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PRODUCTION AND RECOVERY OF HYDROLYZING ENZYMES USING COMPOST SOIL BACTERIAL ISOLATE *BACILLUS CEREUS* STRAIN ABV 13

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ABSTRACT
Enzymes are the biocatalyst which accelerates all types of biological reactions. Upstream and downstream processing of microbial fermentation has gained much popularity in the areas of enzymology. Hydrolase is a class of enzymes (EC-3) catalyses reactions by adding water molecules causing hydrolysis in larger molecules into smaller units. A number of bacteria are found in partially decomposing cow-dung also. Therefore present work was carried with objectives like production, recovery and purification of some Hydrolase enzymes (protease, lipases and amylase) these isolates. Among 14 isolated single potent strains was chosen for further investigation. Biochemical characterizations and 16s r RNA sequencing shows 99.9 % resemblance with *Bacillus cerus* .Partial genome sequence was submitted to NCBI with Accession number KF006372 and named as Bacillus cereus strain ABV 13.Submerged state fermentation was applied of all three kinds of enzymes. Extracted partially purified enzyme molecules were found to have the same optimum temperature at 50-55°C and with pH 8.5-9.0. This creates positive interest in the isolation of such other alklaophilic organisms.

KEYWORDS: Hydrolyzing enzymes, protease, lipases and amylase

INTRODUCTION

Being a key ingredient in organic farming compost is recycled as a fertilizer in soil amendment for organic farming. In Indian tradition it has got lot of interest for sustainable development in agriculture. Compost has rich micro flora of divers kind of micro-organisms comprising even of Actinomycetes. Most of useful bacteria can be well isolated using such samples. Bacteriocin and antibiotic producers with pleomorphic nature are most suited and found in such decaying organic substances. In most of the cases they show variation in their morphology and genetic sequences. Different studies have reveled cause of such existences among bacteria. One of the major causes is adaptation with surrounding environment. Recent studies have revealed that industrially useful bacteria's like antibiotic producers, Enzymes producers and many more could be well isolated from such composting materials. Enzymes (Bio-catalysts) belonging 'Hydrolase class (EC-3)' has gained lot of interest in detergent and tanneries industries. This group includes mainly Protease, lipase and amylases. These enzymes have a wide range of significance even in food processing, candy industries. In textiles industries amylase and lipase play significant role in processes like yarning and enhance texture to cloth. Whereas in cheese making proteases and lipases are added to enhance rancidity and taste (Sharma *et al*., 2001). Industrial approach for strain improvement on such microbial producers seem to be very costly, hence it is a basic need to isolate single potent bacterial strain from a reliable cheap and easily available source. The production of protease and lipase in continuous cultures of *Bacillus species* has been reported by Sangeeta *et al*. (1993). However, fewer reports are found on Upstream and downstream processing of concomitant enzyme productions. Hence interested attempt was taken in producing these enzymes from compost soil isolates using a common, readily available medium with common ingredients (Veerapagu *et al*., 2013).

MATERIALS AND METHODS

Materials

Collection of soil sample
Cow dung was made available from agricultural farm from south Solapur (M.S).

Methods

Bacterial isolation

Collected sample was poured on sterile petri plates upon serial dilution. 0.1ml of each dilution was seeded on separate sterilized nutrient agar plates with slight basic p H and incubated at 50-55°C for 24-48 hrs. Each obtained colonies were examined with their morphological characters Table I.

Characterization of bacterial isolates

Each singly isolated colony was enriched in sterile nutrient broth, streaked on separate medium agar plates (Initially with skimmed agar plate, followed by TAG (Tri Acyl Glycerol) and Starch agar plates. Similar pattern was followed for all isolates.

Purification and identification of isolates

Amongst all 14 isolates, single colony exhibiting more prominent zone of hydrolysis was chosen for further study.

Production and recovery of enzymes

Optimization of fermentation media

Media optimization was carried out by Plackett-Burman Design using seven Variables. Optimized media was chosen for fermentation in all cases (Zhang *et al*., 2012).

