Pure Appl. Biol., 8(2): 1789-1800, June, 2019
http://dx.doi.org/10.19045/bspab.2019.80122

Research Article

Comparative study of alpha amylase producing mutated and unmutated Bacillus strains by taking cost effective measures

Irsa Ramzan, Afeefa Chaudhry, Hamid Bashir*, Muhammad Bilal and Aleena Sumrin

Center for Applied Molecular Biology, 87-West canal Bank Road, University of the Punjab, Lahore-53700-Pakistan
**Corresponding author's email: hamid.camb@pu.edu.pk*

Citation
Irsa Ramzan, Afeefa Chaudhry, Hamid Bashir*, Muhammad Bilal and Aleena Sumrin. Comparative study of alpha amylase producing mutated and unmutated Ba-cillus strains by taking cost effective measures. Pure and Applied Biology. Vol. 8, Issue 2, pp1789-1800. http://dx.doi.org/10.19045/bspab.2019.80122

Received: 22/03/2019 Revised: 20/06/2019 Accepted: 27/06/2019 Online First: 29/06/2019

Abstract

Bacillus species is considered as most appropriate bacterial specie for extracellular production of alpha amylase at commercial level. The alpha amylase is in high demand in a lot of food, beverage, and textile industries. In recent years, researchers have made many attempts to scale up the production of alpha amylase by using various strategies. The current study deals with the isolation, identification of Bacillus species and evaluation of their alpha amylase activity by random mutagenesis. Amylase producing bacteria were isolated from soil sample and further categorized via 16S rRNA technique. Random mutagenesis technique (MNNG and UV radiation) was employed to enhance the production of an enzyme. Mutants were then assessed for alpha amylase production via solid state fermentation using different agro-industrial waste, i.e. potato peels, apple peels, banana peels, wheat bran and rice husk. Different parameters such as incubation temperature, incubation period and pH were optimized to obtain maximum yield. Optimum amylase production (207.84U/ml) was obtained after 48 hours of incubation at 37°C by using MNNG mutant and potato peels. This study reveals that Bacillus cereus has the potential of alpha amylase production. It further suggests that secretion of alpha amylase can further be maximized by improving the strain genetically and optimizing the fermentation conditions.

Keywords: Alpha amylase; *Bacillus*; MNNG; 16S rRNA; UV radiation

Introduction

Alpha amylase (endo-1,4-a-D-glucan glucohydrolase) is a secretory enzyme responsible for the cleavage of 1,4-a-D-glucosidic bonds that link the glucose molecules in a linear amylase chain [1]. This enzyme, contributes to approximately 25% of the global enzyme market owing to its significant utilization in food, textile, brewing, paper and pulp industries [2]. Alpha amylase can be isolated from animals, plants and microorganisms [3]. Microorganisms have become the organism of choice for amylase production due to advantages like high availability, ease of handling and manipulation, cheap nutrient requirement and favorable growth conditions over other production methods [1, 4]. Several Bacillus species like *B. stearothermophilus* [5], *B. subtilis* [6], *B. licheniformis* [7] and *B. amyloliquefaciens* [8] are renowned

alpha amylase producers, and have been exploited widely due to their rapid growth rates, their capability to secrete prolific amount of proteins and general handling safety [9-11]. Several mutation studies have been carried out to achieve higher production of the enzyme as compared to the wild bacterial strains [12-14]. Many researches are now being carried out to cut the cost and time for enzyme production. Utilization of agro-industrial waste as substrates in solid state fermentation offers an alternative value-addition process for alpha amylase production [15, 16]. A number of wastes like coffee waste [16], gram husk, mustard oilseed cake, wheat bran, and rice bran [10] have been utilized as substrates in this respect. This study investigates the effect of UV and MNNG induced mutation in *Bacillus cerus* on the α-amylase production. Moreover, the current work studies the effect of potato peel, apple peel, wheat bran , banana peel, and rice husk as a substrates in case of alpha amylase production by using the mutant strains.

Materials and methods

Sample collection, isolation and screening of bacterial isolates

Soil samples of various origins were collected from starchy fields of Lahore, Pakistan. Alpha amylase producing bacteria were screened from the local soil habitat by plating the samples on Luria Bertani agar containing starch as a source of substrate [17-19]. One gram of the soil sample was added in 10 ml of sterilized distilled water, followed by shaking and incubation 15 min at 80^{o}C. 50µl from above sample was then plated on the agar medium supplemented with starch. Afterwards, the plates were incubated for 48 hours at 37^{o}C. Positive colonies formed clear zones of substrate degradation in the region of them were selected and stored for further processing.

Morphological identification of bacterial isolates

Morphological identification, gram staining, endospore staining, motility test and biochemical test of isolated bacteria were performed in a similar way as mentioned in "Bergey's manual of systemic bacteriology– (2004) [20].

