Characteristic of Amylase Produced by *Bacillus subtilis* Isolated from Stale Rice

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Abstract:

Bacillus *subtilis* is isolated from Stale rice. It had aggregation time 52 mins. It showed protease, lipase, and amylase activity. It passes cell surface hydrophobicity 82.8%; resistance to acidic condition (pH 3 for 90 min) and growing in presence of bile salts (in culture medium containing more than 0.15% bile salt). The thermo stable extracellular amylase was isolated and partially purified, the optimum temperature and pH for it was found to be 55^{0}C and 6.5 respectively. The maximum amylase production was seen with maltose as carbon source while among the nitrogen sources, complex nitrogen sources support for maximum amylase production.

Keywords:
Bacillus, Stale rice, probiotics, thermostable, amylase.

Introduction:

Bacillus species, such as *Bacillus subtilis, Bacillus licheniformis, Bacillus axarquiensis* and *Bacillus pumilus,* produce biosurfactants (Arima et al., 1968; Naruse et al., 1990; Yakimov et al., 1995), compounds that reduce surface and interfacial tension and thus have excellent detergent, emulsifying, foaming and dispersing properties. They are used extensively in the textile, pharmaceutical and cosmetics industries and also in bioremediation (Banat et al., 2000).

Similar to Stale rice in India, Natto is a traditional Japanese food made from soybeans fermented with *Bacillus subtilis*. Due to health benefits it is most popular in the eastern regions of Japan, including Kanto, Tohoku, and Hokkaido but less popular in Kansai. The natto is produced with starter culture containing *Bacillus subtilis*. *Bacillus sp.* are Gram-positive long rods, and classified as Kingdom: *Bacteria*, Phylum: *Firmicutes,* Class: *Bacilli*, Order: *Bacillales,* Family: *Bacillaceae,* Genus: *Bacillus*.

Bacillus subtilis (B-3) is isolated from Stale rice, its 16S rRNA gene amplified by PCR using following primers and standard protocols.
Forward primer: Bac 8f (5'AGAGTTTGATCCTGGCTCAG3')
Reverse primer: Univ592r (5'ACCGCGGCKGCTGGC3')
The sequences obtained was compared to reference 16S rRNA gene sequences available in the Genbank, and found 86% identical with *Bacillus subtilis* subspecies natto.

Isolated *Bacillus subtilis* has been found to be amylase positive as hydrolyzing starch. The amylases are industrially important like microbial amylase, which has higher yield and thermo stability. They are used also in industries like food, fermentation, textile paper and detergent. The efficiency of microbial amylases has been proved to be better than chemical hydrolysis.

Material and methods:

Isolation of Bacillus:

The *Bacillus subtilis* was isolated from Stale riceon medium; rice powder: 0.5%, peptone: 0.5%, K_2HPO_4: 0.2%, $MgSO_4$: 0.05%, $FeCl_3$: traces, and agar: 2%.

Cell surface hydrophobicity test:

It was determined by the method of Rosenberg et al. The strain was harvested after 18h of growth, washed twice and suspended in saline solution to OD of 0.5 at 600 nm. To 3 ml of washed cells, 1 ml of toluene added and mixtures were blended for 90 seconds. The tube was left to stand for 15 min for separation; the OD of the aqueous phase was taken. Hydrophobicity was given by the percentage decrease in the OD of the bacterial suspension due to partitioning of cells into the hydrocarbon layer. Percentage of hydrophobicity = [(OD600 before mixing - OD600 after mixing) / OD600 before mixing] x100 (Handly et al.).

Effect of carbon and nitrogen sources on production of amylase:

For optimization of cultural conditions, following media was used, whose composition as, starch, 10.0 g; yeast extract, 3.0 g; peptone, 5.0 g; NaCl, 3.0 g; $MgSO_4.7H_2O$,0.05 g; dist. water 1 liter & pH adjusted to 7. For study of effect of carbon source in media, starch was replaced by different 1.0% carbon sources as mentioned in Table 3. For effect of nitrogen source in media, peptone, & yeast extract were replaced by different 1% nitrogen sources as mentioned in Table 4.