Fermentation of Extracellular Protease, lipase and amylase

Liquid state fermentation was preferred way to produce extra cellular Protease, lipase and amylase (Ito *et al*., 2001).During fermentation initial pH of medium was maintained slightly basic, and later it has been adjusted to a course of fermentation by optimization.

Following Medias were chosen for production (With p H Range 8-9)

For protease-MGYP Broth

For Lipase-TAG Broth (with 0.01 %Emulsifier)

For Amylase-PD Broth

Recovery Protease, lipase and amylase

All three types of enzymes were recovered using salt precipitate and dialysis technique.

Extracellular protease has been fractionated using 50 % salt where as Amylase was recovered using 60% ammonium salt and lipase was recovered successfully using 55 % for. Purity level was increased by membrane dialysis procedure (Rao *et al.,* 1993).

Enzyme assay

Incerase in Enzyme activity was rchecked by effective digestion assay.

Protease enzyme activity was assayed by Azo -casein Digestion method with slight modification (Jinka *et al*., 2009). optical density was taken at 540 nm. One unit of Protease activity was defined as the amount of enzyme that releasing 1micro mol tyrosin equivalent per minute under the assay conditions.

Activity calculation

$$\text{Enzyme units / ml} = \frac{\text{O.D. of Blank} - \text{O.D. of Sample} \times \text{amount of enzyme used}}{\text{Incubation period}} \times 1\text{ml}$$

Enzyme assay

Lipase enzyme activity was assayed by copper soap colorimetric method with slight modification (Ramesh *et al.,* 2014). Fatty acids liberated during hydrolysis of an olive oil substrate by lipase can be determined colorimetrically using a cupric acetate/pyridine reagent. Fatty acids complex with copper to form cupric salts or soaps that absorb light in the visible range 715 nm. Yielding a blue color. One unit of lipase activity is defined as the amount of enzyme that liberated 1 micro mol free fatty acid in 1min.

Activity calculation

$$\text{Enzyme units / ml} = \frac{\text{O.D. of Blank} - \text{O.D. of Sample} \times \text{amount of enzyme used}}{\text{Incubation period}} \times 1\text{ml}$$

Enzyme assay

Assay system for amylase activity was carried out by measuring the amount of reducing sugar according to the DNSA method. Optical density was taken at 540 nm. One unit of amylase activity was defined as the amount of enzyme that releasing 1micro mol glucose equivalent per minute under the assay conditions (Senthilkumar *et al.*, 2012).

Activity calculation

$$\text{Enzyme units / ml} = \frac{\text{Microgram of glucose produced}}{\text{Volume of enzyme solution x Incubation time}} \times 1\text{ml}$$

Taxonomical studies and identification of the microorganism

The morphological, cultural and physiological characteristics of the isolated bacterium were compared with data from Bergey's Manual of Systematic Bacteriology .The identification of the potent and screened microorganism was performed through rRNA 16S sequence and compared to the sequences deposited at the GENBANK. Sequencing was done with forward and reverse primers. Gathred sequence was submitted through BankIt to NCBI. (Accession number *KC884940*)

Primer forward and reverse sequence

Primer 1 and 2 for Sequencing Reference.
Primer 3 and 4 for PCR Amplification

1.518 F Universal forward CCAgCAgCCgCggTAATACg

2 .800R Universal Reverse TACCAgggTATCTAATCC
3 .27F Universal Forward AgAgTTTgATCMTGGCTCAg
4 .1492R Universal Reverse TACggYTACCTTgTTACgACTT

Partial genome sequence

Sequencing of single selected strain was performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDye TM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Result and Discussion

An overview of literature on pH stability indicates that all three hydrolystic enzymes were generally stable over a range of pH from 7.5-9.5 Sangeeta *et al* (1994).

