Research Journal of Microbiology

ISSN 1816-4935

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 10 (9): 393-420, 2015
ISSN 1816-4935 / DOI: 10.3923/jm.2015.393.420

Molecular Identification of Newly Isolated *Bacillus* Strains from Poultry Farm and Optimization of Process Parameters for Enhanced Production of Extracellular Amylase Using OFAT Method

[1]Ameer Khusro and [2]Chirom Aarti
[1]Department of Plant Biology and Biotechnology, Loyola College, Nungambakkam, Chennai, 600034, Tamil Nadu, India
[2]Department of Biotechnology, M.S. Ramaiah College of Arts, Science and Commerce, Bengaluru, Karnataka, 560054, India

Corresponding Author: Ameer Khusro, Department of Plant Biology and Biotechnology, Loyola College, Nungambakkam, Chennai, 600034, Tamil Nadu, India Tel: 91-8939253216

ABSTRACT

Bacillus sp. has been used as attractive and efficient industrial organisms in the last few decades due to their rapid growth rates and their potential to secrete extracellular enzymes into the medium. Amylase obtained from bacteria has gained immense interest due to its biotechnological potential in various industries. The aim of this experimental context was to isolate novel strains of *Bacillus* sp. from poultry farm, screening and optimization of different parameters for the enhanced production of extracellular amylase using traditional OFAT method (One Factor At a Time). Five hyperamylase producing *Bacillus* strains were identified after preliminary screening of poultry feces soil sample. The strains were showing maximum amylase production at pH ranging from 6-9. On the other hand strains were showing more enzyme activity at 35 and 45°C. The agitation speed of 130 and 150 rpm was found to be suitable for maximum enzyme production by *Bacillus* sp. The amylase activity of each isolate was reduced with increase in incubation time from 24-96 h. Starch and yeast extract were found to be the suitable carbon and nitrogen source in order to enhance the enzyme production. The enzyme obtained from the isolates was stable upto 60°C and pH 9.0 for 4 h of incubation. Percentage GC-content of 16S rRNA sequences showed a significant effect on amylase production at high temperature. The present study clearly indicates that amylase production was 2 fold higher when optimized conditions were used. The crude amylase obtained from the isolates showed stability at high temperature and pH. As to the best of our knowledge this present investigation makes a novel contribution to research on amylase production from new *Bacillus* strains of poultry farm by demonstrating the isolation, molecular characterization and enhanced amylase activity at optimal conditions using OFAT method.

Key words: Amylase, *Bacillus* sp., GC-content, OFAT, poultry farm

INTRODUCTION

Amylases constitute approximately 30% of the enzyme market that have opened new frontiers of many commercial biotechnological processes. Amylases hydrolyze starch molecules and yield various products like dextrins and smaller glucose units. Initially the term amylase was used originally to designate an extracellular enzymes capable of hydrolyzing α-1,4-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked glucose units. The enzyme acts on starches, glycogen and oligosaccharides in a random manner, liberating reducing groups. Amylases

have completely replaced chemical hydrolysis of starch in starch processing industry (De Souza and Magalhaes, 2010). Around 25% of the enzyme market has been obtained from varied sources like plants, animals and microorganisms. Microbial enzymes are widely used in industrial processes because of their low cost, large productivity, chemical stability, consistency, environmental protection, plasticity, easy handling and vast availability (Mishra and Behera, 2008). The microbial source of amylase meets the industrial demand due to the fact that microbes are easy to manipulate to produce the enzymes of desired characteristics. A broad range of microorganisms such as yeast, bacteria and fungi have been reported to produce amylase (Li *et al*., 2007; Liu and Xu, 2008; Gupta *et al*., 2008). Although, there are many microbial sources available for producing amylases, the capacity of bacteria to produce large quantities of enzymes has placed them among the most significant industrial enzyme producers. The production of microbial amylases from bacteria is dependent on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, incubation period, pH, temperature, metal ions and thermostability (Pandey *et al*., 2000). Among bacteria, *Bacillus* species are endowed to produce large quantities of amylase. *Bacillus* sp. is an industrial important microorganism because of its rapid growth rate, secretion of enzyme into the extracellular medium and safe handling (Vijayalakshmi *et al*., 2012). *Bacillus subtilis, Bacillus amyloliquefaciens* and *Bacillus licheniformis* are used as bacterial workhorses in industrial microbial cultivations for the production of a variety of enzymes as well as fine biochemicals (Deb *et al*., 2013).

Fermentation medium optimization plays a critical role in enhancing the production yields of the industrially important enzyme. Optimization for enhanced production of enzyme depends upon medium components like carbon source, nitrogen sources, pH, temperature, agitation and incubation time. The optimization of the fermentation medium by One Factor At a Time (OFAT) is a potential approach in order to analyze the interactive effects of various factors.

In the present day scenario, amylases have a great commercial value in biotechnological applications ranging from food, fermentation and textile to paper industries. As there are very few research activities on amylase production from poultry farm bacteria, hence considering the industrial importance of amylases, the present study was investigated to isolate novel *Bacillus* strains from poultry farm for amylase production and to identify the isolates efficient for maximum amylase production at different parameters using OFAT method. Another part of the present context was also focused on the partial characterization of amylase and sequence analysis of isolated strains using bioinformatics tools.

