

Stability of the Enzyme with Temperature: To detect the enzyme stability at various temperatures about 10mL of the enzyme mixture was placed in a temperature range of 30 to 90°C. The enzyme activity of the samples was noted at 10 minutes interval.

Partial Purification for Enzyme Characterization: The purification and characterization of alkaline protease was done using the method of Abd Rahman *et al.* [14].

Preparation of Crude Extract: The culture was grown in 100mL flasks with Reese broth at 50°C and then shake using rotary shakers at 200 rpm for 72 hours. This culture broth was centrifuged at 10000 rpm for 30 minutes to remove the bacterial cells. The supernatant was used as crude enzyme extract.

Ammonium Sulphate Fractionation: Solid ammonium sulphate was added to the crude extract in 40-80% saturation. The precipitate was collected by centrifugation, dissolved in minimal volume of 0. 05 M carbonate: bicarbonate buffer (pH 9. 25) and dialyzed against same buffer at 4°C.

Gel Filtration Chromatography: Fix the column to a stand vertically 0. 2mL of the sample was added over the column gently. The sample was allowed to drain completely and then 0. 2mL of buffer was added. Then the buffer was allowed to drain completely and then the pooled buffer fractions checked for protease activity. Fractions showing protease activity were checked for homogeneity and to study the enzymatic properties.

Iron Exchange Chromatography: First the column washed with the hot water (90°C). The column was fixed and packed with 2.5mL CM Cellulose and it was equilibrated with 50 mL of 1X equilibration buffer. Samples were added to the column replacing the top and bottom caps and incubated for 1 hour at room temperature with intermittent mixing. After 1 hour the column was fixed to the stand and allows the gel to settle and slowly pipette out or decant the supernatant without disturbing the gel. The column was washed with approximately 30 - 40 mL of 1X wash buffer. Enzyme was eluted from the column using 15 mL of 1X elution buffer. The fractions showing protease activity were pooled and checked for homogeneity to study the enzymatic properties.

Characterization of Purified Alkaline Protease

SDS-Poly Acrylamide Gel Electrophoresis: The homogeneity of the purified enzyme was confirmed by polyacrylamide gel electrophoresis following the method

of Laemm Li [29]. Prepare 7. 5% SDS-Polyacrylamide gel at pH 8. 3 (Tris-Glycine buffer). Protein sample of 50 mg was loaded with sample gel buffer and a constant current supply of 4 MA per gel rod (13×0. 6cm) was applied for 4 h. After electrophoresis run, gel were stained with 1% Comassie brilliant blue R-250 and were destained with acetic acid methanol till the appearance of blue band against a clear background. The molecular weight of the purified protease was measured by SDS polyacrylamide gel electrophoresis using a series of protein with known molecular weight as standard.

Compatibility of Enzyme with Detergents: The compatibility of purified enzyme with commercial detergents was done in accordance to Meenumadhan *et al.* [9] method. Detergents solutions at a concentration of 7mg/mL were prepared in double distilled water. The solutions were boiled for 10 minutes to destroy any protease already present and cooled. Fixed enzyme concentration was added to each detergent solution and the mixture was incubated at 35°C for different time intervals. The activity was then assayed using the method of Ellaiah *et al.* [26].

Immobilization of Enzymes: Purified enzyme storage was done with using immobilization technique.

- Sodium alginate, 0.1g was dissolved in 5 mL of double distilled water and heated to 80°C. After cooling 3 mLmL of enzyme was added and the mixture was dropped into 0. 1M CaCl.
- Three milliliters of enzyme mixture was added to 1.6 % of acrylamide and 0.08 % of bisacrylamide solution. 200 µL of tris buffer, 50 µL of ammonium persulphate solutions and 100 µL of TEMED was added. The polymerized gel was cut into pieces and stored.