Table I Bacterial Isolates with their morphological characters

Sr	Isolate	Size	Shape	Color	Margin	Opacity	Elevation	Consistency	Gram Nature	Motility
1	B- 1	1	Circular	Whitish	Regular	Transparent	Convex	Viscous	Positive	Motile
2	B- 2	3	Irregular	Whitish	Regular	Transparent	Flat	Smooth	Positive	Motile
3	B- 3	6	Irregular	Creamy	Irregular	Translucent	Convex	Sticky	Positive	Motile
4	B- 4	2	Irregular	Whitish	Irregular	Translucent	Convex	Sticky	Positive	Motile
5	B- 5	1	Circular	Whitish	Regular	Translucent	Convex	Smooth	Positive	Motile
6	B- 6	1	Circular	Whitish	Regular	Translucent	Convex	Smooth	Positive	Motile
7	B- 7	2	Circular	Whitish	Irregular	Transparent	Flat	Smooth	Positive	Motile
8	B- 8	2	Irregular	Whitish	Irregular	Transparent	Flat	Dry	Positive	Motile
9	B- 9	1	Regular	Whitish	Regular	Transparent	Flat	Smooth	Positive	Motile
10	B-10	9	Irregular	Creamy	Irregular	Transparent	Convex	Dry	Positive	Motile
11	B-11	2	Irregular	Whitish	Irregular	Translucent	Convex	Sticky	Positive	Motile
12	B-12	2	Circular	Whitish	Regular	Transparent	Flat	Sticky	Positive	Motile
13	B-13	1	Circular	Whitish	Regular	Transparent	Convex	Smooth	Positive	Motile
14	B-14	1	Circular	Whitish	Regular	Translucent	Convex	Sticky	positive	Motile

Sharma *et al.*, (1999) found that the optimum temperature of the lipase and protease ranged from 50 to 55 0 C with casein, TAG and starch as the substrate Zhang *et al*.,(2006).

Characterization of bacterial isolates

Total of 14 different bacterial colonies have been isolated. Each colony has been examined with respect to their morphological characters .But selection of single was done strictly on the basis of its hydrolyzing ability. Table I

B- 8 Isolate was chosen for further study.

Biochemical testing and partial genome sequence

Identification of selected strain was done on the basis of biochemical (*Bergey's Manual of Determinative Bacteriology*) and Partial genomic sequence. Both confirm 90% resemblance with *Bacillus cereus*. Higher GC percent confers thermo stability of strain. Table II.

Upon confirmation isolate was named as *Bacillus sp AV 12* with accession number *KC884940* By NCBI.

Table II Bioinformatics summary report of Bacillus AV 13

Name	Read Length (Normal)	Read Length (Q16)	Read Length (Q20)	GC Content
sample1_contig_1	1475	1393	1393	55.11864406779661
sample1_R	748	735	733	54.81283422459893
sample1_F	916	912	912	55.24017467248908

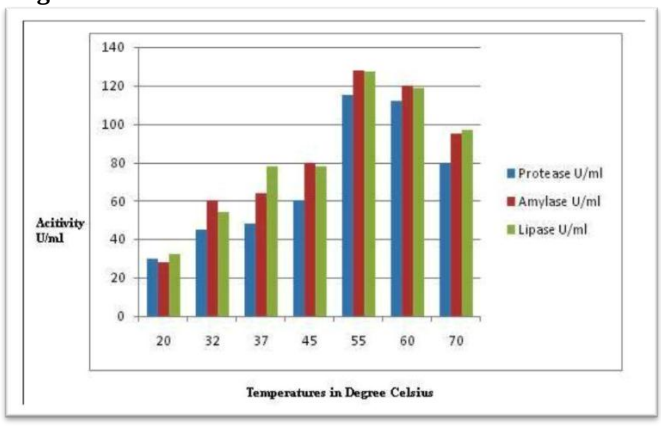
Enzyme assay

All extracted and partially purified enzymes molecules of Protease, Amylase and Lipase showed maximum activity at 55 0 C with optimum p H 8.5. Table III and Graph 1

Table III Optimum temperature profiling

Sr.no	Temperature	Protease U/ml	Amylase U/ml	Lipase U/ml
1	20	30	28	32
2	32	45	60	54
3	37	48	64	78
4	45	60	80	78
5	55	115	128	127
6	60	112	120	119
7	70	80	95	97