Molecular characterization

Molecular characterization of isolates showing maximum amylolytic prospective was performed with the 16S rRNA primers technique [21] for the further confirmation bacterial strains. From fresh bacterial culture the genomic DNA was extracted, according to the method described by [22, 23]. For amplification of the 16S rRNA region, universal primers were used and confirm the sequence by sequencing the product.

Culture improvement by chemical mutagenesis

Overnight grown culture of *Bacillus species* was treated with MNNG. The stock solution of MNNG was prepared as 7.5mg/ml. 1ml of the bacterial cells were shifted to twelve sterile eppendorf tubes. Two of them were taken as control while the remaining tubes were treated with different concentrations (7µl, 14µl, 21µl, 28µl and 35µl) of MNNG from stock. Incubation was done at 37^{o}C for 1 and 2 hours, respectively, followed by centrifugation at 13000rpm for 7min. The cell pellet was resuspended in 1ml LB media and centrifuged at 13000 RPM for 7min. This step was repeated twice, followed by resuspension of cells in LB media.

UV mutagenesis

UV chamber was used to carry out mutations using the method adopted by [24, 25]. Vegetative culture of Bacillus cereus was centrifuged at 8000rpm for 10 min. The supernatant was discarded and cell pellet was resuspended in autoclaved phosphate buffer saline pH: 7. Resuspended cell solution (10ml) was then exposed to UV irradiation for 30-90 minutes with constant interval of

10min. 0.2 ml of the irradiated culture was spread on starch agar plates. The plates were subjected to incubation at 37°C for 24-48 h.

Screening of mutants

Colonies that showed largest starch hydrolysis zones were selected from UV and MNNG treated culture and were named as BCU1 and BCM1 respectively. These two colonies were preserved further for fermentation studies.

Vegetative inoculum preparation

LB (Luria-Bertani) medium was prepared using Tryptone1%, NaCl 0.5%, and yeast extract 0.5% in Erlenmeyer flasks of 250ml adjusted the pH 7.0 and autoclave the media. Culture from the fresh slants of BCU1, BCM1 and wild strains of *Bacillus subtilis* was inoculate in the media and Incubated in shaking incubator at 37ºC for 18 hours, at 200RMP.

Solid-state fermentation

Optimization of *amylase* enzyme production was carried out using solid state fermentation in 250ml Erlenmeyer flask. In this study five different fermentation media were prepared using agro-industrial wastes such as apple peel, banana peel, potato peel, rice husk and wheat bran. Following fermentation media (g/l) were evaluated alpha amylase for production: M1: Apple peel, Na_2HPO_4 (18g/L), KH_2PO_4 (9g/L), NaCl (1.5g/L), NH_4Cl (3g/L) M2: Banana peel, Na_2HPO_4 (18g/L), KH_2PO_4 (9g/L), NaCl (1.5g/L), NH_4Cl (3g/L) M3: Potato peels, Na_2HPO_4 (18g/L), KH_2PO_4 (9g/L), NaCl (1.5g/L), NH_4Cl (3g/L) M4: Rice husk, Na_2HPO_4 (18g/L), KH_2PO_4 (9g/L), NaCl (1.5g/L), NH_4Cl (3g/L) M5: Wheat bran, Na_2HPO_4 (18g/L), KH_2PO_4 (9g/L), NaCl (1.5g/L), NH_4Cl (3g/L). 1 % (v/v) vegetative culture of BCU1, BCM1 and wild strains of *Bacillus* was inoculated in each fermentation medium under aseptic conditions. All the cultures were incubated in shaking incubator at 37ºC for 18 hours, at 200RMP. After incubation supernatant of the culture was used to observe the of alpha amylase production.

Enzyme assay

The activity of *amylase* was determined by the method described **[26]**. 500µl of 20mM phosphate buffer (pH: 6.0) along with 1% starch solution was added to 500µl of enzyme solution. The tubes were incubated at 37ºC for 30 min. 0.5 ml of DNS reagent was added to 500µl of the reaction mixture in order to stop the reaction. The tubes were again incubated in water bath at 100ºC for 5 minutes. 4ml of water was added in each tube. Amount of reducing sugar produced was calculated to measure the absorbance at 540nm, with reference to the glucose standard. Assays were performed in triplicate and average values of all assays were reported. "One unit (U) of amylase is described as the amount of enzyme that releases 1 µmol of glucose as reducing sugar per minute under the assay conditions."

Results and discussion

Isolation, identification and screening of amylase producer

Selection of an appropriate strain is imperative for enhanced production of alpha amylase. In current research work soil samples were obtained from diverse starchy fields of Lahore. Screening of potent strains was done by spotting them on nutrient starch medium and the diameter of starch hydrolysis zones was measured that were formed around the colonies. Isolates showing a large zone of hydrolysis were selected for further study. Morphological and biochemical characteristics of the isolate are summarized in (Table 1).

