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Research Article

Screening and Optimization of Physical Parameters for Enhanced Alkaline Protease Production by Alkaliphilic Bacillus Subtilis SH2 Isolate

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Abstract

The present investigations dealt with the optimization of the physical parameters for production of alkaline protease by alkaliphilic *Bacillus subtilis* SH-2 isolated from slaughter house soil of Warangal, Telangana State, India. Primary screening of four different samples revealed one potent isolate. Morphological and Biochemical characterization followed by Molecular signature of 16s rRNA homology confirmed that the isolate SH-2 belongs to *Bacillus subtilis*. *Bacillus subtilis* SH-2 was screened on four different reported mediums (M1213, M660, Horikoshi and Halophilic Bacillus medium) under shake culture conditions. Maximum alkaline protease production (500 EU/ml) obtained on M1213 and Horikoshi mediums. Further optimization of physical parameters by OVAT method revealed that mean generation time (41.18 min), 4% level inoculum, incubation time 72 hrs, pH 10, temperature 350C and agitation 150 rpm are ideal for enzyme production. OVAT method resulted in 2.2 fold increased production of alkaline protease production (1100 EU/ml).

Keywords: Alkaliphiles; *Bacillus subtilis* SH-2; Alkaline protease; Physical parameters optimization and OVAT method

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Introduction

Proteases (EC: 3.4. 21-24, 99) are ubiquitous, about 2% of genes code for proteases enzymes in organisms [1]. Proteases represent one of the three largest groups of industrial enzymes and account for about 59% of the total worldwide sales of enzymes [2]. The inability of the plant and animal proteases to meet current global demands has led to an increased interest in microbial proteases. Bacterial alkaline proteases are characterized by their high tolerance at alkaline pH10, and their broad substrate specificity. Alkaline proteases of alkaliphilic bacteria are the promising source of extremozymes with billion dollars of international trade market in industrial sector.

In light of the above mentioned advantages of alkaliphiles we made an attempt to exploit the indigenous soils of Warangal for alkaliphilic bacterial isolates. The present investigations dealt with the optimization of the physical parameters of alkaline protease production by an alkaliphilic *Bacillus subtilis* SH-2 from slaughter house effluents.

Material and Methods

Sample collection

In the present investigations samples from habitats of slaughter house effluents were collected from a depth of 5 to 10 cm. The samples were carefully transported in an ice pack and later stored, if necessary, in refrigerator at 4^0C for future investigations.

Enrichment

a. **Preparation of composite sample and enrichment:** About 10 gm of sample was taken in to the sterile Erlenmeyer flask, in aseptic conditions under laminar airflow and the contents were suspended in 100 ml of the Horikoshi broth with a pH of 12.0±5, thoroughly vertexed for about 10 minutes to make uniform suspension and incubated for 6 to 24 hours.

b. **Sample processing:** Osmo-stable solution is a diluting fluid which preserves the alkaliphilic and alkali tolerant cells in their original viable condition. About 1mL of the enriched composite sample was serially diluted with the 9 mL osmo-stable solution (Nacl 8.5g/L; KCl 0.20 g/L; $CaCl_2$ 0.2 g/L; $Na_2CO_3$10g/L Final pH set to 12) up to 108 dilution and the contents were incubated at the room temperature for an hour, later the desired dilution was used to evaluate the viable count of the alkaliphiles by the method described by the Horikoshi et al. (1979) [3].

Plating and isolation

Isolation of the alkaline protease producing bacteria was made on ASMA (Alkaline Skimmed Milk Agar: poly peptone 5g/l; yeast extract 5g/l; glucose 10g/l; KH_2PO_4 1.0g/l; $MgSO_4$ 0.02g/l; skimmed milk 2g/l; agar 18g/l; Na_2CO_3 10g/l). Glucose, skimmed milk and Na_2CO_3 were separately sterilized at 121^0C/15min/15lbs after cooling and added the contents in sterile aseptical conditions. The pH of the medium was set to 12 with addition of the Na_2CO_3 medium. Appropriately 0.1ml of the diluted sample was over layered by sterile spreader in an aseptic condition. The plates were well labeled and incubated for 24 to 72 hours at 25 – 30^0C.