RESULTS

Isolation of Microorganism: Soil was collected from nearby dairy unit and the *Bacillus* species were isolated by serial dilution technique (10^{-4}, 10^{-5}, 10^{-5} dilution). The colonies were confirmed as *Bacillus* species by characteristics of motile, Gram positive rod shaped bacterium, arrangement in chains, creamy colour colony, no pigment, methyl red positive, Voges- Proskauer positive, indole negative, catalase positive, lactose fermentation positive, sucrose fermentation positive, dextrose fermentation positive, starch hydrolysis positive, hydrogen sulphide production negative, litmus milk reduction positive, urea hydrolysis negative and citrate utilization test positive.

Random Mutagenesis of *Bacillus* spp.: The isolates were subjected to UV irradiation for 15 minutes and 0.1 mL of sample was inoculated into the plates of Reese medium. Out of 48 isolates, six were selected and named as BW, BM1, BM2, BM3, BM4, BM5 and BM6. The strains were inoculated in to Reese medium (pH 8. 5) and incubated under dark for 16 hours.

Enzyme Production under Submerged Fermentation: Table (1) reveals that the enzyme production reached its maximum at a temperature of 50°C by a strain BM2. The 316 U/mL of enzyme was produced in wild type at 50°C; at the same time BM2 produced 464 U/mL. The second optimal temperature is 40°C, the maximum enzyme production for BW is 224 U/mL and the BM2 produced 396±1. 25 U/mL. The least production of enzyme was seen in the temperature of 80 and 90°C.

Optimum Time for Enzyme Production: BW produced the maximum amount of enzyme at 72hours. The enzyme produced at 24 hours was 189± 0. 87U/mL at 40°C and maximized to 246±0. 98 U/mL in 72 hours and in the case of mutant cells (BM1) enzyme production were increased to maximum 263±1. 4 in 72 hours at 50°C (Table 2).

Effect of pH: The enzyme production reached its maximum at pH of 8.0 for both wild and mutant cells. 111. 6±1.24 U/mL of enzyme produced from wild and the BM2 produced 295. 6±0. 47 U/mL (Table 3).

Whereas BW produces the maximum enzyme production of 190.33±4.4 U/mL when compared to the other mutant types at pH ranges of 6 and 6.5. From the pH 7.0 -9.0, the mutant types dominate the wild type in enzyme production.

Selection of a Strain: On the basis of the enzyme production the BM2 was selected for the enzyme production in submerged fermentation.

Enzyme Production by Solid Substrate Fermentation: Wild and mutant cells were inoculated with different ratio of substrates like groundnut shell and paddy husk for 72 hours. The maximum enzyme production was 147.6±0. 816 U/g on the substrate by BM4 while BW produced only 72. 66 ±1. 24 U/g (Table 4).

Table 1: Enzyme production under submerged fermentation

	Temp in °C (u/mL±SD)						
Strains	30	40	50	60	70	80	90
Bacillus wild	223.37± 125	224 ± 0.816	316 ± 1.69	213.33± 2.4	316 ± 1.63	113.33 ± 1.8	243.66 ± 2.62
Bacillus M1	264.66 ± 1.25	296.33 ± 1.25	217.66 ± 2.05	224.33 ± 1.24	224 ± 0.81	262.66 ± 2.2	257.66 ± 1.24
Bacillus M2	223.33 ± 1.24	396.37 ± 1.25	464 ± 2.62	302.66 ± 2.49	302.3 ± 2.05	334.33 ± 1.64	184.66 ± 3.68
Bacillus M3	222.33 ± 4.19	275.66 ± 2.05	223 ± 2.16	163.33 ± 1.24	222.33 ± 0.942	244.66 ± 1.25	274.66 ± 1.24
Bacillus M4	222 ± 2.16	294.66 ± 1.25	184.66 ± 1.24	312.66 ± 2.49	293.33 ± 2.05	364 ± 1.41	182.33 ± 2.05
Bacillus M5	316.66 ± 3.09	226.33 ± 1.247	246.33 ± 1.25	247 ± 2.16	214.33 ± 1.69	248 ± 1.63	165.33 ± 2.86
Bacillus M6	224.0 ± 0.816	152 ± 1.63	362.66 ± 1.7	286.33 ± 2.5	161.66 ± 1.24	262.66 ± 2.05	169.66 ± 1.247

