Journal of Biotechnology Research
ISSN(e): 2413-3256, ISSN(p): 2413-8878
Vol. 4, Issue. 8, pp: 58-65, 2018
URL: http://arpgweb.com/?ic=journal&journal=16&info=aims

Academic Research Publishing Group

Original Research **Open Access**

Optimization of Amylase Production from Bacillus Cereus Using Solid State Fermentation

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Abstract

Aim: Amylases are the extracellular enzymes which catalyze the hydrolysis of carbohydrates contain glucose as monomers and attracted a worldwide enzyme market due to their potential applications in various industries like food, pharmaceutical, textile industries etc. The present study was conducted to isolate and characterize the high yielding amylase producer isolated from marine water sample using various natural and synthetic carbon sources for fermentation. Methodology and Results: *Bacillus cereus* was isolated from marine water (Visakhapatnam, India) for the production of extracellular amylase enzyme. The isolate was examined for the extracellular amylase production using starch agar media and results showed a large clearance zone in the starch agar plate which indicates that it was able to produce amylase in a considerable quantity. The production of enzyme by the *B.cereus* was initially detected using Pikovskaya's medium at temperature 37^{o}C, pH 7.0 for 24h of incubation time. The various process parameters were optimized, pH, temperature, various natural and synthetic carbon sources respectively. The optimal conditions for the production of amylase enzyme were found at temperature 37^{o}C (1890 µg/ml) and pH 6.0 (2000 µg/ml). Among the various synthetic carbon sources tested for optimum production lactose sugar will much enhances the production 5520µg/ml at 1% sugar concentration and in natural carbon sources maximum amount was produced with jaggery 2140 µg/ml at 5% concentration. Conclusion, Significance and Impact of Study: From the study it was concluded that *Bacillus cereus* was the efficient α-amylase enzyme producer by utilization of various synthetic and natural carbon sources for fermentation. A considerable interest can be given in using jaggery, wheat flour and barley as an alternative sources of carbon for α-amylase production by *Bacillus cereus* and makes the production economically feasible at industrial level.

Keywords: Alpha-amylase; Bacillus cereus; Synthetic carbon sources; Natural carbon sources.

1. Introduction

Alpha-amylases cleaves the 1,4-α-D-glycosidic linkages that are present between the adjacent glucose units of linear starches, glycogen, and oligosaccharides in a random manner [1]. Microbes account more than 90% of the marine biomass and majority of life remains unknown because it is difficult to observe in nature and many of the marine microbes have failed to grow on synthetic media but our knowledge on the microbial diversity of oceans is still in untapped. These marine microbes contain different types of enzymes like protease, amylase, lipase and oxidase etc. Among them starch degrading amylolytic enzymes have a great commercial value in biotechnological applications. Spectrum of applications of amylase has widened in many sectors such as industrial, food, medical and analytical chemistry. Amylases also used in fermenting, baking, brewing, detergent, textile, paper and distilling industries. Although amylases can be derived from several sources such as plants, animals and microbes, the microbial amylases meet industrial demands. Amylase has been derived from several fungi, yeasts, bacteria, and actinomycetes members. However, enzymes from fungal and bacterial sources have dominated applications in industrial sector [2]. Many marine treasures of enzyme producing microorganisms produce this enzyme, which include *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Aspergillus niger* and *Pencillium*. Earlier reports are also in agreement with the fact that most of the *Bacillus* species, namely, *Bacillus licheniformis* and *Bacillus stearothermophilus*, are the most effective producers of alpha-amylase [3, 4]. Amylases are used in preparation of chocolate syrups, in which the chocolate starch in dextrizing and thus syrup does not become thick. Bacillus cereus is an endemic, soil-dwelling, Gram-positive, rod-shaped, beta hemolytic microorganism. Some strains are harmful to humans and cause food borne illness; while other strains can be beneficial as probiotics for animals and it's have a broad industrial importance. It is common mesophile and saprophyte and widely distributed in nature in soil, fresh water and sea water. It secrets some extracellular enzymes and hydrolyze the proteins, carbohydrates and lipids. Agrowastes like wheat bran, rice bran, and coconut oil bran have replaced the high cost media generally used in submerged fermentation for alpha-amylase preparation because of their simplicity, low cost,

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easy availability, better productivity, and lesser water output [5, 6]. Additionally it solves the pollution problem occurring due to their disposal in the surrounding [2]. High starch content of almost all agrowastes (60–70% by weight) can be effectively utilized as a major nutrient source by microorganisms like bacteria, fungi, and so forth, for the synthesis of inducible alpha-amylase which is under the control of catabolic repression.