Molecular characterization

In this study, 10 isolates were selected for sequencing with16S RNA to validate their molecular properties. To confirm the exact sequence blast all the sequence results in the gene databank with BLASTN2 software. Among the selected isolates, the isolate with the highest zone of hydrolysis shows a simi-

larity index of 94% with Bacillus cereus strain A65 (sequence ID: gi|1114444182|KX057546.1), and thus used further in mutation and fermentation studies. While the other isolates that were selected for 16S sequencing based on their hydrolytic activity against starch were found to be different strains of Bacillus subtilis. The sequencing results revealed that isolate with the highest zone of hydrolysis was the member of the genus Bacillus. The reason behind it might be due to highly prevalent these bacillus species in soil samples [27]. Anto *et al*. Also reported that Bacillus cereus has the ability to produce alpha amylase [28].

Mutagenesis

Amylase is a commercially valuable enzyme which is used in a lot of industrial processes. Mutations are thus induced to hyper activate the enzyme production in bacteria. Studies have proved that mutations not only increase the rate of cell growth, but they also augment the enzyme production ability of bacteria [29]. In this study maximum zone of hydrolysis with MNNG treatment was obtained when cells of bacterial strain Bacillus cereus strain A65 were treated with 7 µl of MNNG for 2hours.In (Table 2 & Figure 1) represents the zone of hydrolysis shown by the MNNG mutants. When *Bacillus cereus A65* was exposed to UV treatment from 30 to 90 minutes, highest zone was observed in a culture that was treated for 80 min shown in (Table 3 & Figure 2). After that, growth of culture decreases drastically along with reduction in starch hydrolysis shown in (Figure 2: plate 7, 8 & 9). Both the UV and MNNG mutant strain show a considerable amount of increase in starch hydrolysis zone as compared to wild strain. *Bacillus cereus* was observed to have (157.45 U/ml) of amylase activity, whereas BCM1 and BCU1 were found to produce 207.84 U/ml and 196.8U/ml of *amylase* activity respectively. From these studies, we found MNNG as a better mutagenic compound.

Table 1. Microscopic, macroscopic and biochemical properties of bacillus cereus

Morphological and biochemical characteristics of *Bacillus cereus*	
Characteristics	**Results**
Gram stain	Positive
Shape	*Bacilli*, rods
Sporulation	Positive
Growth in nutrient broth	Flocculent
Growth on nutrient agar	Abundant, cream to off-white color
Catalase test	Positive with effervescence
Starch hydrolysis	Positive
Casein hydrolysis	Positive

Pure Appl. Biol., 8(2): 1789-1800, June, 2019
http://dx.doi.org/10.19045/bspab.2019.80122

Table 2. Zone of starch hydrolysis shown by MNNG treated cells after 1 and 2 hours of incubation

Incubation time	MNNG conc. (µl)	Zone (mm)
0 hour	0 µl	15
1 hour	7 µl	16
	14 µl	17
	21 µl	17
	28 µl	17
	35 µl	17
2 hour	7 µl	18
	14 µl	16

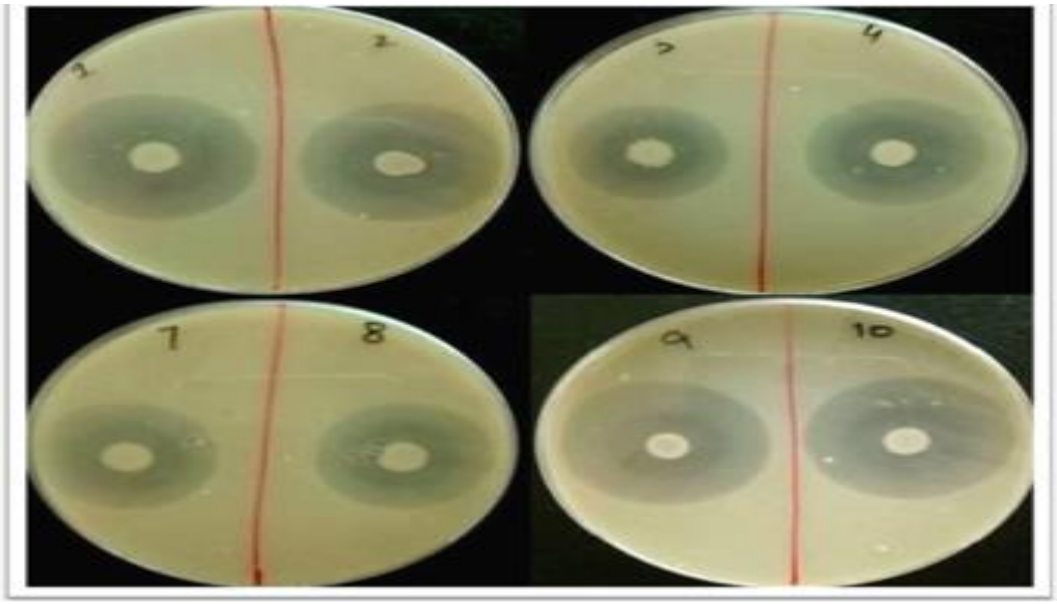

Figure 1. Zone of hydrolysis by selected colonies from 1 and 2 hours treated MNNG culture plates labeled as 1, 2, 3, 4 and 7 shows colonies selected from 1 hr treated culture and plates labeled as 9 and 10 are colonies from 2 hr treated cultures. No: 8 is untreated culture