MATERIALS AND METHODS

Isolation of bacteria from feces soil sample: Poultry feces soil sample was collected from poultry farm of Guduvanchery, Tamil Nadu (India). Feces soils were brought to the laboratory in aseptic condition. One gram of sample was suspended in 9 mL of saline and mixed vigorously to make uniform suspension. After that soil samples were serially diluted up to 10^{-5} and 0.1 mL of aliquots were spread over nutrient agar plates from 10^{-5} dilution. The plate was incubated at 37ºC for 24 h. Colonies showing different morphology were picked out and purified by repeated streaking on nutrient agar plates. Cultures were streaked on slants and kept in incubator at 37°C for 24 h and were preserved in slants at 4±2°C. Subsequently, the selected isolates were maintained in 20% glycerol at -80°C for further use.

Screening of bacteria for extracellular amylase production: Starch agar media (Starch 10 g L^{-1}, agar 20 g L^{-1} and pH 7) were prepared and transferred to the sterile petri plates

aseptically. The solidified media was punched with 5 mm of sterile cork borer in order to make wells of uniform diameter. Overnight grown bacterial isolates were centrifuged at 8000 rpm for 10 min. The supernatants (0.1 mL) of the isolates were collected and transferred to the agar wells. The supernatant was allowed to diffuse in the agar media overnight at 37°C. After overnight incubation, amylase producing bacterial strains was observed by staining the plates with iodine solution. Only the hyperamylase producing isolates were selected for further optimization process.

Organism identification: Isolates showing potential amylase production was characterized by cultural, morphological and biochemical analysis following standard procedures according to the Bergey's Manual of Systemic Bacteriology (Sneath, 1994).

Genomic DNA isolation: Two milliliter of hyperamylase producing bacterial cultures were centrifuged at 6000 rpm for 5 min. The supernatant was discarded. One milliliter of UniFlex™ Buffer 1 and 10 μL of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 min at 37°C in a water bath. To the lysed samples 1 mL of 1:1 phenol:chloroform was added and mixed well. The samples were centrifuged at 10,000 rpm for 15 min at room temperature. The aqueous layers were separated in a fresh 1.5 mL vial. To the aqueous layer 1 mL of UniFlex™ Buffer 2 was added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 min at room temperature. The supernatant was discarded. To the pellet 500 μL of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded. The pellet was air dried for about 10-15 min till the ethanol evaporate. The pellet was resuspended in 50-100 μL of UniFlex™ Elution Buffer. DNA was stored at -20ºC.

PCR amplification and sequence of 16S rRNA: The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with *Taq* DNA polymerase and primers 27F (5` AGTTTGATCCTGGCTCAG 3`) and 1492R (5`ACGGCTACC TTGTTACGACTT 3`). The conditions for thermal cycling were as follows: Denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining.

Purification of amplified product: PCR sample is taken in fresh vial and 5 μL of 3 M sodium acetate solution (pH-4.6) and 100 μL of absolute ethanol were added into it. The vial was mixed thoroughly. The vial was kept at -20ºC for 30-40 min to precipitate the PCR product. Then it was centrifuged at 10,000 rpm for 5 min. About 300 μL of 70% ethanol was added to the pellet, without mixing and the centrifugation was repeated at same rpm. The pellet was air dried until the ethanol effervescence is removed. The pellet was suspended in 10 μL of sterile distilled water (Shah *et al*., 2013).

Sequencing of PCR product and BLAST search: The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems and USA). The same primers as above were also used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http://www.ncbi.nlm.nih.gov/.

Accession number: The sequences were submitted to GenBank and the following accession numbers were assigned for the isolates; *Bacillus subtilis* strain KPA (KC918878), *Bacillus licheniformis* strain 018 (KC342225), *Bacillus subtilis* strain AK (KC414759), *Bacillus tequilensis* strain ARMATI (KC424491) and *Bacillus licheniformis* strain BIHPUR 0104 (KC424492).

Amylase assay

Inoculum preparation: Fifty millilitre of nutrient broth (pH- 7.0) was prepared in 250 mL of conical flask and cotton plugged. The flask was sterilized at 121°C, 15 lb pressure for 15 min. A loopfull of bacteria was inoculated aseptically into the cooled medium and kept for incubation overnight at 37°C in a rotatory shaker.

Shake flask fermentation and cell-free supernatant preparation: Five hundred microliter of overnight grown each bacterial inoculum was inoculated into 50 mL of fermentation medium. The fermentation medium consisted of: [(w/v) 0.6% peptone, 0.05% $MgSO_4$, 0.05% KCl, 1% starch; pH 7]. The flasks was kept for incubation in rotatory shaker at 37°C. After overnight incubation the broth cultures were centrifuged at 8000×g for 10 min. The supernatants were collected and the quantitative assay for amylase production was performed according to standard procedure.

Estimation of amylase activity: Amylase activity was measured according to Miller (1959) with some modifications. The supernatant obtained from overnight bacterial broth culture was used as crude enzyme. One milliliter of enzyme solution was added to the test tube containing 1 mL of 1% solubilized starch solution. The reaction mixture was incubated at 60°C in water bath for 10 min. The reaction was stopped by adding 1 mL of dinitrosalicylic acid (DNSA) into it. The tubes were incubated in boiling water bath for 5 min. After cooling, the solution was centrifuged at 8000×g for 5 min and the supernatant was analyzed for absorbance at 540 nm. Maltose (100-1000 μg mL^{-1}) was used as the standard. One unit (U) of enzyme activity is defined in all cases as the amount of enzyme releasing 1 μg of reducing sugar as maltose per minute, under assay conditions.