Primary screening: The qualitative screening of the alkaline protease producing bacteria was performed by the method suggested by Horikoshi et al. (1999) [4], and the proteolytic potentiality of the isolates was calculated. The isolates showing highest degree of proteolytic efficiency i.e R/r ≤ 2.0 were selected for further screening. Proteolytic efficiency was calculated by the method suggested by Chandra et al. (2016) [5]. Proteolytic potential was calculated by the formula:

Hydrolytic zone HZ = (R = Total zone diameter – Colony diameter(r)) /r (Colony diameter)

The selected efficient isolates were further screened to find out the production capabilities. The colonies were maintained on Horikoshi medium (Peptone 5g/l; yeast extract 5g/l; glucose 10g/l; KH_2PO_4 1 g/l; $MgSO_4$ 0.2 g/l; agar 16 g/l; Na_2CO_3 10g/l) at 4^0C. Monthly subcultures were made and proteolytic potential was tested.

Cultural and biochemical Characterization: Isolates maintained on Horikoshi medium were observed for the colony morphological characteristics such as margin, elevation, consistency and pigmentation. Their biochemical characterization was carried out as per the guidelines of the Bergey's Manual of Determinative Bacteriology [6].

About 100 ml of ASMA medium with varying concentrations of the carbon, nitrogen and inducer sources was taken in flasks. Incubation was carried out for 24 hr under the defined conditions. The cell-free supernatant recovered by centrifugation (10000 g, 10 min) was used as enzyme source. Extracellular alkaline protease activity was determined by the modified method of Yang et al. (1994) [7].

Evaluation of the significant environmental factors: The influence of environmental factors on growth and alkaline protease production was investigated by culturing the cells in Horikoshi broth at wide-ranging temperatures i.e. 20, 25, 30, 35, 40, 45, 50, 55 and 60oC, respectively for 72 hours. For alkaliphily test, different pH values, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5 and 12 were used. The medium pH was adjusted and after inoculation the cultures were grown at 30oC for 72 hours. At the end of the incubation period, the final pH, biomass, enzyme production were determined by standard methods in the respective ranges.

Enzyme assessment: About 5 mg Hammerstein casein in 0.9 ml of Glycine-NaOH buffer was taken and 0.1 ml of enzyme source was added. Reaction mixture was incubated for 30 min. To terminate the reaction 2 ml of 5% Tri-Chloro acetic Acid (TCA) was added, to remove denatured proteins the contents were passed through No.2 filter paper (Whatman), centrifuged at 10000 g for 10 min. The resultant supernatant fraction was read at 275 nm. One unit of protease activity was defined as the amount of enzyme required to produce an increase 0.001 in the absorbency at 275 nm per min under the assay conditions. Specific activity was expressed as enzyme units per mg protein. Simultaneously, a blank without enzyme was used for comparison purpose.

Results and Discussion

A total of 4 samples from the habitats which included; slaughter house and blood soaked soils located in and around the Warangal were selected. After isolation and preliminary screening 09 isolates of bacteria showing alkaline protease production were selected (Table-1, Fig-1).

Table 1 Primary screening and Proteolytic potentials of nine isolates

Sample code	Location	Isolate code	Morphology	Nonproteolytic	Proteolytic	Percentage	r	Tz	R	R/r
SM	Mulug	SH1	C,E,R, Umb, Smt	18	4	22.22	2	8	6	3
SH	Hanamko	SH2	C,E,R, Mcd,Wht	5803	10.3	17.66	2	16	14	7
SA	Atmakur	SH3	C,E,R, Umb, Smt	57.2	18.2	31.81	2.3	11.5	9	4.5
SP	Parkal	SH4	C,E,R, YE	32.7	5.7	17.43	1.5	7.5	6	4
SB	Bhoopalp	SH5	C,E,R, Umb, Wht	73	16	21.91	2	10	8	4
SR	Regonda	SH6	Irr,Lb,Flt,Trslu	24	4	16.66	3	15.6	12.6	4.2
SE	Eturnagar	SH7	Irr,Lb,Flt,Smt	42	10	23.8	2	12	10	5
ST	Tadvai	SH8	Irr,Lb,Flt,Tnslu	93.8	17.8	18.97	2	10.4	8.4	4.2
SG	Govindar	SH9	C,E,R, Umb, Smt	14	4	28.57	2	8	6	3

Characterization and identification of alkaliphilic protease producers

Colonies exhibiting high proteolytic zone (R/r ≥ 3) were picked up and further screened for their proteolytic activity on ASMA medium plates, Out of nine, one isolate with maximum proteolysis was selected for the further studies and they are designated according to the habitat of selection and are being maintained laboratory, Department of Microbilogy, Kakatiya University, Warangal. The detailed study of cultural, morphological, physiological and biochemical characteristics would fit all the strains tentatively in the genus *Bacillus* [6].