Based on the prior knowledge and literature survey on amylase production, the present study was initiated and conducted to isolate and characterize the high yielding amylase producer isolated from marine water sample using different natural and synthetic carbon sources.

2. Materials and Methods

2.1. Sample collection

The marine water sample were collected from the coastal area of Visakhapatnam in a sterile BOD bottle and kept under sterilized conditions, and labeled by the date of collection and transported to the laboratory for further study.

2.2. Isolation of bacteria

The collected marine water samples at a concentration of 0.1ml were inoculated and spread on the surface of the starch agar plate and incubated for 24 hours at 37^{o}C. After incubation, the plates were observed for colony characteristics such as size, surface texture, pigmentation, opacity features etc. Gram staining and spore staining methods were performed to observe the morphological characteristics. The isolates were used for bio chemical studies. The various biochemical tests like IMViC tests (Indole, methyl red, Voges-proskauer and citrate utilization test), starch hydrolysis, urease, sugar fermentation, H_2S production, oxidative fermentation and nitrate reduction etc. were performed for the identification of bacterial isolate. All the cultures were incubated at 37°C for 24-48hrs [7].

2.3. Screening and Selection of Amylase Producer

All the isolates were examined for the extracellular amylase production using starch agar media containing soluble starch as carbon source. After incubation the plates were flooded with 1% iodine solution for 1 min until the entire media perfectly colored in blue. Appearance of clear zone around the colonies in blue colored medium was taken for further studies [7].

2.4. Production of Amylase

Overnight grown pure culture of amylase producer was inoculated into 1L of standard Pikovskaya's medium which contains Dextrose 10 g, $(NH_4)_2SO_4$ 0.5g, $Ca_3(PO_4)_2$ 5.0g, $MgSO_4.7H_2O$ 0.1g, $MnSO_4.7H_2O$ 0.0001g, $FeSO_4$ 0.0001g, KCl 0.2g and Yeast extract 0.5g. Fermentation was performed at 37^{o}C, pH 7.0 for 24 hours.

2.5. Extraction of Enzyme

After incubation, the broth was subjected to centrifugation at 5,000 rpm for 15min at room temperature. The supernatant was collected in sterile test tubes and the pellet was discarded. The supernatant was used for the assay of amylase.

2.6. Amylase Assay

The amylase activity was determined by DNS method using starch as substrate. Maltose is produced by the hydrolytic activity of amylase. Amylase breaks the α-1,4 linkage present in the polysaccharide (starch or glycogen) to yield maltose. It is reduced by 3,5-Dinitrosalicylate to an orange-red colored 5-nitro 3-amino salicylate which can be measured calorimetrically at 520nm. 1ml of culture filtrate was boiled in water bath for 20 min. Boiled samples were cooled suddenly and used as killed enzyme sample. Both killed and live samples were taken for the assay. The reaction mixture consists of 1mL of enzyme, 2.5mL of substrate; 2.5mL of sodium phosphate buffer and 1mL of sodium chloride, was incubated in a water bath at 37^{o}C for 15 min. Then, the reaction was terminated by adding 1mL of DNS, and immersing the tubes in boiling water bath. The absorbance was measured at 520nm. The OD value of killed enzyme was subtracted from the live enzyme value. One unit of enzyme activity was defined as the amount of enzyme releasing 1µmol of reducing sugars per minute under the standard assay conditions. So the amount of maltose liberated was determined using standard curve and expressed the activity of amylase in µg of maltose liberated per ml of supernatant [8].