Table 2: Optimum times for enzyme production

	At 40°C (u/mL±SD)			At 50°C(u/mL±SD)		
Strains	24	48	72	24	48	72
BW	189 ± 0.56	108 ± 1.05	246 ± 0.98	46 ± 1.1	116 ± 0.95	212 ± 0.76
BM1	29 ± 2.4	69 ± 0.81	191 ± 1.61	39 ± 2.4	108 ± 1.1	263 ± 1.4
BM2	34 ± 1.9	74 ± 0.53	208 ± 0.81	34 ± 1.6	106 ± 2.3	248 ± 0.81
BM3	46 ± 0.56	81 ± 2.06	196 ± 0.64	42 ± 0.81	98 ± 0.61	161 ± 1.8
BM4	39 ± 0.6	56 ± 1.8	186 ± 1.91	49 ± 1.54	66 ± 0.46	121 ± 1.4
BM5	44 ± 0.89	69 ± 1.64	193 ± 0.84	53 ± 2.6	68 ± 0.31	201 ± 0.56
BM6	52 ± 2.4	71 ± 0.71	201 ± 1.64	43 ± 1.6	73 ± 0.86	195 ± 1.47

Table 3: Enzyme production at different pH U/mL

	Different pH U/mL±SD					
Strains	6	6.5	7	8	8.5	9
BW	190.33 ± 4.4	180 ± 4.54	294.66 ± 1.24	111.66 ± 1.24	224.66 ± 3.4	282.33 ± 1.7
BM1	24.66 ± 1.53	189.33 ± 5.43	196.33 ± 3.68	262.66 ± 2.1	147 ± 2.16	171.66 ± 1.24
BM2	127.33 ± 3.85	177.66 ± 4.5	305.33 ± 4.1	295.66 ± 0.47	4.86.16 ± 1.7	293 ± 1.63
BM3	93.66 ± 2.62	129.66 ± 4.18	284.61 ± 3.68	282.33 ± 2.05	162.66 ±2.05	176.66 ± 1.67
BM4	122.33 ± 1.7	182.33 ± 4.78	286 ± 0.86	369.66 ± 2.62	143.33 ± 1.25	224.37 ± 1.24
BM5	159.66 ± 2.86	150.6 ± 3.09	223.66 ± 2.62	284.37 ± 1.65	182 ± 1.63	262.33 ± 12.49
BM6	125.66 ± 4.5	155.33 ± 4.11	359.33 ± 2.49	295.33 ± 3.68	181.32 ± 1.86	290 ± 0.816

Table 4: Enzyme productions in solid substrate fermentation µ/g+SD

Stains	Paddy husk	Ground nut shell	Paddy husk: Ground nut shell (2:2)	Padddy husk: Ground nut shell (2:3)	Paddy husk: Ground nut shell (3:2)
BW	56.12 ± 0.561	47 ± 0.816	44.1 ± 1.81	48.1 ± 0.67	54.6 ± 0.86
BM1	26.44 ± 1.58	47.1 ± 2.1	52.1 ± 2.71	52.7 ± 2.81	59.4 ± 0.86
BM2	28.52 ± 1.71	39.5 ± 0.87	41.87 ± 1.81	42.7 ± 1.06	47.1 ± 1.81
BM3	27.2 ± 2.8	42.7 ± 0.71	45.1 ± 1.34	46.8 ± 1.4	49.1 ± 3.4
BM4	48.1 ± 0.86	72.66 ± 2.4	52.3 ± 2.7	54.2 ± 1.8	133 ± 2.16
BM5	26.2 ± 0.91	42.1 ± 1.89	44.31 ± 1.61	44.86 ± 4.81	46.09 ± 1.4
BM6	28.1 ± 3.2	38.1 ± 2.1	40.1 ± 2.61	41.3 ± 0.95	43.7 ± 1.31