Amylase assay:

For amylase assay 0.5 ml of 1 % starch in 0.1M phosphate buffer (pH 6.5) and adding 0.5 ml of enzyme were incubated for 30 min at room temperature i.e. 37^0C. While the reaction was stopped by adding 1.0 ml of dinitrosalicylic acid reagent, heated on boiling water bath 5 min and then to it 10 ml dist. water was added. Absorbance was checked at 540 nm against blank. The blank was the same as above without incubation. One unit of the amylase activity was defined as the amount of enzyme that liberated one µmole of reducing sugar under experimental condition.

Partial purification of amylase:

From Stale rice total 32 *Bacillus* were screened based on aggregation time, cell surface hydrophobicity, co-aggregation, tolerance to bile salts and

acidic condition and finally selected *Bacillus subtilis* (B-3). The results showed that ithas amylase, lipase, and protease activity. This isolate was selected for partial purification of amylase. The inoculums was prepared from slant culture by transferring a loop-full of cells in inoculums media 50ml in 250ml fermentation flask and incubating at room temp in a rotary shaker at 120 rpm for 48 h. The fermentation medium was inoculated with 0.1% inoculums (medium 100ml in 250ml flask) and incubated for 72 hrs. On 48 hrs of fermentation, broth was centrifuged at 6000 rpm for 15 min at 4^0C. The partial purification of enzyme was carried out by ammonium sulphate precipitation (40%). Bradford method was used to estimate enzyme protein using bovine serum albumin as standard (Kotiranta et al).

Effect of pH and temperature on amylase:

Effect of pH was from pH 2.0 to 12.0 (using HCl/KCl buffer for pH 2; glycine/HCl buffer for pH 2.5 to 3.5; acetate buffer for pH 4 to 5.5 phosphate buffer for 6 to 7.5; tris/HCl buffer for pH 8 to 9; glycine/ NaOH buffer for 11 to 12). Effect of temp was examined from 5^0 to 80^0C.

Results and discussion:

The morphological and physiological characteristics of the *Bacillus subtilis* are shown in Table 2. The cells of the *Bacillus subtilis* subspecies natto are Gram-positive, aerobic, rods, occurring as single cell or chains. They exhibit swarming motility. The endospores are mainly oval and lie in central positions in non-swollen sporangia. When grown on TSA the colonies are cream-coloured, large and spreading. In liquid medium a pellicle is formed at the surface whilst the rest of the medium is slightly cloudy. The bacterium grows within a temperature range 5 to 40^0C and pH 5 to 10. Optimum growth is at 35^0C, pH 7.2. It is catalase and oxidase positive. It reduces nitrate aerobically. Starch, Tween 20, Tween 80, gelatin, and casein are hydrolysed. Voges–Proskauer test, methyl red, citrate, and H_2S from cysteine, are positive. It grows in the presence of lysozyme and in media without yeast extract. Tyrosine and urea are not hydrolyzed. Tryptophan and phenylalanine deaminase, pigment after growth on tyrosine medium, H_2S from sodium thiosulphate, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase, and indole are negative. Acids are produced from the following sugars: glycerol, glucose, ribose, D- galactose, mannose, fructose, mannitol, sorbitol, lactose, sucrose, raffinose, melibiose, inulin, methyl α-D-glucoside, maltose, trehalose, starch, glycogen, methyl-β-D-xyloside, cellobiose, gluconate and N-acetyl glucosamine. Acids are not produced from erythritol, inositol, D-lyxose, xylitol, D-arabinose, xylose, L-arabinose, sorbose, rhamnose, fucose, arabitol, gentiobiose, and dulcitol,. It is susceptible to amoxycillin, clindomycin, chloramphenicol, erythromycin,

imipenem, kanamycin, nalidixic acid, norfloxacin, rifampicin, tetracycline, tobramycin, trimethoprim, and vancomycin.

The optimum pH and temperature for its amylase activity was found to be 6.5 and 55^{0}C (Fig. 5 & 6), respectively. The maltose is found to induce amylase activity to 0.5624 U, followed by fructose, raffinose, sucrose, xylose, galactose, and ribose but starch, and arabinose have very low inducing effect. In addition to soluble starch the lactose, glucose and dextrin were also found suitable for amylase production.

This selected *Bacillus subtilis* gives higher yield of amylase with complex nitrogen sources than with simple nitrogen sources as given in Table 4. Further enzyme purification is required for more characterization.