Estimation of total soluble protein: Estimation of total extracellular protein was performed through Bradford (1976). Bradford method is a simple and rapid method to estimate the protein content in a sample based on the ability of protein to bind with the dye Coomassie Brilliant Blue G250. The unbound dye has an absorption maxima of 495 nm, on binding with the protein, the absorption maxima becomes 595 nm. Thus from the absorbance at 595 nm, the protein in the sample solution can be estimated. Bovine Serum Albumin (BSA) was used as standard. Hundred microliter of bacterial supernatant was pipetted out into test tubes. Volume of the tubes was made up to 1 mL using sterilized distilled water. Five milliliter of the Bradford reagent were added to all the tubes and mixed thoroughly. One milliliter of distilled water with 5 mL of Bradford reagent was used as blank. Absorbance at 595 nm was recorded against blank. Protein content per milliliter of test samples was determined against the standard curve.

Optimization of process parameters by One Factor At a Time (OFAT) method and growth kinetics of bacteria: In order to study the growth kinetics and amylase activity of bacteria, the organisms were grown in the liquid media. Growth was estimated by measuring optical density of culture broth at various parameters. Using OFAT method, the production media was optimized with various fermentation parameters like pH, temperature, agitation, incubation period,

additional carbon sources and nitrogen sources after working out a series of experiments for the bacterial strain. Effect of pH on amylase activity was assessed by cultivating the isolates (1% inoculum) in the production media of varied pH ranging from 5-10. The influence of pH on amylase activity over starch was investigated in 100 mM phosphate buffer with desired pH. The influence of different fermentation temperature such as 30, 35, 40, 45 and 50°C on amylase activity was evaluated under optimized pH (using 100 mM phosphate buffer) of media for specific bacterial isolate (1% inoculum). The fermentation medium with optimized pH was incubated at optimized temperature in order to study enzyme activity at different agitation. The fermentation was carried out at varying agitation speed such as 120, 130, 140, 150 and 160 rpm in an orbital shaking incubator. The bacterial inoculum (1%) was added to 50 mL of fermentation medium in each of 250 mL of Erlenmeyer flasks. The flasks were incubated at optimized pH, temperature and agitation for 12-96 h. The effect of various carbon and nitrogen sources on the extracellular amylase production was studied at optimized parameters. Approximately 1% (w/v) of carbon sources (Glucose, starch, xylose, sucrose, lactose, mannose and CMC) and nitrogen sources (Peptone, yeast extract, beef extract, tryptone, KNO_3, ammonium sulphate and ammonium chloride) were added separately to the production media. After 24 h of incubation, cultures were centrifuged at 6000×g for 10 min at 4°C and the supernatants were collected. The extracellular amylase activity was estimated as described earlier.

Estimation of biomass (Analytical study): Two milliliter sample was collected in a pre-weighed eppendorf tube and centrifuged at 8000 rpm for 10 min. Supernatant was discarded and the pellet was washed thrice with sterile distilled water, followed by drying the pellets at 95°C till constant weight and expressed in dry cell weight (mg mL^{-1}).

Partial characterization of crude amylase

Temperature stability: The temperature stability of the enzyme was studied by using crude enzyme. To evaluate thermal stability, the enzyme solution was incubated at temperatures of 30-70°C for up to 4 h. The relative enzyme activity was recorded at 1 h intervals during 4 h incubation. The enzyme activity was determined as described earlier.

pH stability: pH stability was measured by incubating the enzyme at pH 5-10 in different buffers (0.1 M) such as sodium phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0, 9.0) and carbonate-bicarbonate (pH 10.0). To evaluate the stability of the enzyme at each pH, the crude enzyme was incubated into the respective buffer over a pH range of 5.0-10.0 for up to 4 h at optimum temperature. The relative enzyme activity was determined at 1 h interval during the 4 h period of incubation. The enzyme activity was determined as described earlier.

Multiple Antibiotic Resistances (MAR) index and RNA secondary structure prediction of the 16S rRNA sequences: The antibiotic susceptibility pattern of the bacterial isolates was performed as per standard procedure. A homogeneous bacterial lawn was prepared on Mueller Hinton agar plates using sterile cotton swabs. The sterile discs of 6 mm diameter were soaked with 25 μL of antibiotics. Using an ethanol dipped and flamed forceps the standard antibiotic and soaked discs were aseptically placed over the agar plates sufficiently separated to avoid overlapping of zone of inhibition. Plates were incubated at 37°C for 24 h. After 24 h, diameter of zone of inhibition was measured in millimeter and results were recorded. The MAR index was calculated by the ratio of

number of antibiotics ineffective over the organisms to the number of antibiotics exposed (Krumperman, 1983). The antibiotics used in this study were ampicillin (AMP-10 μg), kanamycin (K-30 μg), nalidixic acid (NA-30 μg), streptomycin (S-10 μg), cephotaxime (CTX-30 μg) and penicillin (P-10 μg).

RNA secondary structure was generated using bases of 16S rRNA of each bacterial isolate. RNA secondary structure prediction at 37ºC was performed to determine the stability of chemical or biological molecules or entities of the isolates. Minimum Free Energy (MFE), mountain plot and entropy of each bacterial sequence were calculated by RNA fold web server (Mathews *et al*., 2004). This program predicts the lowest free energy structure for an input sequence. Fast Fourier Transform based Gene Prediction Server (FTG) tool was used to determine the GC-content (% G+C) in 16S rRNA sequences of each isolate (Issac *et al*., 2002).