Table 3. Data representing the zone of measurement and O.D of UV treated cultures

	wet mass (g)	O.D. (595nm)	zone (mm)	
			after 15 hrs	after 24 hrs
0 min	0.03	0.674	13	15
30 min	0.05	0.691	15	16
40 min	0.05	0.697	15	16
50 min	0.06	0.737	15	16.5
60 min	0.06	0.746	15.5	16.5
70 min	0.06	0.757	16	17
80 min	0.08	0.947	16	18
90 min	0.02	0.651	12	14

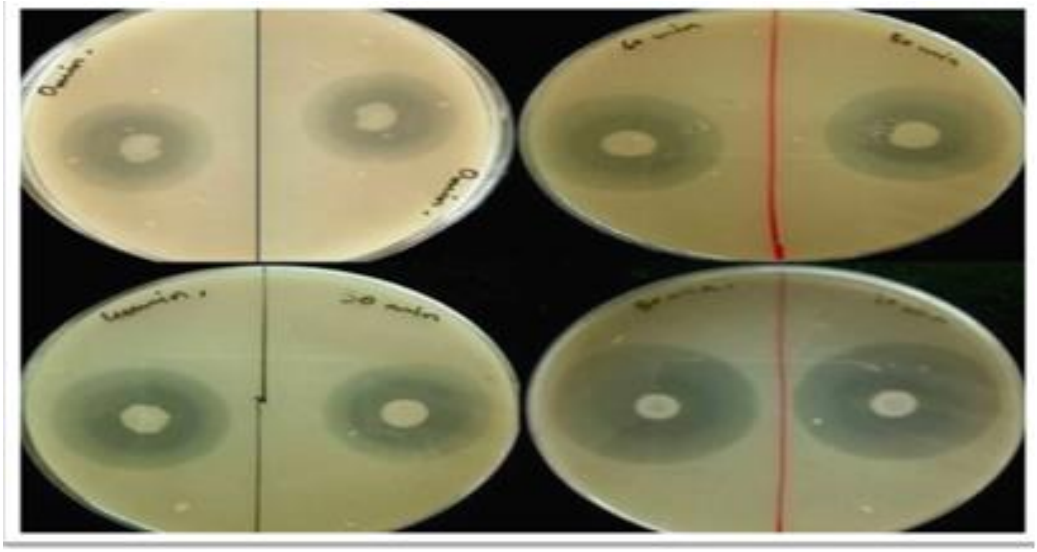

Figure 2. Plates representing zone of hydrolysis after UV treatment from 30 to 90 min

Screening of fermentation media

The choice of an appropriate medium plays a major task in the production of enzymes. Different fermentation media (M1–M5) were evaluated for the production of α-amylase by Bacillus cereus A65. Among all used media, M3 medium comprised of potato peels, Na_2HPO_4, KH_2PO_4, NaCl, and NH_4Cl gave the highest amylase production (207.84 U/ml), as compared to other media shown in (Table 4 & Figure 3). It may be due to the fact that potatoes are rich in starch. Figure:4 shows the results of SDS-PAGE analysis of different fermentation medium with wild, UV and MNNG mutant of Bacillus cereus.

Impact of incubation period

The effect of time of incubation on the production of alpha amylase was investigated (Figure 5). Fermentation medium was incubated for a total time period of 72 hrs at 37^{o}C and culture was drawn after every 12 hrs to determine the time period at which

amylase production got highest. Results depict that maximum *amylase* (207 U/ml) was produced at 48 hours of incubation. After that *amylase* production decreases. Decrease in activity after a particular time may be due to the reason that media run lack of nutrients. It was described by Reese et al. that after a particular time soluble substrate in the medium was completely consumed by microbes and the left over was crystalline portion, due to which the production decreases [30].