2.7. Partial Purification of Amylase Enzyme

The total protein content from the sample was determined using Bradford method. The partial purification of amylase enzyme was performed by ammonium sulphate precipitation followed by dialysis. The sample was centrifuged and the cell free extract was saturated with ammonium sulphate up to 70%. The mixture was stored in cold room for 24 hours to precipitate all the proteins, and the precipitation was separated by centrifugation at 10,000rpm for 10min. Then carefully the aqueous phase was discarded, and the remaining precipitate was dissolved with 5mL of 1M citrate phosphate buffer (pH 5). The dialysis bag containing precipitated protein was freely suspended in a beaker containing 1M citrate phosphate buffer, and was stirred slowly using magnetic stirrer. The entire setup was positioned in cold room for 48 hours and the buffer was changed every 12 hours periodically. After the dialysis, the sample was shifted into a clean lyophilized flask.

2.8. Optimization of Fermentation Parameters

Various physico-chemical parameters which increases the production of amylase enzyme were studied in the optimization studies which include temperature (28°C - 48 °C), pH (5.0-9.0) and carbon sources which include both natural and synthetic carbon sources listed in Table 1. The synthetic and natural carbon sources are used as supplements at a concentration ranging from 1 to 5%.

3. Results and Discussion

3.1. Isolation and Screening of Amylase Producer

The organism *Bacillus cereus* showed smooth, shiny, glistering, slightly elevated, irregular colonies on nutrient agar culture plates. The organism was Gram-positive Streptobacilli (Figure 1), motile and spore forming. The organism was positive for catalase production, Voges-proskauer test, and citrate utilization test and able to ferment carbohydrates like glucose, fructose, glycerol, maltose, and ribose. It showed beta haemolysis on blood agar and positive for esculin hydrolysis (Figure 2).

3.2. Starch Hydrolysis

The starch agar plate was inoculated with *B. cereus* and kept for 48 hours at 37°C. The plate was flooded with iodine and clear zone of starch hydrolysis (Figure 3) has been observed. This ensures that this microorganism secretes amylase that is capable of starch hydrolysis. The isolated organism showed a large clearance zone in the starch agar plate which indicates that it was able to produce amylase in a considerable quantity. From the industrial point of view one of the essential important features of any microbial strain is able to produce large quantity of the enzyme within a short period of fermentation time. Our isolated microbial strain produced very high amount of amylase, 2750µg/ml in only 24h which is much higher than that reported by earlier studies [9, 10].

3.3. Effect of Temperature on Amylase Production

To optimize the optimum temperature for better production, various temperatures were monitored. The temperature requirement of the organism for its optimum growth is 37°C and production of the enzyme (1890µg/ml) was also found to be higher at 37°C (Figure 4). This identifies the exclusive feature of the *Bacillus* organism that grows as mesophile at 37°C, but the enzyme it produces will be active and stable at higher temperatures (>50°C) . Similar reports were reported by [11, 12].

3.4. Effect of pH

The effect of pH was studied by varying the pH of the organism to determine the optimum production of amylase enzyme. It was found that, pH 6.0 is the optimum pH for the organism and produces maximum amount of amylase (2000µg/ml) (Figure 5). The amylase enzyme activity was higher than at acidic pH than at alkaline conditions. Generally at alkaline conditions the organism produces more proteins that may degrade or inactivate the enzyme. In such cases, bacterium should be maintained at acidic conditions to achieve maximum amylase production. The optimum pH for production and growth was found to be 6.0 and the enzyme will stable up to pH 5–7 [13].