Table 5: Enzyme stability at different temperature (u/mL ±SD)

Time	30°C	40°C	50°C	60°C	70°C	80°C
10 min	183.66 ± 2.62	186.33 ± 1.88	155.33 ± 0.94	155.33 ± 2.49	14.33 ± 1.69	4.61 ± 1.52
20 min	102 ± 2.44	152 ± 2.1	98.6 ± 2.05	44.3 ± 1.69	-	-
30 min	82.33 ± 12.06	142.33 ± 2.49	63 ± 1.63	36 ± 2.65	-	-
40 min	49 ± 2.94	95.66 ± 2.05	46.3 ± 1.24	8 ± 2.6	-	-
50 min	14.33 ± 2.06	53.66 ± 1.69	36.6 ± 1.69	-	-	-
60 min	12.33 ± 2.06	24 ± 2.16	24 ± 2.16	-	-	-

Table 6: Purification and Characterization of enzymes through chromatography

	Volume (ml)	Total activity (U/mL)	Total protein (mg/ml)	Specific activity (U/mg)	Fold purification	Recovery (%)
Culture filtrate	250	30.0	7.2	6.09	1.0	100
Ammonium sulphate (20-40%)	25	74.5	4.3	19.09	7.36	84.30
CM cellulose fraction	49	88.9	2.4	63.48	13.5	45.55
Sephadex G-100	35	92.3	1.6	113.6	47.14	35

Table 7: Compatibility of crude enzyme with detergents

Enzyme activity at different time	SURF (%)	RIN (%)	ARIEL (%)	WHEEL (%)
10 min	84.9	56.6	5	57.73
20 min	24.5	25	0	52.08
30 min	8	16	0	10
40 min	4	2	0	1
50 min	0	0	0	0

Enzyme production on using paddy husk substrate resulted in a low productivity compared to groundnut shell. BW cell produced 56.12±0.561 U/g whereas mutant BM4 produced 48.1±0.86 U/g. While combination of groundnut shell and paddy husk at 3:2 ratio showed the maximum enzyme production of 133±2.16 U/g in BM4 but in wild cells BW produced only 54.6±0.86 U/g.

Enzyme Stability at Different Temperature: The enzyme extract were placed in a range of 30 to 80°C temperatures. The activity of the enzyme was gradually decreased in the range of 30 to 50°C. But in 60°C the enzyme activity was retained only for 40 minutes and enzyme activity were lost after 10 minutes in 70 and 80°C. While the whole the enzyme was stable at 50°C for 1 hour (Table 5)

Purification and Characterization of Enzyme: A summary of purification steps for alkaline protease from *Bacillus* species in given in Table 6. The purification of alkaline protease resulted in 7 fold purification with 84% recovery by ammonium sulfate precipitation. The purification of crude enzyme through DEAE cellulose column chromatography gave 47 fold increases in purity with 35% recovery of alkaline protease from *Bacillus* species.

The molecular weight of purified enzyme was determined by SDS-PAGE was found to be 31 kDa. The appearance of the purified enzyme was single band and SDS-PAGE further suggested the enzyme to be monomeric. By using iron exchange chromatography with CM cellulose, an overall 97% fold increase specific activity. The purified enzyme got eluted as a single peak on Sephadex-G-100 column and had a specific activity of 113. 6 $\mu . mg^{-1}$.

Compatibility of Enzyme with Detergent: Enzyme activity and stability in the presence of some available commercial detergents was studied with a view to exploit this enzyme in detergent industry. After 10 minutes of incubation, maximum activity was observed in commercial detergents like Surf (84. 9%), Ariel (5%), Wheel (57. 73%) and RIN (56. 6%) while the enzyme activity was completely lost after 10 minutes in Ariel detergent. The enzyme retained 4, 2 and 1% activity after 30 minutes of incubation respectively in the presence of Surf, RIN and Wheel. After 40 minutes, the enzyme lost its activity in the all four detergents (Table 7)

Immobilization of Enzymes: The enzyme stored in 25 and 4°C, lost their activity at 4 and 30 days respectively. On immobilization with sodium alginate, the days of stability extended for 6 days at 25°C and 41 days in 4°C.