Table 4. Alpha amylase activity of Bacillus cereus and its UV and MNNG mutant (BCU1 and BCM1), using different substrates

	***Amylase* activity (U/ml/min)**				
	Apple	**Potato**	**Banana**	**Rice husk**	**Wheat bran**
Wild	86.4	157.45	59.78	55.2	87.5
UV	92.45	196.8	110.59	59.74	92.43
MNNG	110.4	207.84	111.32	63.21	98.70

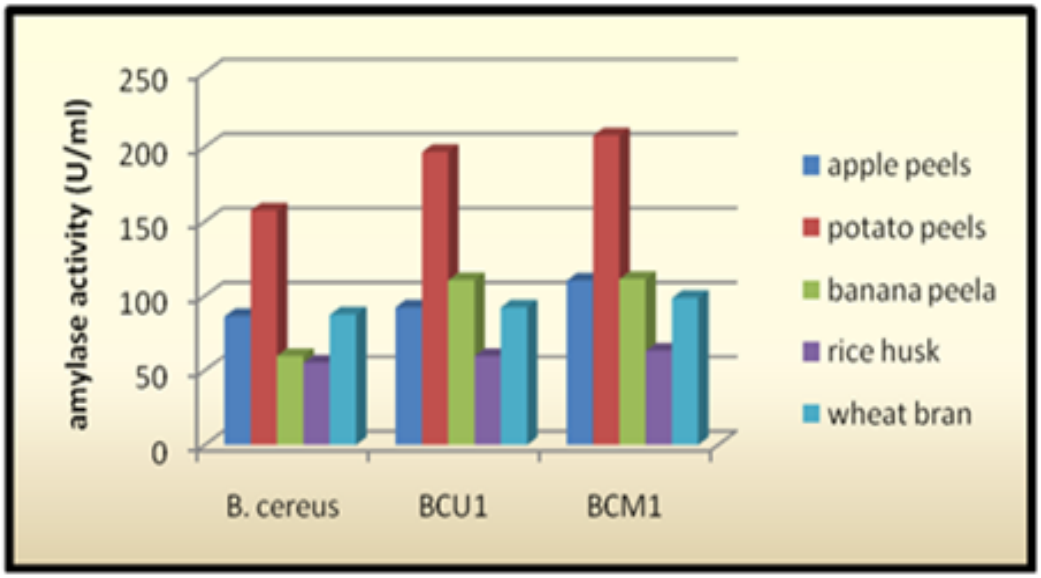

Figure 3. Effect of different substrates on wild and mutant strains of Bacillus cereus

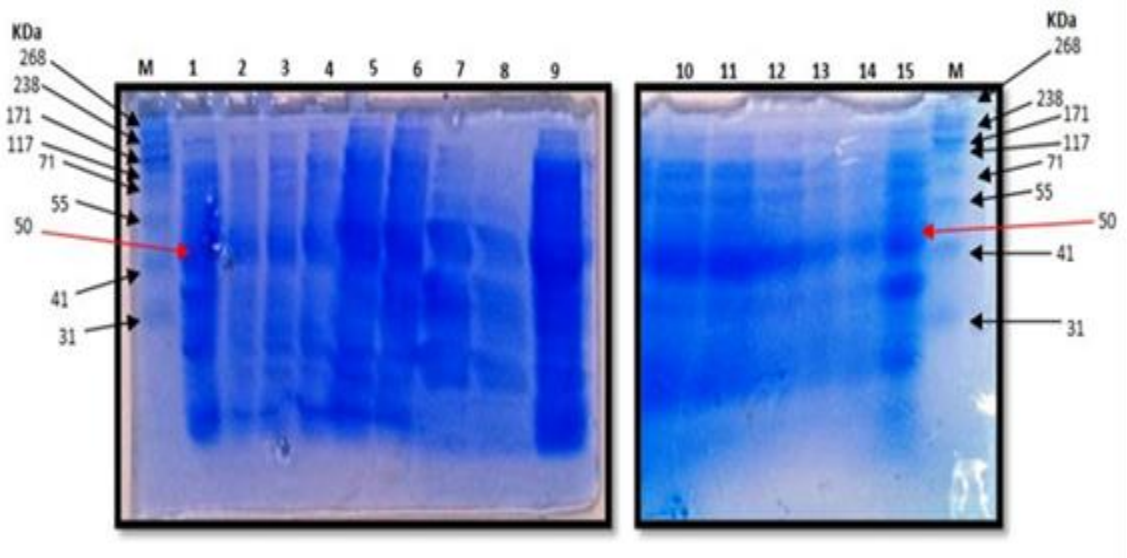

Figure 4. SDS PAGE analysis of different fermentation medium with wild UV and MNNG mutant of Bacillus cereus, Lane M is protein marker; Lane1- M1 media with BCM1; Lane2- M1 media with BCU1;Lane3- M1 media with wild Bacillus cereus; Lane 4- M2 media with wild Bacillus cereus; Lane 5-M2 media with BCM1; Lane 6- M2 media with BCU1; Lane7- M3 media with UV mutant; Lane 8- M3 media with wild Bacillus cereus; Lane 9- M3 media with MNNG mutant; Lane10-M5 media with MNNG mutant; Lane 11-M5 media with UV mutant; Lane 12-M5 media with wild Bacillus cereus; Lane 13-M4 media with wild Bacillus cereus; Lane 14-M4 media with UV mutant; Lane 15- M4 media with MNNG mutant