Primary screening of alkaline protease producers

The selected strain viz, SH-2 was screened further for alkaline protease production on four different reported mediums (M1213, M660, Horikoshi and Halophilic Bacillus medium) at pH 11 under shake culture condition of 150 rpm for 72 hours. After 72 hours the growth in terms of OD and enzyme production in enzyme units and pH changes were recorded and these are presented in Table-2.

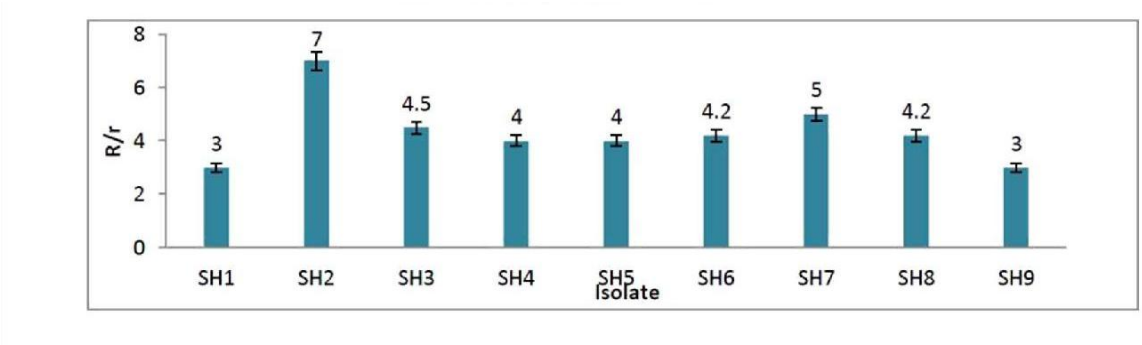

Fig. 1 Proteolytic potentials of nine selected isolates

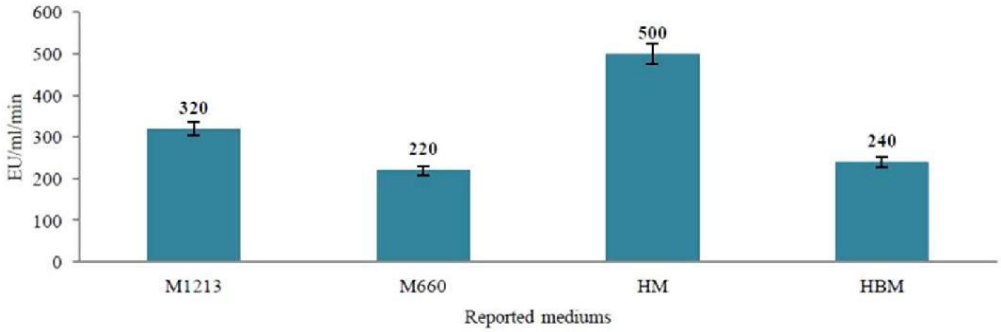

Fig. 2 Alkaline protease production by Bacillus subtilis SH2 on four different repoted mediums

An appraisal of the Fig-2 reveals that M1213 and Horikoshi media supported maximum enzyme production under batch type of shake culture technique. Interestingly, highest production about 500 EU/ml obtained. It is evident from the present studies (Fig-2) that both M1213 and Horikoshi mediums are the good mediums for the maximum enzyme production by all the strains.

Studies on optimization of cultural conditions for alkaline protease production by OVAT method

A large quantity of alkaline protease production is possible only after successful optimization of cultural and environmental factors and their cumulative influence. Conventional practice of single variable optimization is by maintaining other variables involved at a constant level. The major advantage of this

"one-variable-at-a-time" method is that it gives information about the effects among the variables [8-11]. Hence, in the present investigations an attempt was made to understand the influence of environmental factors that contribute to optimization of alkaline protease production on reported medium.

Determination of mean generation time

Preliminary studies reveal that SH2 strain grows with mean generation time (41.18 min not shown here). The present studies on mean generation time are almost similar to the observations made on alkaliphilic *Bacillus firmus* OF4 by Michel et al .1994[12].