Phylogenetic tree analysis: Phylogenetic relationship of the isolates with other *Bacillus* species were inferred from phylogenetic comparison of the 16S rRNA sequences. The partial 16S rRNA sequences were retrieved on NCBI server using BLAST tool. Sequences similar to isolates sequences were downloaded in FASTA format from NCBI server. Phylogenetic trees were inferred using the Neighbor-Joining (NJ) algorithm in Molecular Evolution Genetic Analysis (MEGA) software version 4.0 (Tamura *et al*., 2007). The evolutionary history was inferred using NJ method.

Statistical analysis: All the analytical experiments were conducted in triplicates and data presented is Mean±SD.

RESULTS

Isolation, screening and selection of amylase producing bacteria: Ten bacteria were isolated from feces soil samples of poultry farm, collected from Guduvanchery. These isolates were further screened for amylase production. Of 10 isolates, 5 showed the production of amylase as indicated by appearance of clear halo zone around the agar wells after Iodine solution staining at 37°C. These five amylase positive isolates were further selected for cultural, morphological and biochemical characteristics.

Cultural, morphological and biochemical test analysis: The cultural, morphological and biochemical characteristics of the isolates were studied (Table 1). All the bacterial cultures showed white and spreading type colonies. The microscopic observation showed the morphology of cultures as rod shaped with endospore. The isolated bacterial strains were identified as *Bacillus* sp. based on the taxonomical characteristics.

PCR amplification and sequencing of 16S rRNA: Genomic DNA of the isolates was visualized under UV. The amplicon of 653 bp (KC342225), 483 bp (KC918878), 733 bp (KC414759), 740 bp (KC424491) and 712 bp sequences (KC424492) was observed using PCR amplification. In the present study, 16S rRNA gene sequencing of the isolates was investigated. The isolates were identified as *Bacillus licheniformis* strain 018 (KC342225), *Bacillus subtilis* strain KPA (KC918878), *Bacillus subtilis* strain AK (KC414759), *Bacillus tequilensis* strain ARMATI (KC424491) and *Bacillus licheniformis* strain BIHPUR 0104 (KC424492) by comparing the similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST. The comparison showed that the similarities of 16S rRNA gene sequences were 100% for KC342225, KC414759 and KC424491. On the other hand, the similarities of 16S rRNA

Table 1: Morphological and biochemical characteristics of *Bacillus* sp.

	Strains				
Tests	KPA	018	AK	ARMATI	BIHPUR 0104
Surface	Smooth	Smooth	Smooth	Smooth	Smooth
Colony colour	White	White	White	White	White
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Gram reaction	Gram (+)	Gram (+)	Gram (+)	Gram (+)	Gram (+)
Cell shape	Rod	Rod	Rod	Rod	Rod
Endospore	+	+	+	+	+
Motility	+	+	+	+	+
Glucose test	-	+	-	+	+
Lactose test	-	+	-	+	+
Mannitol test	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+
Tween 80	-	-	-	+	-
Casein	+	+	+	+	+
Indole	-	-	-	-	-
Methyl red	+	+	+	-	+
Voges Proskauer	+	+	+	+	+
Citrate utilization	+	+	+	+	+
Urease test	-	-	-	-	-
Catalase test	+	+	+	+	+
Oxidase test	-	+	-	+	+
Aerobic growth	+	+	+	+	+

+: Positive, -: Negative

gene sequences were 99% for KC918878 and KC424492. The identities of strains were determined by comparing them with the available sequences of the strains and with high scored rRNA sequences in BLAST search. The novel isolated sequences were deposited in GenBank, maintained by NCBI, USA.

Effect of pH on amylase activity: Amylase activity was markedly affected by change in pH. pH played a major role in the secretion and production of amylase by isolates (Fig. 1). During optimization study pH was changed from 5-10 for each isolate. Among all isolates, maximum amylase activity of 68.641 U mL^{-1} was estimated by strain AK at acidic pH (pH 6). On the other hand strain KPA showed minimum amylase production with the activity of 21.012 U mL^{-1} at acidic pH (pH 5). Strain ARMATI showed maximum amylase activity of 50.045 U mL^{-1} at pH 6. The amylase activity was more for strain 018 at alkaline pH. Surprisingly maximum amylase activity by another strain of *B. licheniformis* was found to be at pH 7.

Effect of temperature on amylase activity: The fermentation temperature also plays a very important role in the production of amylase by bacterial isolates. Figure 2 shows the amylase activity of novel isolates at different temperatures under optimized pH for each isolate. Maximum enzyme activity of 66.782 U mL^{-1} was observed by strain AK at 35°C. Among all the isolates tested, strain 018 showed minimum amylase production of 25.11 U mL^{-1} at 50°C. On the other hand the enzyme production was increased for the same strain at 35°C. Strain ARMATI showed maximum amylase activity at 40°C. Enzyme activity of 60.551 and 60.103 U mL^{-1} was estimated by strain KPA and strain BIHPUR 0104 respectively at 35°C.

Effect of agitation speed on amylase activity: The influence of varying agitation for amylase activity was evaluated in Fig. 3. Amylase activity for strain 018 (75.521 U mL^{-1}) and strain BIHPUR 0104 (72.957 U mL^{-1}) was found to be increased at 150 rpm. Other strains showed