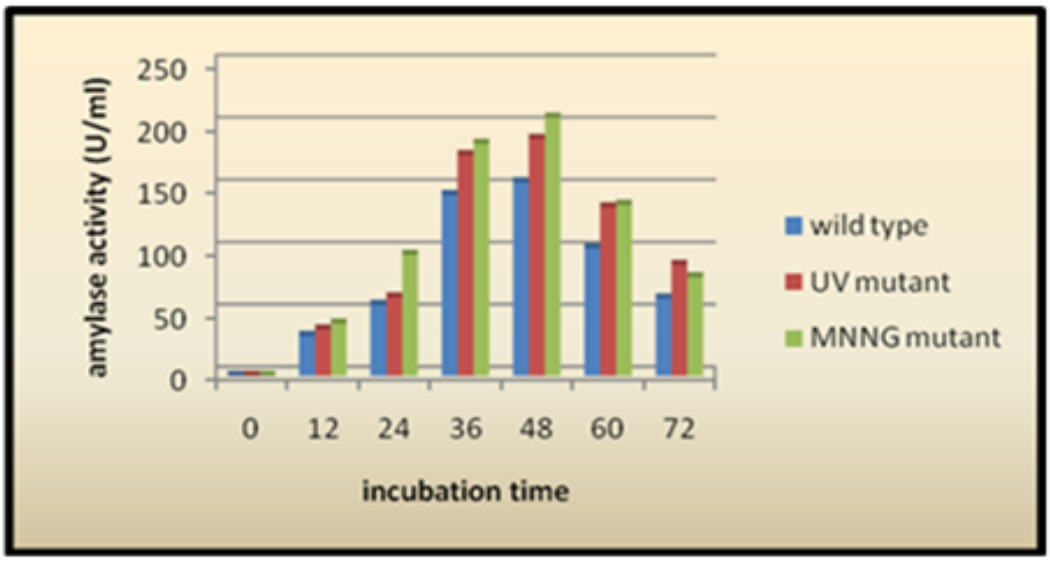

Figure 5. Effect of duration of incubation on amylase activity

Effect of incubation temperature

Temperature is one of the most important factors which not only maintains the microbial growth but also affects the enzyme production. In the current research, the effect of incubation at different temperature (17-57°C) was investigated (Figure 6).Maximum *amylase* production was obtained as 210 U/ml, during the incubation at 37ºC. Our results matched with Raul et al. who also reported maximum α-amylase production at 37 °C for 48 h of incubation [31]. At high incubation temperature enzyme production decreases mainly due to the fact that bacterial growth got suppressed due to elevated temperature, and finally, inhibit enzyme production [32].

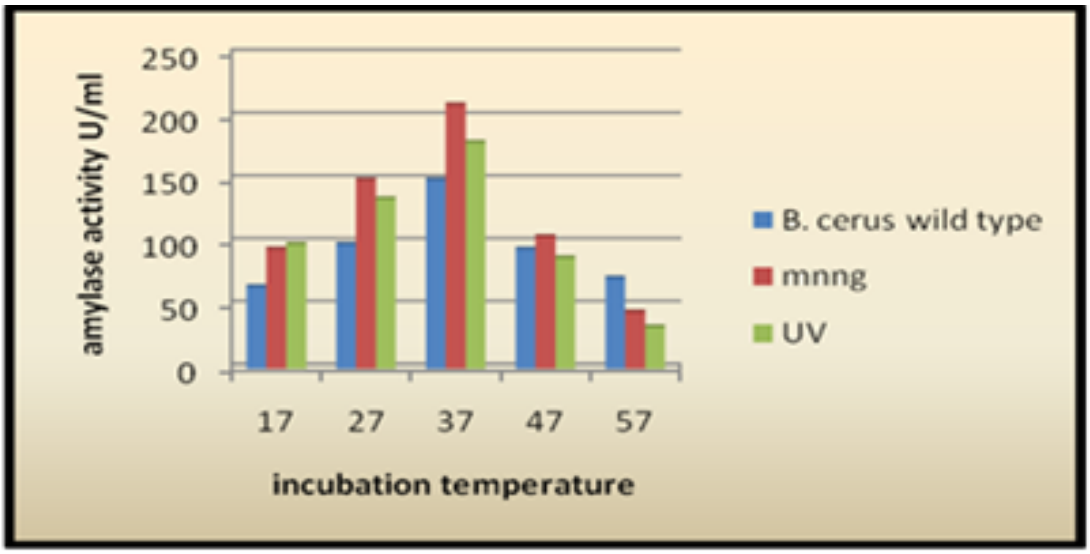

Figure 6. **Effect of incubation temperature on amylase production**

Effect of pH of fermentation medium

During culturing process pH of culturing medium is one of the most vital parameter because it can bring changes in culture as well as in secretion of enzymes. The influence of different pH (5–10) on the biosynthesis of amylase was studied (Figure 7). The maximum *amylase* production (205.4 U/ml) was observed at pH 7. These results are identical with Pavithra et al who reported that the enhanced activity of alpha amylase was observed at pH 7.0 [33]. At high pH enzyme production decreases because due to the high concentration of hydrogen ions in the bacterial cell growth suppresses which results in the decrease of enzyme production [34].