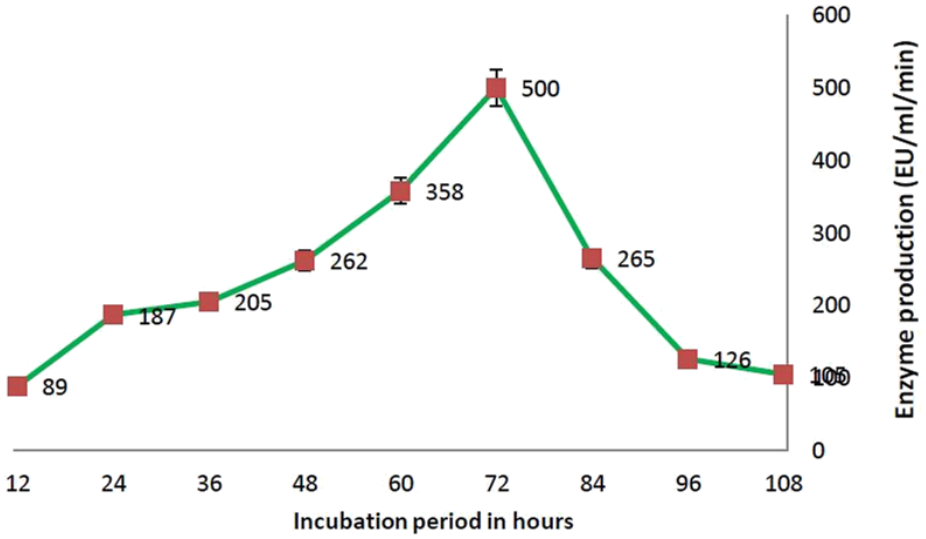

Fig. 3 mean generation time and Alkaline protease production by Bacillus subtilis SH2

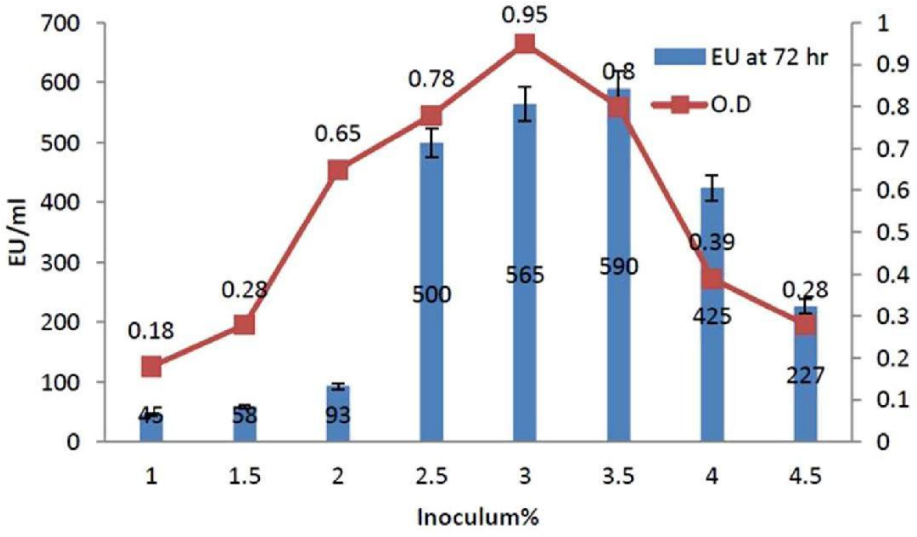

Fig. 4 Influence of inoculum percentage on alkaline protease production by *Bacillus subtilis* SH2

Time course Vs enzyme production

In continuation of growth studies, the enzyme production vis-a-vis growth was also studied and presented in Fig. 3. The results reveal that increase in the time course correlated with the increase in enzyme production. Significantly, 72 hours of incubation time was found to be favorable for SH2 strains

irrespective of their growth. SH2 strain steadily increased upto 500 EU/ml of enzyme production with growth 1.3 OD, pH change from 11 to 10.0. Further, these studies indicated that the enzyme productions have steadily increased from the 12 hours to 72 hours and subsequently there was sudden decline ((Fig-3). The enzyme unit curves obtained (Fig-3) indicated that the sharp increase in alkaline protease EU/ml reached with time line. The stable biomass production at 60 hours to 84 hours is recorded. Alkaline protease production by the present strains was noticed in the late exponential phase or in early stationary phase. The present investigations are in accordance with those obtained by Hadeer et al. (2009), Jignasha and Satya (2007) and Moreira et al. (2002) [13-15]. They found that the optimum protease production starts in early stationary phase of growth and reached to maximum by 72h. Thus, it is clear that 72 hours time is ideal for the production of enzyme by the *Bacillus subtilis* SH2.