Graph 1 Temperature profiling



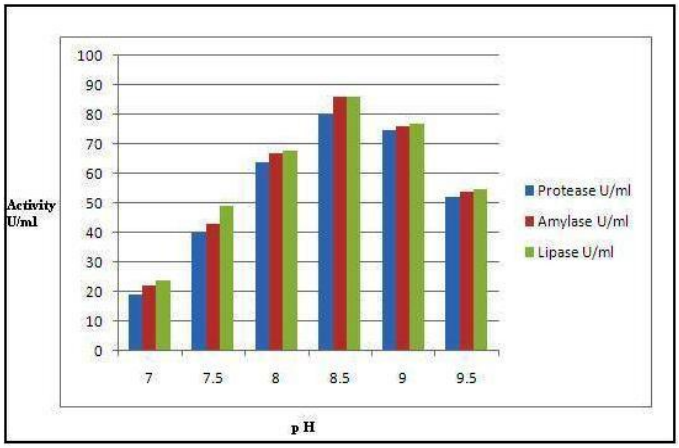
pH profile

As similar to p H profile obtained by Sangeeta *et al*., (2010) maximum hydrolytic activity was also noted in the range of pH 8 and 9 with an optimum pH of 8.5 Table IV and Graph 2

Table IV Optimum p H profiling

Sr.no	pH	Protease U/ml	Amylase U/ml	Lipase U/ml
1	7.0	19	22	24
2	7.5	40	43	49
3	8.0	64	67	68
4	8.5	80	86	86
5	9.0	75	76	77
6	9.5	52	54	55

Graph 2 p H profiling



Conclusion

Bacillus AV 13 was isolated from cow dung sample and the organism was studied for the production of hydrolyzing enzymes like protease, amylase and lipase. All extracted partially purified enzyme molecules were found to have the same optimum temperature and pH. This creates an interest in the isolation of other alklaophilic organisms, opening a new way for studying thermo-stable, alklophilic, lipase sand amylases.

REFERENCES

Ito T., Kikuta H., Nagamori E., Honda H., Ogino H., Ishikawa H and Kobayashi T. (2001). Lipase production in two-step fed-batch culture of organic solvent-tolerant Pseudomonas aeruginosa LST-03. *J. Biosci Bioeng*. 91: 245–50.

Jinka R., Ramakrishna V., Rao S and Rao R. (2009). Purification and Characterization of cysteine Protease from Germinating Cotyledons of Horse Gram.*BMC Biochem*.10(28).

Ramesh S., Rahul R., Devi R.A and Balakrishnan K. (2014). Isolation of a Lipase Producing Bacteria for Enzyme Synthesis in Shake Flask Cultivation. *Int.J.Curr.Microbiol.App.Sci* .3(3): 712-719

Rao M., Tanksale A., Ghatge M. and Despande V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev*. 62(3): 597-635

Sangeetha R., Geetha A. and Arulpandi I. (2010). Concomitant Production of Protease and Lipase By *Bacillus licheniformis* vsg1: Production, Purification and Characterization. *Braz. J. Micro*. 41: 179-185

Senthilkumar P.K., Uma C and Saranraj P. (2012). Amylase Production by *Bacillus* sp. Using Cassava as Substrate. *Int. J. Pharma. Biol Archi*. 3(2):300-306

Sharma R., Chisti Y. and Banerjee U. (2001). Research review on Production Purification Characterization and Applications of Lipases. *Biotech. Adva*.627–662.

Veerapagu M., Narayanan A.S., Ponmurugan K and Jeya K.R. (2013). Screening Selection Identification Production And Optimization OF Bacterial Lipase From Oil Spilled Soil. *Asian. J Pharm Clin Res.* 6(3):62-67

Zhang Y., Wang. Y., Wang Z.G., Wang. X, Guo H.S., Meng D.F., and Wong.P.K. (2012). Optimization of Fermentation Medium for the Production of Atrazine Degrading Strain Acinetobacter sp. DNS32 by Statistical Analysis System. *J. Biomed. Biotech.* 1-7.