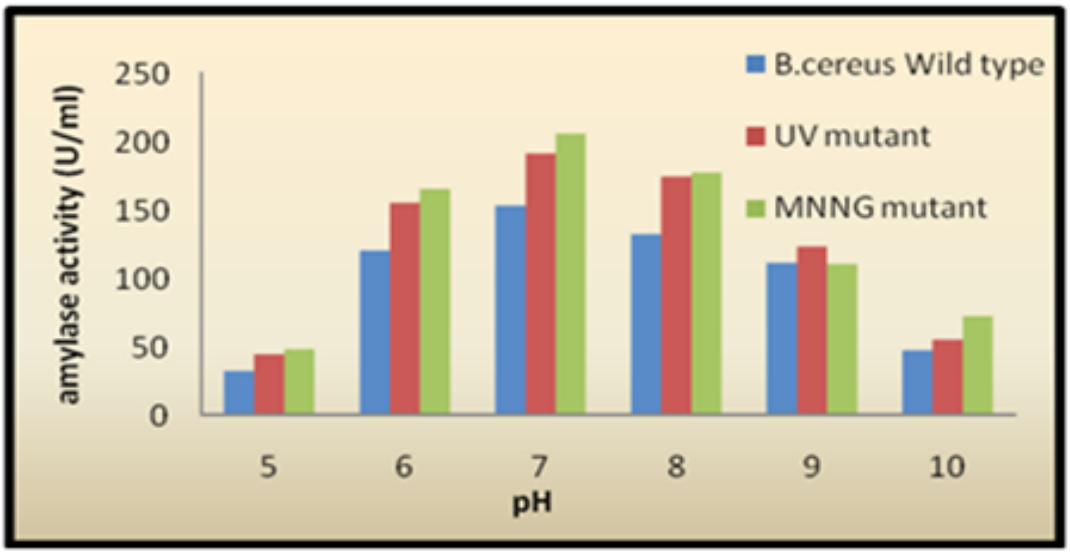

Figure 7. Effect of pH on production of alpha amylase

Conclusion

The present study suggests *Bacillus cereus* as a promising and economical organism for industrial utilization in the field of alpha amylase production. However, studies should be conducted to extract enzymes from thermophiles by using different genetic engineering techniques because amylase used in industrial process should be temperature stable. Moreover, scale-up studies are also needed for the establishment of a cost-effective bioprocess for commercial utilization of this organism for alpha amylase production.

Authors' contributions

Conceived and design the experiments: H Bashir & A Sumrin, Performed the experiments: I Ramzan, Analyzed the data: H Bashir, M Bilal & I Ramzan, Contributed reagents/material /analysis tools: M Bilal & A Chaudhry, Wrote the paper: I Ramzan, H Bashir & A Chaudhry.

References

1. Nigam P & Singh D (1995). Enzyme and microbial systems involved in starch processing. *Enzyme Microb Tech* 17(9): 770-778.
2. Reddy N, Nimmagadda A & Rao KS (2003). An overview of the microbial α-amylase family. *Afr J Biotechnol* 2(12): 645-648.
3. Sundarram A & Murthy TPK (2014). α-amylase production and applications: a review. *J of Appl & Environ Microbiol* 2(4): 166-175.
4. Souza PMD (2010). Application of microbial α-amylase in industry-A review. *Braz J Microbiol* 41(4): 850-861.
5. Srivastava R & Baruah J (1986). Culture conditions for production of thermostable amylase by *Bacillus stearothermophilus*. *Appl Enviro Microb* 52(1): 179-184.
6. Liu YH, Lu FP, Li Y, Yin XB, Wang Y & Gao C (2008). Characterisation of mutagenised acid-resistant alpha-amylase expressed in Bacillus subtilis WB600. *Appl Microbiol Biot* 78(1): 85-94.
7. Liu YH, Lu FP, Li Y, Wang JI & Gao C (2008). Acid stabilization of Bacillus licheniformis alpha amylase through introduction of mutations. *Appl Microbiol Biot* 80(5): 795-803.