3.5. Effect of Synthetic and Natural Carbon Sources On the Production of Amylase Enzyme

By using various synthetic carbon sources as supplements to the basal medium Pikovskaya's medium to enhance the production of alpha-amylase enzyme, the results revealed that (Table 2) lactose sugar will much enhances the production 5520µg/ml at 1% sugar concentration (Figure 6) followed by galactose 4480 µg/ml at 5% sugar concentration (Figure 7) and sucrose 4400 µg/ml (Figure 8) at 1% sugar concentration. However when natural carbon sources were used as supplements for the production of alpha-amylase, the production is not much higher when compared to pure sugars used for amylase production. Among various natural carbon sources used for the production (Table 3) maximum amount was produced with jaggery 2140 µg/ml at 5% concentration (Figure 9) followed by barley 1850 µg/ml at 5% concentration (Figure 10) and wheat flour 1780 µg/ml at 3% concentration (Figure 11). In the current study jaggery was found to be the best carbon source for the production of alpha-amylase enzyme and based on previous literature it was found that this was the first report on the amylase production from jaggery as a substrate for amylase production. The results also found that wheat flour also showed opulent production which was similar to the reports of Ikram-Ul-Haq, *et al.* [14] showed wheat bran as the best substrate for α-amylase production by *Bacillus licheniformis* species. Shatta, *et al.* [15] utilized many agro-industrial wastes like wheat bran, rice bran, yellow maize meal, dry yeast, brewery yeast and corn steep liquor in basal medium as sole carbon source for the production of amylase by *Streptomyces species* where as Kammoun, *et al.* [16] reported amylase production on wheat gruel based medium using *Aspergillus oryzae*.

4. Conclusion

The present study showed that the production of α-amylase enzyme by utilization of various synthetic and natural carbon sources employing *Bacillus cereus* as the fermenting organism. A considerable interest can be given in using jaggery, wheat flour and barley as an alternative sources of carbon for α-amylase production by *Bacillus cereus* and makes the production economically feasible at industrial level.

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Appendix

Table-1. Various natural and carbon sources used for amylase production

Natural Carbon Sources	Synthetic Carbon Sources
Metroxylon rumphii (Sagu)	Maltose
Triticum vulgare (Wheat flour)	Xylose
Oryza sativa (Rice flour)	Starch
Triticum vulgare (Maida)	Lactose
Zeamays (Corn flour)	Galactose
Eleusine coracana (Ragulu)	Sucrose
Saccharum officinarum (Jaggery)	Fructose
Hordeum vulgare (Barley)	Dextrose
Solanum tuberosum (Potato)	Mannitol
Colacasia esculenta (Chema)	Cellulose

Table-2. Production of Amylase by *Bacillus cereus* using synthetic carbon Sources

Sugars	Amylase Production (µg/ml) With Different Percentages of Sugar Concentration				
	1%	2%	3%	4%	5%
Maltose	960	880	2120	2960	2800
Xylose	3480	1040	1040	1280	1040
Starch	3890	160	2300	520	560
Lactose	5520	0	1800	3780	980
Galactose	1300	1580	2300	2020	4480
Sucrose	4400	1100	1760	1950	1300
Fructose	1680	1840	200	3040	3580
Dextrose	1440	1360	2880	920	2240
Mannitol	160	320	440	120	480
Cellulose	40	420	440	200	640

Table-3. Production of Amylase by *Bacillus cereus* using natural carbon sources

Natural carbon sources	Amylase Production (µg/ml) With Different Percentages of Natural Carbon Source Concentration				
	1%	2%	3%	4%	5%
Metroxylon rumphii (Sagu)	320	1040	1440	1160	1000
Triticum vulgare (Wheat flour)	1320	600	1780	920	710
Oryza sativa (Rice flour)	480	700	360	160	1060
Triticum vulgare (Maida)	440	320	160	640	1200
Zeamays (Corn flour)	800	1080	320	720	640
Eleusine coracana (Ragulu)	160	280	490	600	880
Saccharum officinarum (Jaggery)	1480	1840	1980	1750	2140
Hordeum vulgare (Barley)	210	600	720	990	1850
Solanum tuberosum (Potato)	450	560	920	800	1360
Colacasia esculenta (Chema)	1100	320	400	510	503