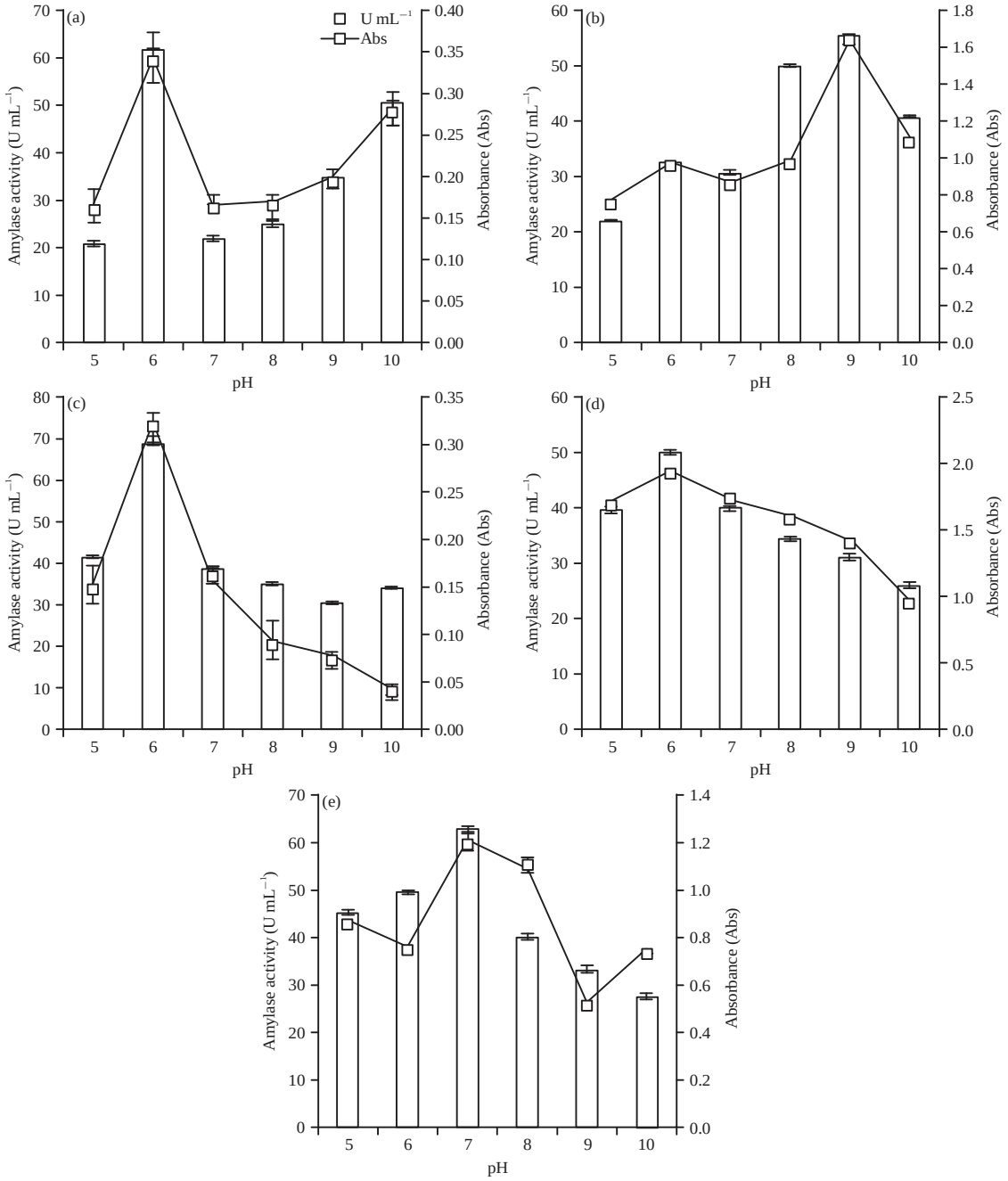

Fig. 1(a-e): Effect of pH on amylase activity, (a) *Bacillus subtilis* strain KPA was showing maximum amylase activity at pH 6, (b) *Bacillus licheniformis* strain 018 was showing maximum amylase activity at pH 9, (c) *Bacillus subtilis* strain AK was showing maximum amylase activity at pH 6, (d) *Bacillus tequilensis* strain ARMATI was showing maximum amylase activity at pH 6 and (e) *Bacillus licheniformis* strain BIHPUR 0104 was showing maximum amylase activity at pH 7

maximum enzyme activity at 130 rpm. The agitation speed lower and higher than 130 rpm affected the enzyme activity for other strains.