Inoculation ratio

An attempt was made to understand the relationship between the inoculum ratio and enzyme production and these results are presented in Fig-4. The results reveal that as the proportion of inoculum increases the enzyme production also increases for all the three strains under investigation. Further, some interesting observations could be made. For instance, SH2 produced maximum amount of enzyme. As expected, at 4% level inoculum, the growth was more. In addition, to more growth more enzyme production was noticed. The present investigations are substantiating with the finding of Mukesh et al. (2012) and Elibol and Moreira (2005) [16, 17].

Table 2 Influence of pH on growth and enzyme production *OF Bacillus subtilis* SH2

pH	5	6	7	8	9	10	11	12
EU at60hr	35	48	67	100	450	495	410	172
O.D	0.18	0.23	0.56	0.68	0.89	0.80	0.33	0.20
EU at72hr	45	58	91	500	565	590	425	227
O.D	0.18	0.23	0.65	0.78	0.95	0.80	0.39	0.28

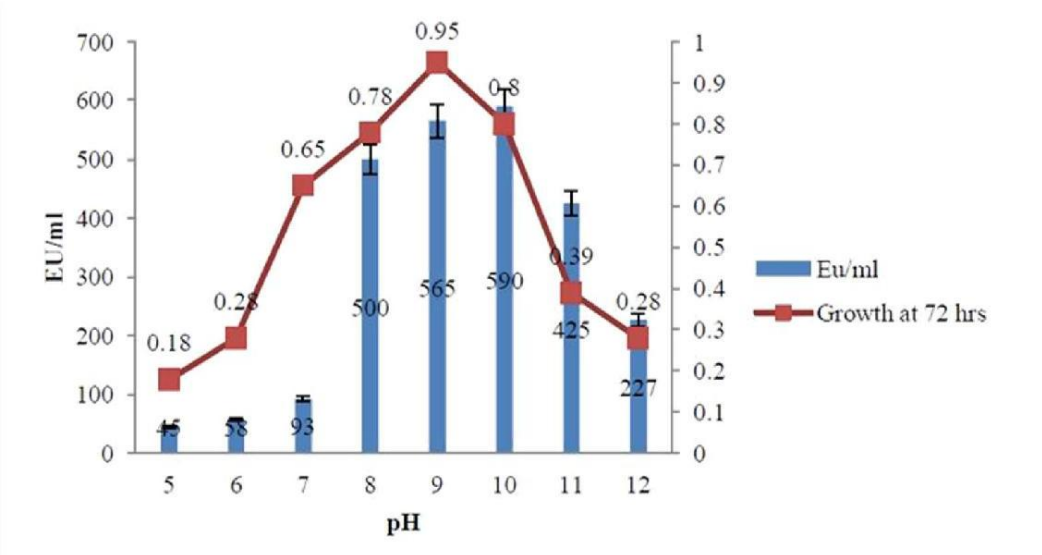

Fig. 5 Influence of pH on alkaline protease production by *Bacillus subtilis* SH2

Media pH

An attempt was made in the present investigations to screen the superior strains for optimal pH against a broad pH range of 5 to 11 with at least two different time intervals (60 and 72 hours) and results were summarized (Table 2). A critical perusal of the Table-2 reveals that for SH2 strain as the initial pH of the medium pH was enhanced, both enzyme production and biomass increased correspondingly. At 72 hours incubation maximum enzyme production was achieved at pH 10.0 (Fig 5). However, A steady increase in growth and enzyme production indicates that enzyme production is correlated with maximum at late exponential phase, further it also confirms that the strain is an alkaliphilic in nature. Generally alkaline proteases activity is pH dependent and they are more active in the broad range of pH 8-10 [18-20].However, the present strain producing alkaline protease with broad range of pH 8-11. Several workers have reported that the cell growth and enzyme production are strongly dependence on the pH [21, 22]. Moon et al. (1991) also reported the strong influence of the culture medium pH on enzymatic processes [23].

However, Aunstrup (1980) felt that for increased protease production the pH of the medium must be maintained constantly above 7.5 throughout the process. The results in the present study are also in accordance with Darani et al. (2008) who reported the production of protease at pH 10 by *Bacillus* sp. 2-5 [24]. Yu et al. (2006) have reported alkaline protease production at pH 10.0 by *Bacillus licheniformis* [25]. Nadeem (2008) has grown *Bacillus licheniformis* N2 at pH 10 for protease production [26].