Figure-1. Gram staining of *Bacillus cereus*

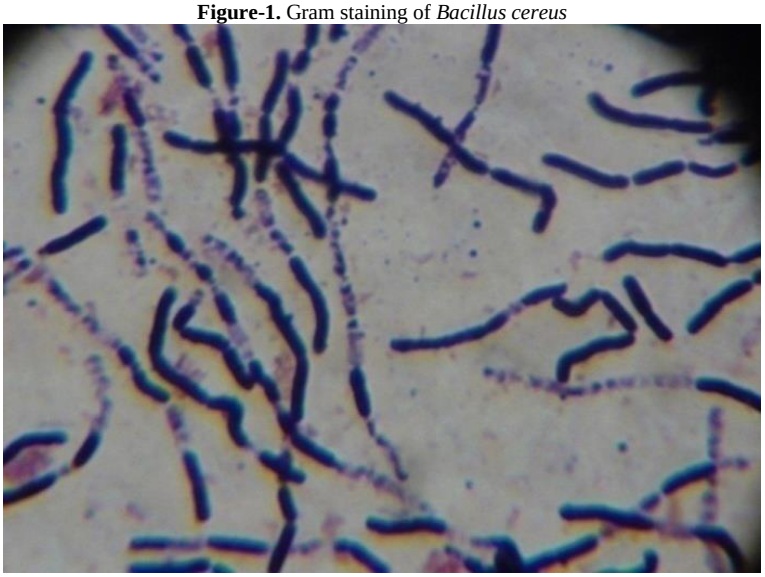

Figure-2. Esculin hydrolysis by *Bacillus cereus*

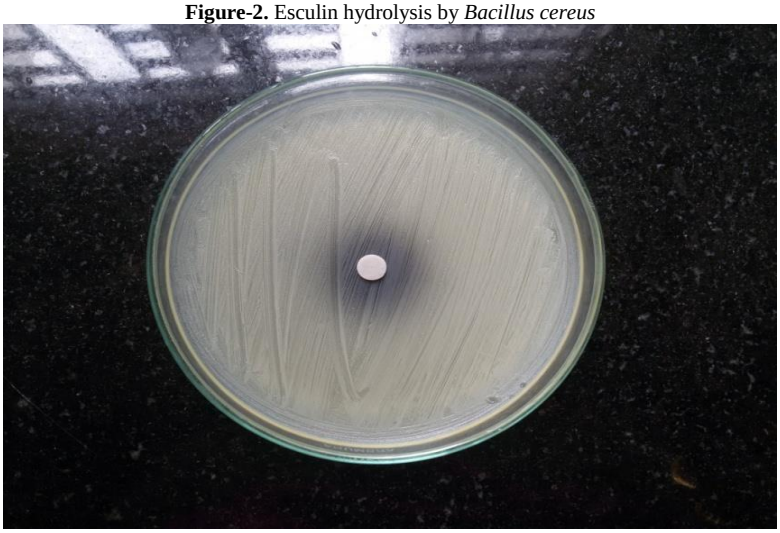

Figure-3. Screening for amylase production in starch agar plate

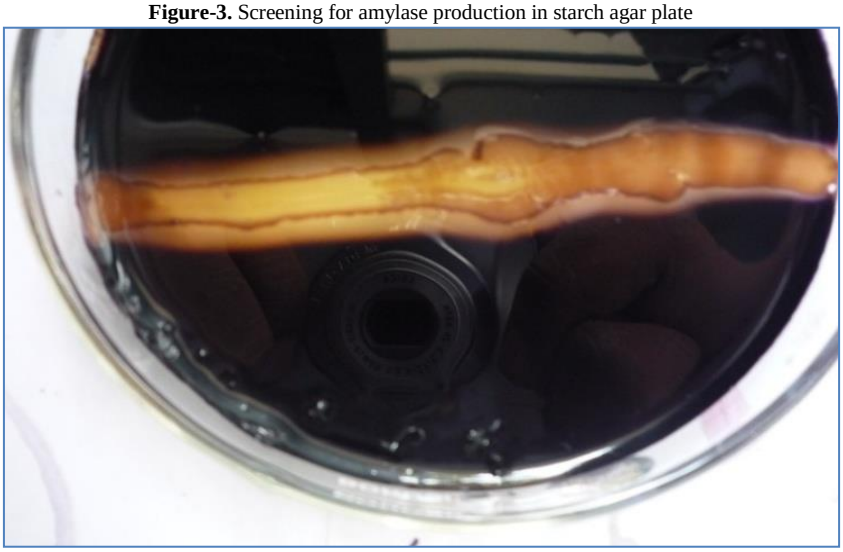

Figure-4. Effect of temperature on the production of amylase enzyme by *B.cereus*