8. Deb P, Talukdar SA, Mohsina K, Sarker PK & Sayem SA (2013). Production and partial characterization of extracellular amylase enzyme from Bacillus amyloliquefaciens P-001. *Springer Plus* 2(1): 1.
9. Soni SK, Goyal N, Gupta JK & Soni R (2012). Enhanced production of α-amylase from *Bacillus subtilis* subsp. spizizenii in solid state fermentation by response surface methodology and its evaluation in the hydrolysis of raw potato starch. *Starch Starke* 64(1): 64-77.
10. Saxena R & Singh R (2011). Amylase production by solid-state fermentation of agro industrial wastes using Bacillus sp. *Braz J Microbiol* 42(4): 1334-1342.
11. Van Dijl J & Hecker M (2013). Bacillus subtilis: from soil bacterium to super-secreting cell factory. *Microb Cell Fact* 12(1): 1.
12. Yoneda Y & Maruo B (1975). Mutation of Bacillus subtilis causing hyperproduction of alpha-amylase and protease, and its synergistic effect. *J Bacteriol* 124(1): 48-54.
13. Chi MC, Chen YH, Wu TJ, Lo HF & Lin LL (2010). Engineering of a truncated α-amylase of Bacillus sp. strain TS-23 for the simultaneous improvement of thermal and oxidative stabilities. *J Biosci and Bioeng* 109(6): 531-538.
14. Niu D, Zuo Z, Shi GY & Wang ZX (2009). High yield recombinant thermostable α amylase production using an improved Bacillus licheniformis system. *Microb Cell Fact 8*(1): 1.
15. Singhania RR, Patel AK, Soccol CR & Pandey A (2009). Recent advances in solid state fermentation. *Biochem Eng J* 44(1): 13-18.
16. Murthy PS, Madhava Naidu M & Srinivas P (2009). Production of α-amylase under solid-state fermentation utilizing coffee waste. *J Chem Technol Biot* 84(8): 1246-1249.
17. Demirkan E, Sevgi T & Başkurt M (2017). Optimization of physical factors affecting the production of the α-amylase from a newly isolated Bacillus sp. M10 strain. *Karaelmas Fen ve Mühendislik Dergisi* 7(1): 23-30.
18. Kaur A, Kaur M, Samyal ML & Ahmed Z (2017). Isolation, characterization and identification of bacterial strain producing amylase. *J of Microbiol and Biotechnol Res* 2(4): 573-579.
19. Mishra S & Behera N (2008). Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. *Afr J Biotechnol* 7(18).
20. Garrity GM, Bell JA & Lilburn TG (2004). Taxonomic outline of the prokaryotes. Bergey's manual of systematic bacteriology. Springer, New York, Berlin, Heidelberg.
21. Sambrook J (2001). Molecular cloning: a laboratory manual/Joseph Sambrook, David W. Russell: Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
22. Saito H & Miura KI (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim et Biophysica Acta (BBA)-Specialized Section on Nucleic Acids and Related Subjects* 72: 619-629.
23. Wilson K (1987). Preparation of genomic DNA from bacteria. *Current Protocols in Mole Biol* 2.4. 1-2.4. 5.
24. Gibson T & Gordon R (1974). Genus I. Bacillus. *Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore* 529-550.
25. Mao Z, Yu C & Xin L (2015). Enhancement of Phenol Biodegradation by Pseudochrobactrum sp. through Ultraviolet-Induced Mutation. *Inter J Mol Sci* 16(4): 7320-7333.

26. Kim CH, Sata H, Taniguchi H & Maruyama Y (1990). Cloning and expression of raw-starch-digesting α-amylase gene from Bacillus circulans F-2 in Escherichia coli. *Biochim et Biophysica Acta (BBA)-Gene Structure and Expression* 1048(2-3): 223-230.
27. Huang CJ, Wang TK, Chung SC & Chen CY (2005). Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. *BMB Reports* 38(1): 82-88.
28. Anto H, Trivedi U & Patel K (2006). α-Amylase production by Bacillus cereus MTCC 130 using solid-state fermentation. *Food Technol and Biotech 44*(2): 241-245.
29. Mobini-Dehkordi M, Jafari-Dehkordi S & Saffar B (2017). Characterization and increment of amylase production in mutant strains of Iranian native Bacillus licheniformis. *Biological J of Microorg* 6(21): 67-81.
30. Reese E, Lola J & Parrish F (1969). Modified substrates and modified products as inducers of carbohydrases. *J Bacteriol* 100(3): 1151-1154.
31. Raul D, Biswas T, Mukhopadhyay S, Kumar Das S & Gupta S (2014). Production and partial purification of alpha amylase from Bacillus subtilis (MTCC 121) using solid state fermentation. Biochem Res Inter 2014.
32. Nusrat A & Rahman SR (2007). Comparative studies on the production of extracellular α-amylase by three mesophilic Bacillus isolates. *Bangla J of Microbiol* 24(2): 129-132.
33. Pavithra S, Ramesh R, Aarthy M, Ayyadurai N, Gowthaman MK & Kamini NR (2014). Starchy substrates for production and characterization of Bacillus subtilis amylase and its efficacy in detergent and breadmaking formulations. *Starch Starke* 66(11-12): 976-984.
34. Gupta A, Gupta V, Modi D & Yadava L (2008). Production and characterization of α-amylase from Aspergillus niger. *Biotechnol 7*(3): 551-556.