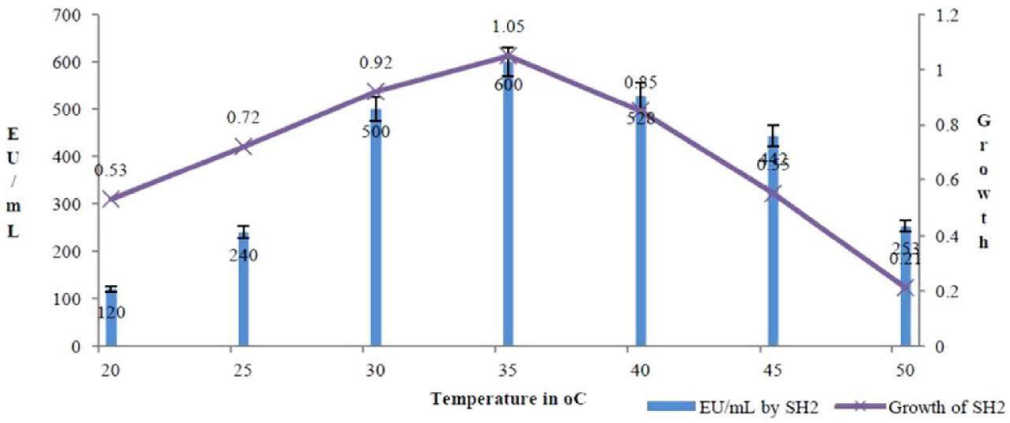

Fig. 6 Influence of Temperature on alkaline protease production by *Bacillus subtilis* SH2

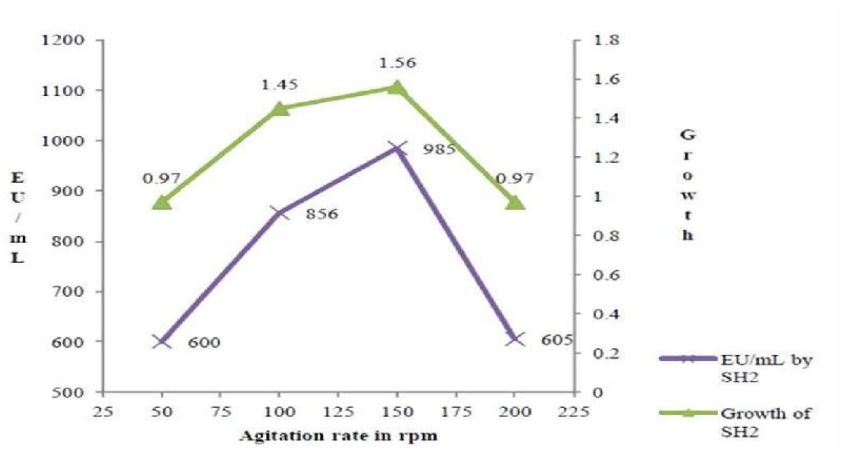

Fig. 7 Influence of Agitation on alkaline protease production by *Bacillus subtilis* SH2

Temperature

Microorganisms grow slowly at a temperature below or above the normal growth temperature because f a reduced rate of cellular production [27]. Both the growth and maximum production of the enzyme primarily depend on the adaptability of the strain and stable cardinal temperature optima. At lower temperature, substrate transport across the cells is suppressed and lower product yields are attained, whereas at higher temperature, the energy requirement for maintenance of cellular growth is high due to thermal denaturation of enzymes of the metabolic pathway [28].

In the present study, influence of temperature on alkaline protease production by the isolate was investigated and the results are presented in Table-3. The observation of the present investigations reveals that the temperature optimum for alkaline protease production is at 35^{o}C and further increase in temperature resulted in decreased enzyme production (Fig-6). In contrary, De Silva et al. (2007) reported protease production by *Bacillus* sp. strain SMIA-2 at 70^{o}C [29]. The present study indicates that the protease is thermotolerant in nature. Similar observations were made by Kim et al. 2001[30], Kumar et al. 2004 [31]. Joo and Chang reported that the optimum temperature for protease production by *Bacillus* sp. I-312 to be 32 ℃ [32].