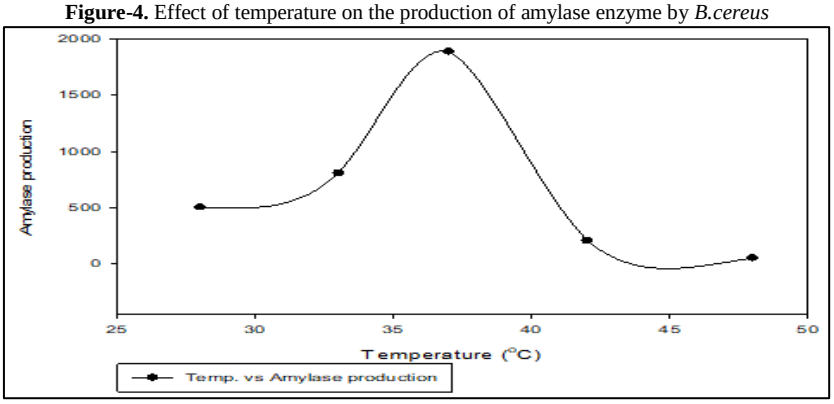

Figure-5. Effect of pH on the production of amylase enzyme by *B.cereus*

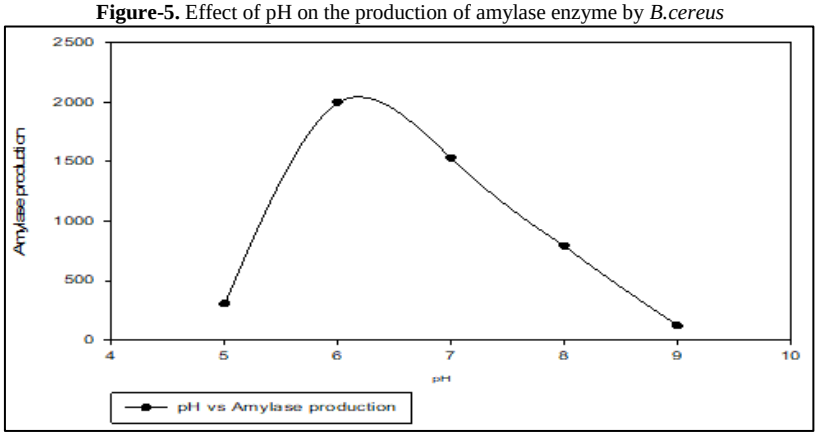

Figure-6. Production of alpha-amylase by *B.cereus* using lactose as carbon source

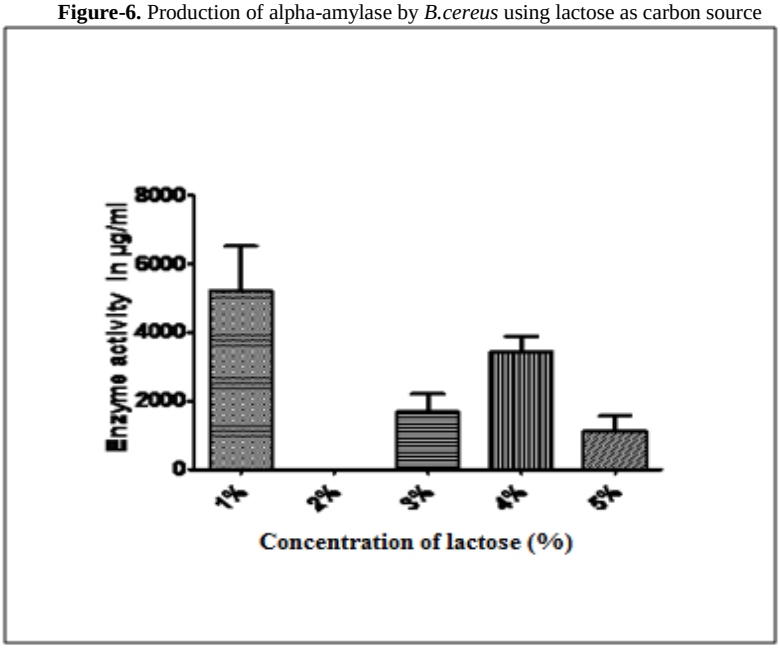

Figure-7. Production of alpha-amylase by *B.cereus* using galactose as carbon source