DISCUSSION

In the present study, the isolated *Bacillus* species were mutated to enhance the production of alkaline proteases. Six strains of *Bacillus* were selected by random mutation. The wild and mutant strains were inoculated into Reese medium for submerged fermentation. *Bacillus* M2 showed maximum activity compared to other strains. This is due to the mutagenic effect and it is useful for the selection of strain for the better production of enzymes. These results were strongly confirmed by Meenumadhan *et al.* [9] and Abd Rahman [14].

The enzyme production reached the maximum at temperature of 50°C for 72 hours. It is greatly differs from the production of alkaline proteases from *Bacillus polymyxa* at 70°C [9]. The temperature optima of 70°C have also been reported for alkaline proteases from some thermophillic 1 *Bacillus* [30], Kim *et al.* [31] and Kaur *et al.* [15]. Morimura *et al.* [32] reported growth and protease production was ceased at 50°C while, production was good at 45°C from *Aspergillus usami*. Kalpana Devi *et al.* [33] reported maximum production of

protease (89.1 U/mL) was obtained at 45°C from *Aspergillus niger*. It was revealed that environmental temperature not only affects growth rates of organism but also exhibit marked influence on the levels of protease production.

Another important factor significantly affecting the production of protease is the medium pH. Protease production by *Bacillus* species was observed in the pH range from 6 to 9. The maximum of enzyme production was observed at pH 8. 0. Majority of organism producing alkaline proteases showed growth and enzyme production under alkaline conditions. A pH 9. 0 has also been reported as optimum for production of alkaline proteases [15] and *Bacillus sterothermophilus* [34]. Kalpana Devi *et al.* [33] reported maximum protease production from *A. niger* at pH 8.5 and production was ceased at pH 9. Optimum pH 8.4 has been reported for alkaline protease of *Conidiobolus coranalis*. Likewise pH 7 has been reported to be optimum for *Aspergillus flavus* [35].

Comparatively the maximum enzyme production was at pH 8.0 on 50°C. These results were similar to the reports of Meenumadhan *et al.* [9]; Ellaiah *et al.* [26].

When natural substrates in different combination were tried to increase the enzyme yield with reduced cost of production under solid substrate, the combination of paddy husk + ground nut shell (3:2) in fermentation condition presented satisfactory results. Nehra *et al.* [36] reported rice bran, rice husk and gram hull are the natural substrate for the production of alkaline protease from *Aspergillus niger*. Commercial wheat bran was reported to be efficient substrates for alkaline protease production by *A. flavus* [37] while *A. fumigatus* produced higher amount of protease on pigeon pea residue compared to wheat bran under SSF [38], similarly rice hull and rice bran in 7:2 ratio was most suitable combination on for alkaline protease production by *A. oryzae* [39].

In the present study, the *Bacillus* species produced 59U/g of enzyme on 3:2 ground nut shell and paddy husk substrate. But the alkaline protease production in *Aspergillus* species was very huge i. e. 4100 U/g in the same substrates [36]. Based on the present results the *Bacillus* is the poor enzyme producer when compared with *Aspergillus spp.*

But in contrary, in submerged fermentation, the *Bacillus* strain produces maximum of 492 U/mL, but the *Aspergillus* produces only 40 U/mL to 120U/mL [36]. On comparison of both solid substrates fermentation and submerged fermentation, the *Bacillus* strains are not doing better in solid substrate fermentation. The results were also similar to *Conidiobolus coronatus* [35].

The enzyme produced from *Bacillus* strain was fully stable and showed 100% activity up to 40 minutes at 50°C. There after it declined to 61% at 60 minutes. However the enzyme was completely inactivated at 80°C in 10 minutes.