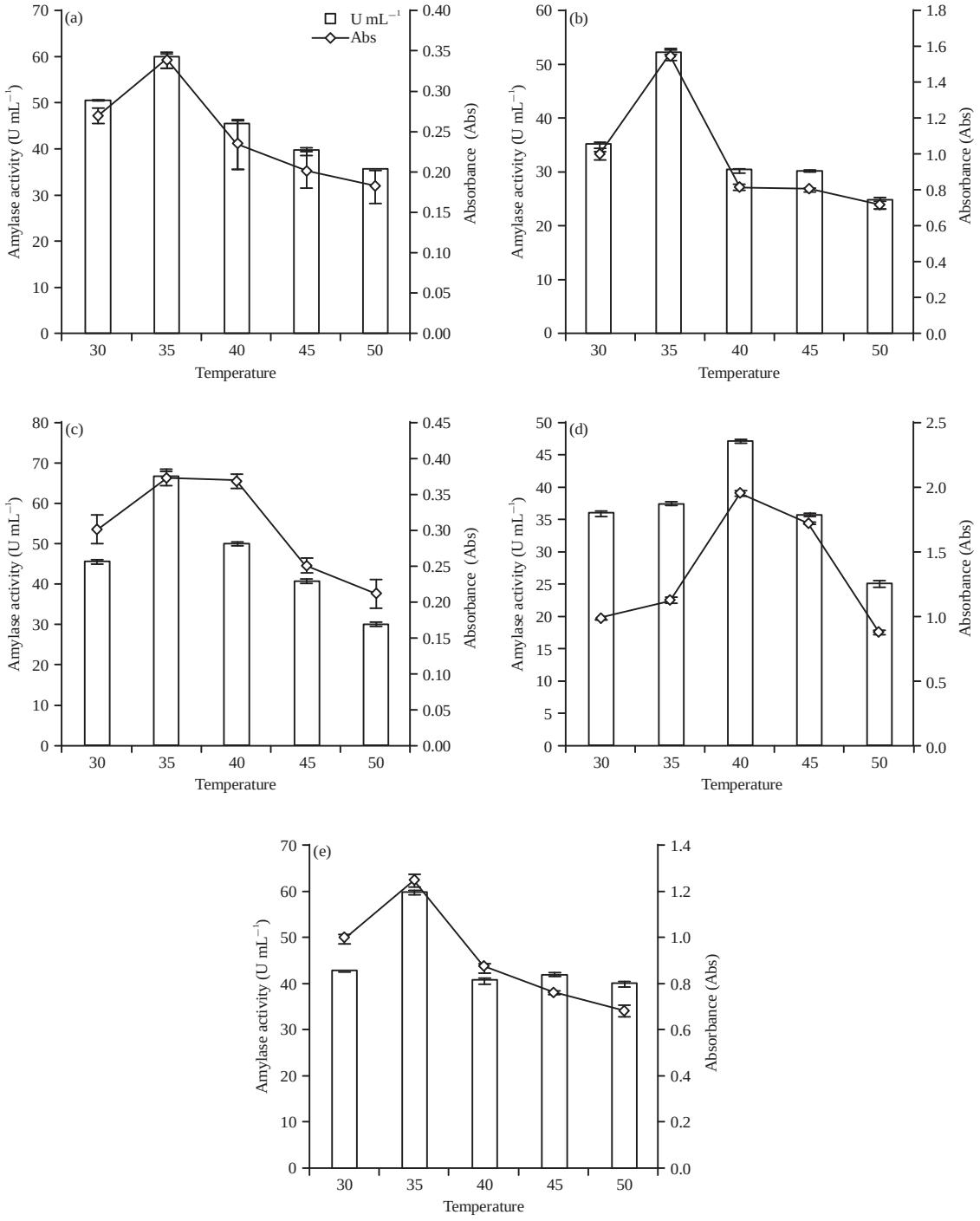

Fig. 2(a-e): Effect of temperature on amylase activity, (a) *Bacillus subtilis* strain KPA was showing maximum amylase activity at 35°C, (b) *Bacillus licheniformis* strain 018 was showing maximum amylase activity at 35°C, (c) *Bacillus subtilis* strain AK was showing maximum amylase activity at 35°C, (d) *Bacillus tequilensis* strain ARMATI was showing maximum amylase activity at 40°C and (e) *Bacillus licheniformis* strain BIHPUR 0104 was showing maximum amylase activity at 35°C