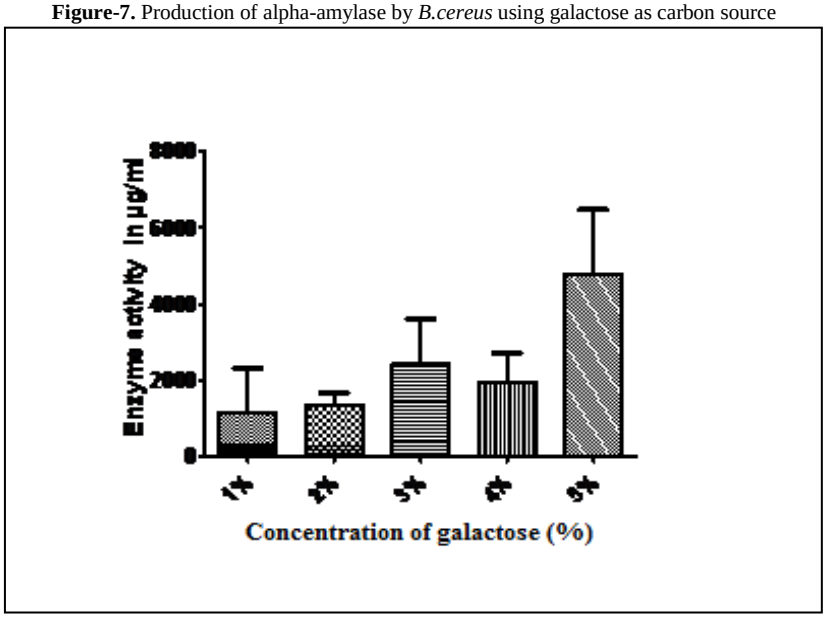

Figure-8. Production of alpha-amylase by *B.cereus* using sucrose as carbon source

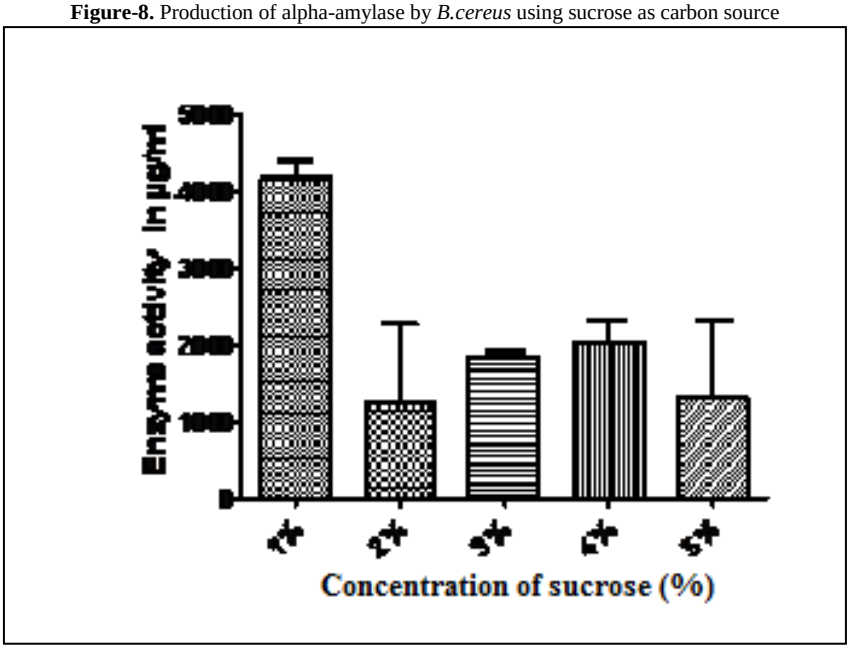

Figure-9. Production of alpha-amylase by *B.cereus* using jaggery as carbon source