This enzyme was more stable than subtilism BPN' and Carlsberg and resembled protease BYA from *Bacillus* spp. which was stable at temperature up to 55°C and showed about 90 and 30% of the native activity at 60 and 70°C respectively [9].

A summary of purification steps for alkaline protease from *Bacillus* species in given in Table 6. The purification of alkaline protease resulted in seven fold purification with 84% recovery by ammonium sulfate precipitation. The purification of crude enzyme through DEAE cellulose column chromatography gave 47 fold increases in purity with 35% recovery of alkaline protease from *Bacillus* species. The similar observation was reported by Ogundero and Osunlaja [40] for *Aspergillus clavatus* and Kalpana Devi *et al.* [33] for *Aspergillus niger*.

Sodium dodecylsulphate polyacrylamide gel electrophoresis was run to determine the molecular weight of protease. Mixture of marker proteins with known molecular weight was loaded as standard. Molecular weight marker in the range of 17-150 kDa was used to detect the molecular weight of alkaline protease. The molecular weight of purified enzyme was 31 kDa. The appearance of single band on SDS-PAGE further suggested the enzyme to be monomeric. The molecular weight was similar to that reported earlier for alkaline protease from its wild type parent *B. polymyxa*[15]. Molecular weight in the range of 30-32 kDa were reported for the enzyme from other *Bacillus* species strain OK 3A, 1 B-18 and BAH-101 [41, 42]. The molecular weight in the range of 32-33kDa has been reported for the enzyme from *Malbranchea inlchella*[43] and 38 kDa has been reported for *A. niger*[33]. Leigh[44] had reported an alkaline serine protease with molecular weight of 19 kDa, determined by SDS-PAGE. Durham *et al.* [45] had reported the molecular weight of serine protease to be 27kDa. Similarly the molecular weight of serine protease was found 30kDa, 28kDa and 19 kDa by Koki [46]. Patel *et al.* [47] also reported a single band protease with the molecular weight of 30kDa whereas; other halophilic and alkaline proteases have molecular weight in range from 40-130 kDa[48, 49]. The protease enzyme retained 4 - 84. 9% of its original activity in various detergents. The maximum enzyme activity was observed in commercial detergent like Surf in 10 minutes (84. 9%) and 10% in wheel at 30 minutes. The enzyme activity was completely lost in commercial detergent Ariel after 10 minutes as reported by

Meenumadhan *et al.* [9] and the enzyme activity retained to 77% at 10 minutes, 25% at 50 minutes and completely lost at 70 minutes. The same author reports that the enzyme activity was completely lost in the detergent, Surf but in contrary our results showed maximum enzyme activity on the same detergent was 84. 9% in 10 minutes and 4% at 30 minutes. Phadatare *et al.* [50] studied the compatibility of alkaline protease of *Conidiobolus coronatus* with commercial detergents. They observed the enzyme protease retains more than 80% of its activity in the presence of detergents viz. Snow white, Nirma, Revel and more than 56% of its activity in the presence of Wheel and Surf when incubated for 1 hour. Similarly among the three protease isolated from *Tritirachium album* proteinase R and T were reported to retain 90 and 89% activity respectively up to 1 hour in the presence of detergents like ERA plus and Dyanamo, while BPN' was highly unstable in all the detergents and retained just 4% activity even after 10 minutes [51].

The above results showed that the enzyme isolated from mutant *Bacillus* species was stable at high pH and temperature and in the presence of detergents may be exploited as an additive in the detergent industry.

CONCLUSION

It is evident from this examination of the possible applications of enzyme technology in detergent industries which are manufacturing at high pH (8. 0) at a temperature 50°C. Thus, the application of the technologies such as random mutagenesis, developing the low cost substrate from agro waste and microbial selection could be used to create desired microorganisms that would then be self-replicating units within the rural community. So entrepreneurs who wish to start the detergent industry, they can produce the enzymes which are essential. This also used to apply for the processing of food materials which may be useful for the unemployed youth in both urban and rural communities.

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