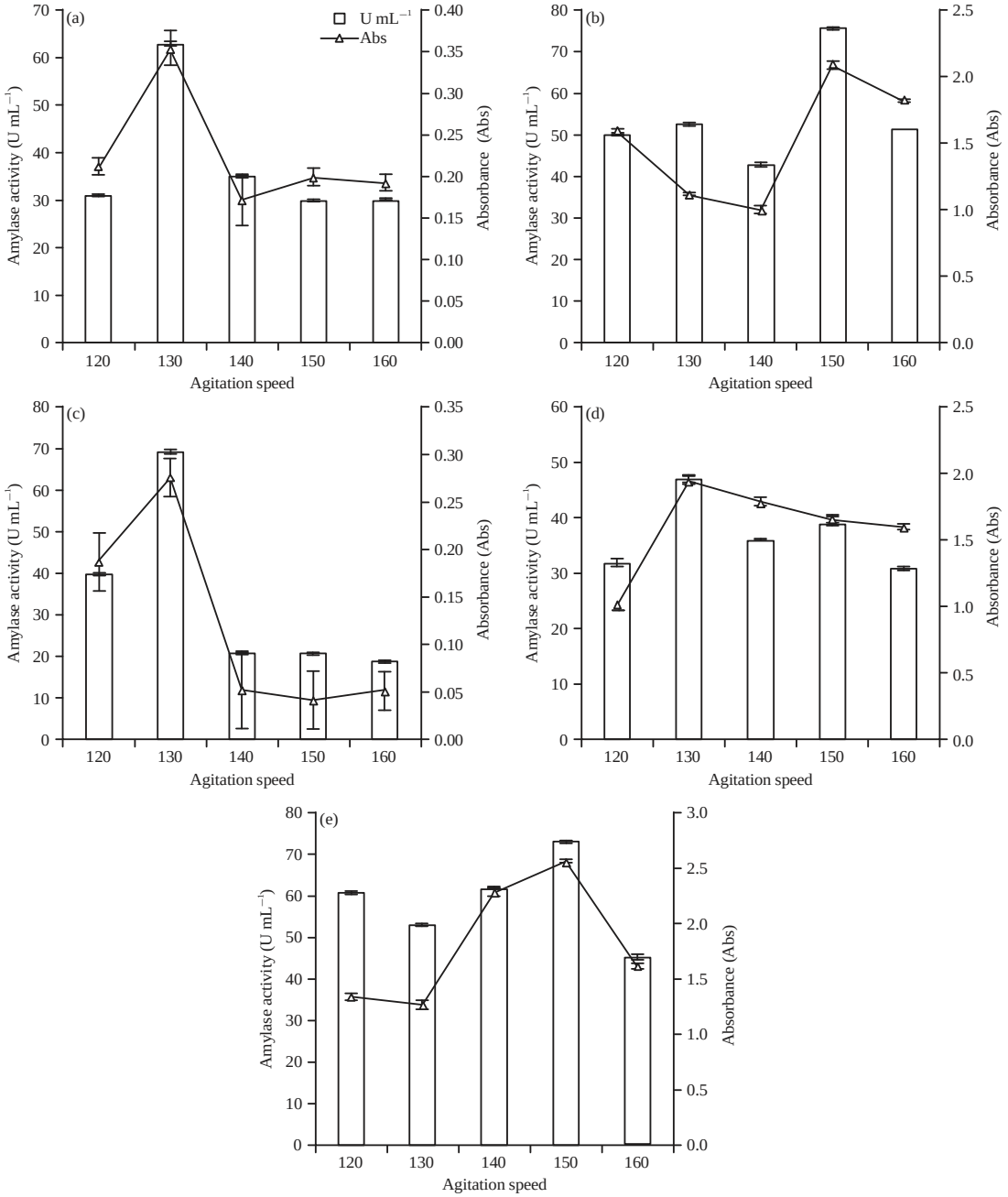

Fig. 3(a-e): Effect of agitation on amylase activity, (a) *Bacillus subtilis* strain KPA was showing maximum amylase activity at 130 rpm, (b) *Bacillus licheniformis* strain 018 was showing maximum amylase activity at 150 rpm, (c) *Bacillus subtilis* strain AK was showing maximum amylase activity at 130 rpm, (d) *Bacillus tequilensis* strain ARMATI was showing maximum amylase activity at 130 rpm and (e) *Bacillus licheniformis* strain BIHPUR 0104 was showing maximum amylase activity at 150 rpm

Effect of incubation time on amylase activity: Time period plays a very critical role in the enzyme activity. In the present investigation amylase activity was determined from 12-96 h. The

obtained results indicated that the highest yield of amylase was 75.399 U mL^{-1} after 24 h of incubation by strain 018 followed by enzyme activity of 74.09, 70.628, 65.86 and 50.327 U mL^{-1} by strain BIHPUR 0104, strain AK, strain KPA and strain ARMATI, respectively. All the isolates showed maximum amylase production after 24 h of incubation. After 24 h, the amylase activity was drastically decreased till the 96th h in a constant manner (Fig. 4).