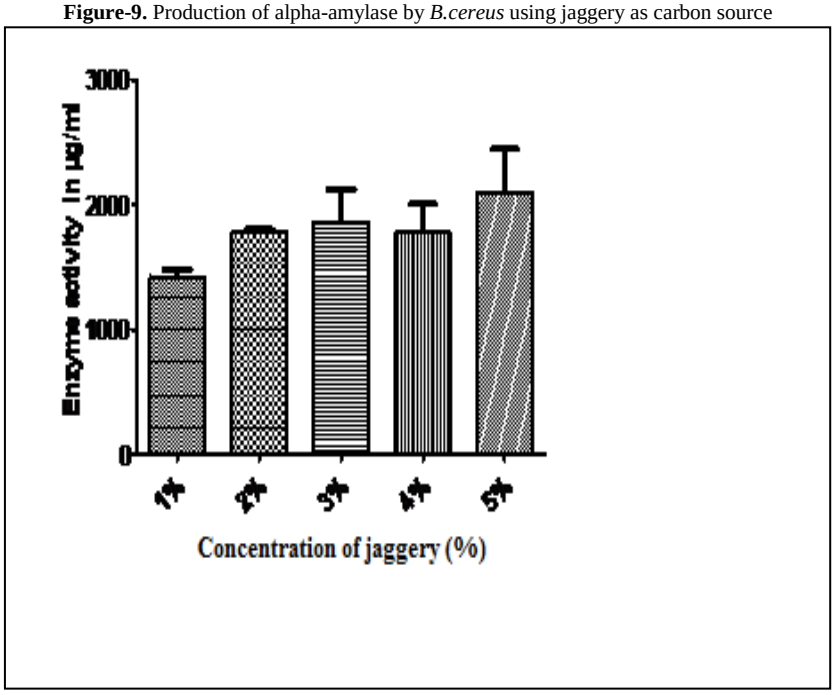

Figure-10. Production of alpha-amylase by B.cereus using barley as carbon source

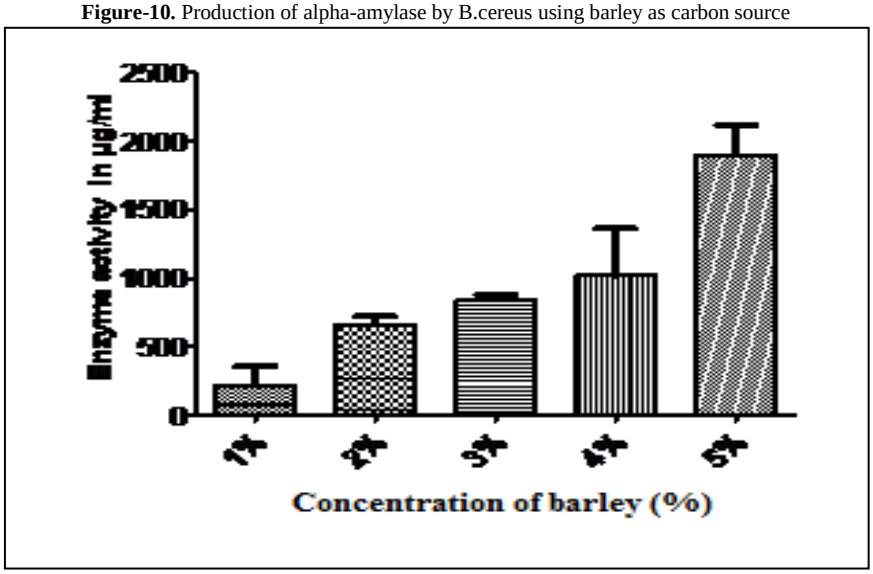

Figure-11. Production of alpha-amylase by *B.cereus* using wheat flour as carbon source