Table. 1- Attributes of *B.subtilis*

Parameter	
Amylase activity	+
Protease activity	+
Lipase activity	+
Aggregation time (min)	52
Cell surface hydrophobicity	82.8%

Table. 2- B.*subtilis*-characteristics

Parameters	**Characteristics**
Morphology	Gram+ve,Straight rods, single or chain Oval endospore
Motility	Swarming
TSA Agar	Cream colored , large spreading
Growth temp.	Opt growth at 35^{0}C range 5-40^{0}C
pH	Opt growth at 7.2 range 5-10
Gelatinase	Positive
Casein hydrolysis	Positive
Amylase	Positive
Catalase	Positive
Indole test	Negative
VP test	Positive
Urease	Negative
Nitrate reduction	Positive
Methyl red test	Positive
Citrate utilization	Positive

Table. 3- Effect of carbon sources on amylase production

Carbon source	**Activity (□mole/min/ml)**
Arabinose	0.0611
Dextrin	0.1543
Fructose	0.3612
Galactose	0.2996
Glucose	0.1401
Glycerol	0.0219
Lactose	0.0110
Maltose	0.5624
Mannitol	0.1105
myo-Inositol	0.1401
Raffinose	0.3251
Ribose	0.2932
Sod. Acetate	0.0074
Sod. Citrate	0.1520
Starch	0.0741
Sucrose	0.3021
Xylose	0.3014

Table. 4- Effect of various nitrogen sources on Amylase production

Nitrogen sources	Amylase activity (□mole/min/ml)	Protein (□g/ml)	Specific activity (U/mg)
*$(NH_4)_2SO_4$	0	8	0
*$(NH_4)_2NO_3$	0	2.4	0
*NH_4Cl	0	7.4	0
*$(NH_4)H_2PO_4$	0	2.1	0
*CH_3COONH_4	0	3.3	0
*L-Glutamic acid	0	2.2	0
*KNO_3	0.08	0.001	80
*Urea	0.08	0.002	40
#Peptone	0.36	0.12	3
#Yeast extract	0.39	0.14	2.79
#Tryptone	0.32	0.41	0.78
#Soybean meal	0.29	0.48	0.510
#Beef extract	0.34	0.36	0.944
#Gelatine	0.12	0.21	0.571

***Simple Nitrogen Source, #Complex Nitrogen Source**

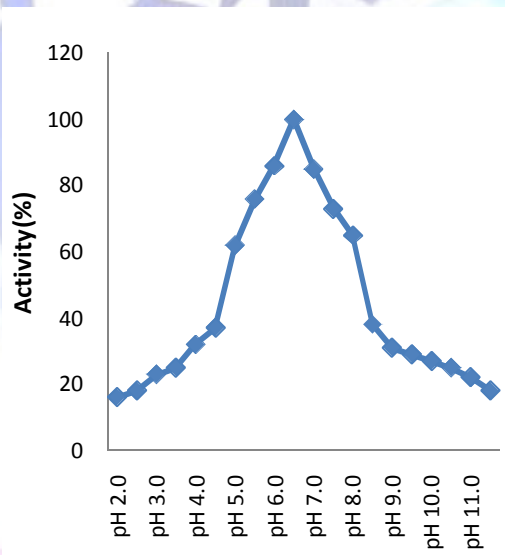

Figure.5-Effect of pH on Amylase activity

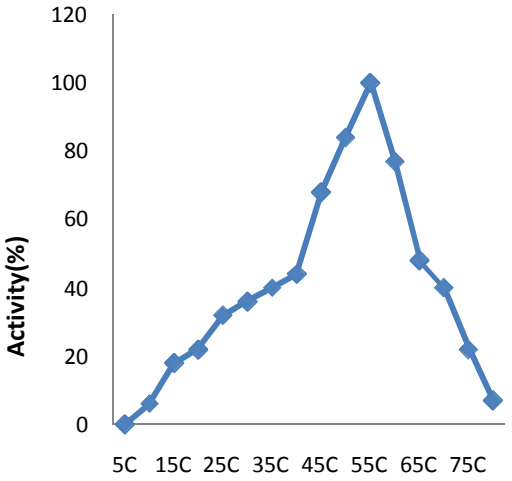

Figure.6-Effect of temp on Amylase activity

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