Table 3 Influence of temperature on growth and enzyme production *Of Bacillus subtilis* SH2

Temp	**20**	**25**	**30**	**35**	**40**	**45**	**50**
EU at60hr	95	1041	267	330	453	435	210
O.D	0.18	0.23	0.56	0.68	0.89	0.80	0.33
EU at72hr	120	240	500	600	528	442	253
O.D	0.53	0.72	0.92	1.05	0.85	0.55	0.21

Table 4 Influence of agitation rate on growth and enzyme production *OF Bacillus subtilis* SH2

Growth and EU/ml	**Agitation rate rpm**			
	50	**100**	**150**	**200**
EU at 60hr	295	1041	267	330
O.D	0. 87	0.97	1.40	0.87
EU at 72hr	600	856	985	605
O.D	0.97	1.45	1.56	0.97

Agitation speed

Agitation speed considered as an essential physical factor known to increase the dispersion of the fermented mass uniformly and enhance the oxygen transfer to actively growing microbial cells. Thus, maintains all the cells at synchronized growth state for the extra cellular production in batch type of shake culture. The results pertaining to the influence of rate of agitation are summarized in Table -4. A critical perusal of the table reveals that the production of alkaline protease increased with the rate of agitation and maximum being recorded at 150 rpm (Fig-7). Patil et al. (2011) investigations on *Pseudomonas aeruginosa* MTCC7926 revealed that agitation speed at 50 rpm was more ideal for protease production [33]. Nadeem et al., 2009 anticipated that higher agitations (600-700 rpm) reduce the cell growth and alkaline protease production due to sheer stress and heterogeneous mixing effects [34].

Conclusions

In light of the idea that the occurrence of alkaliphilic bacteria are widely disseminated in nature this might be due to the transient conditions prevailing. Present investigation enlighten that the environmental conditions have significant effect on process optimization. It is very important to state that further enhancement shall be performed by mutagenesis, studies in this direction helpful for industrial exploitation. Further strain stability and process development is under progression.

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References

1. Barrett AJ, Rawlings ND, Woessner JF. (eds) The Handbook of Proteolytic Enzymes, 2nd ed., ***Academic Press***. 2003. http://dx.doi.org/10.1110/ps.8.3.693
2. Gaur S, Agrahari S, Wadhwa N. Purification of Protease from Pseudomonas thermaerum GW1 Isolated from Poultry Waste Site. ***Open Microbiol J.*** 2010, 4:67-74
3. Horikoshi K. Production and industrial applications of beta cyclodextrin. ***Proc Biochem***. 1979, 14:26-30
4. Horikoshi K. Alkaliphiles: some applications of their products for biotechnology. ***Microbiol Mol Biol Rev***. 1999, 63(4):735-750
5. Chandra SJ, Pallavi P, Anuradha BS, Reddy SR. Optimization of bioprocess for enhanced production of alkaline protease by a *Bacillus subtilis* SHmIIIa through Plackett-Burman design. ***Afr J Microbiol Res***. 2015, 9(28):1738-1744
6. Holt JG, Krieg NR, Sneath P HA, Staley JT, Williams ST. Bergey's manual of determinative microbiology. ***Williams and Wilkins, Maryland***. 1994
7. Yang SS, Huang I. Protease production by amylolytic fungi in solid state fermentation. ***J Chinese Agric Chem Soc.*** 1994, 32:589-601
8. Sathya G, Naidu N, Panda T. Application of response surface methodology to evaluate some aspects on stability of pectolytic enzymes from *Aspergillus niger*. ***Bioche Eng J.*** 1998, *2*(1):71-77
9. Rao K J, Kim CH,Rhee SK. Statistical optimization of medium for the production of recombinant hirudin from *Saccharomyces cerevisiae* using response surface methodology. ***Proc Bio.*** 2000, *35*(7):639-647
10. Teng Y, Xu Y. Culture condition improvement for whole-cell lipase production in submerged fermentation by *Rhizopus chinensis* using statistical method. ***Biores Technol.*** 2008, 99(9):3900-3907
11. Satyanarayana T, Pradeep K. Microbial glucoamylases: characterstics and applications. ***Critic Rev Biotechnol.*** 2009, 29:225-255
12. Chu WH. Optimization of extracellular alkaline protease production from species of Bacillus. ***J Ind Microbiol Biotechnol.*** 2007, 34(3):241-245

13. Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, Nasri M. Two detergent stable alkaline serine-proteases from Bacillus mojavensis A21: purification, characterization and potential application as a laundry detergent additive. ***Biores Technol.*** 2009, 100(13):3366-3373
14. Jignasha T, Satya T, Singh P. Secretion of an alkaline protease from a salt tolerant alkaliphilic, *Streptomyces clavuligerus* strain MIT. ***Braz J Microbiol.*** 2007, 38:766-772
15. Moreira KA, Albuquerque BF, Teixeira MFS, Porto ALF, Lima Filho JL. Application of protease from Nocardiopsis sp. as a laundry detergent additive. ***World J Microbiol Biotechnol***. 2002, 18(4):309-315
16. Kumar DM, Venkatachalam P, Govindarajan N, Balakumaran MD, Kalaichelvan PT. Production and purification of alkaline protease from *Bacillus* sp. MPTK 712 isolated from dairy sludge. ***Glob Vet***. 2012, 8:433-439
17. Elibol M, Moreira AR. Optimizing some factors affecting alkaline protease production by a marine bacterium *Teredinobacter turnirae* under solid substrate fermentation. ***Proc Bio.*** 2005, 40(5):1951-1956
18. Hiraga K, Nishikata Y, Namwong S, Tanasupawat S, Takada K, Oda K. Purification and characterization of serine proteinase from a halophilic bacterium, *Filobacillus* sp. RF2-5. ***Biosci biotechnol biochem.*** 2005, 69(1):38-44
19. Gupta A, Roy I, Patel RK, Singh SP, Khare SK, Gupta MN. One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. ***J chroma***. 2005, *1075*(1):103-108
20. Patel RK, Dodia MS, Joshi RH, Singh SP. Production of extracellular halo-alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp. isolated from seawater in Western India. ***World J Microbiol Biotechnol***. 2006, 22(4):375-382
21. Kumar CG, Takagi H. Microbial alkaline proteases: from a bioindustrial viewpoint. ***Biotechnol adv***. 1999, 17(7):561-594
22. Esakkiraj P, Immanuel G, Sowmya SM, Iyapparaj P, Palavesam A. Evaluation of protease-producing ability of fish gut isolate *Bacillus cereus* for aqua feed. ***Food bioproc technol.*** 2009, 2(4):383-390
23. Moon SH, Parulekar SJ. A parametric study ot protease production in batch and fed - batch cultures of Bacillus firmus. ***Biotechnol bioeng.*** 1991, 37(5):467-483
24. Khosravi-Darani K, Falahatpishe HR and Jalai M. Alkaline protease production on date waste by an alkalophillic *Bacillus* sp. 2-5 isolated from soil. ***Afr J Biotechnol.*** 2008, 7(10):1536-1542
25. Yu Y, Kim J, Hwang S. Use of real - time PCR for group - specific quantification of aceticlastic methanogens in anaerobic processes: population dynamics and community structures. ***Biotechnol bioeng.*** 2006, 93(3):424-433
26. Nadeem M, Qazi JI, Baig S. Effect of Aeration and Agitation Rates on Alkaline Protease Production by *Bacillus licheniformis* UV-9 Mutant. ***Turk J Biochem.*** 2009, 34(2):89-96
27. Dutta JR, Banerjee R. Isolation and characterization of a newly isolated *Pseudomonas* mutant for protease production. ***Braz arch of Biol Technol.*** 2006, 49(1):37-47
28. Aiba S, Humphrey AE, Millis NF. *Biochemical Engineering* 2nd edn, ***Academic Press Inc***. New York. 1973, 358-362
29. Carvalho RV, Côrrea TL, Silva JC, Mansur LR, Martins ML. Properties of an amylase from thermophilic *Bacillus* SP. ***Braz J Microbiol.*** 2008, 39(1):102-107
30. Kim S, Kim Y, Rhee IK. Purification and characterization of a novel extracellular protease from *Bacillus cereus* KCTC 3674. ***Arch Microbiol.*** 2001, 175(6):458-461

31. Kumar CG, Joo HS, Koo YM, Paik SR, Chang CS. Thermostable alkaline protease from a novel marine haloalkalophilic *Bacillus clausii* isolate. ***World J Microbiol Biotechnol.*** 2004, 20(4):351-357
32. Joo HS, Chang CS. Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. ***Proc Bio***. 2005, 40(3):1263-1270
33. Patil U, Chaudhari A. Optimal production of alkaline protease from solvent-tolerant alkalophilic *Pseudomonas aeruginosa* MTCC 7926. ***Ind J Biotechnol.*** 2011, 10(3):329-339
34. Nadeem M, Qazi JI, Baig S. Enhanced production of alkaline protease by a mutant of *Bacillus licheniformis* N-2 for dehairing. ***Braz Arch Biol Technol.*** 2010, 53(5):1015-1025