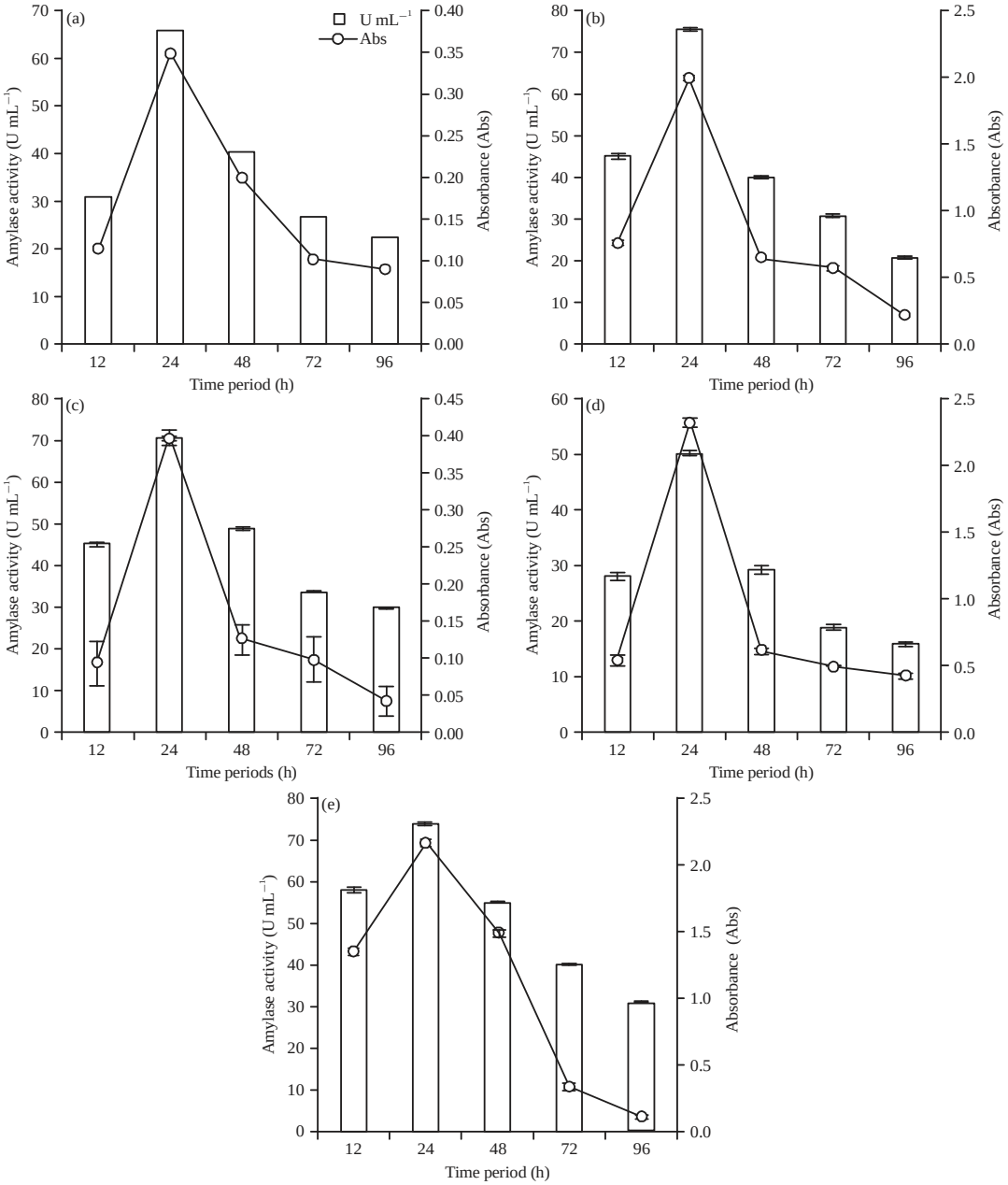

Fig. 4(a-e): Effect of incubation time on amylase activity. The isolates showed maximum amylase activity after 24 h of incubation, (a) Strain KPA, (b) Strain 018, (c) Strain AK, (d) Strain ARMATI and (e) Strain BIHPUR

Effect of carbon and nitrogen sources: The effect of various carbon and nitrogen sources on extracellular xylanase production is shown in Fig. 5 and 6, respectively. All bacterial isolates show enhancement in the enzyme production when grown in the media supplemented with 1% (w/v)

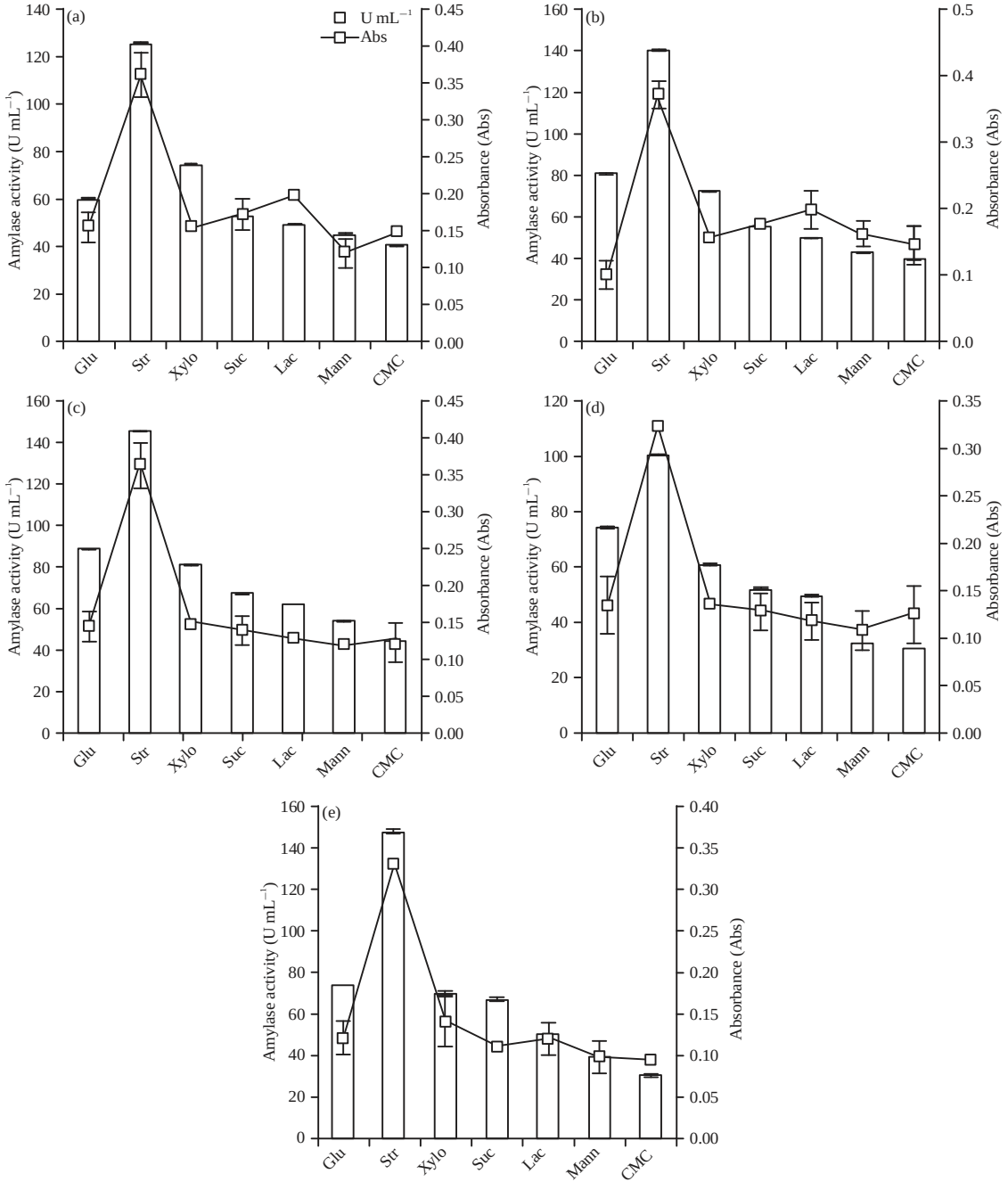

Fig. 5(a-e): Effect of carbon sources on amylase production. The isolates showed enhanced amylase activity in the presence of starch, (a) Strain KPA, (b) Strain 018, (c) Strain AK, (d) Strain ARMATI and (e) Strain BIHPUR, Glu: Glucose, Str: Starch, Xylo: Xylose, Suc: Sucrose, Lac: Lactose, Mann: Mannose, CMC: Carboxy Methyl Cellulose

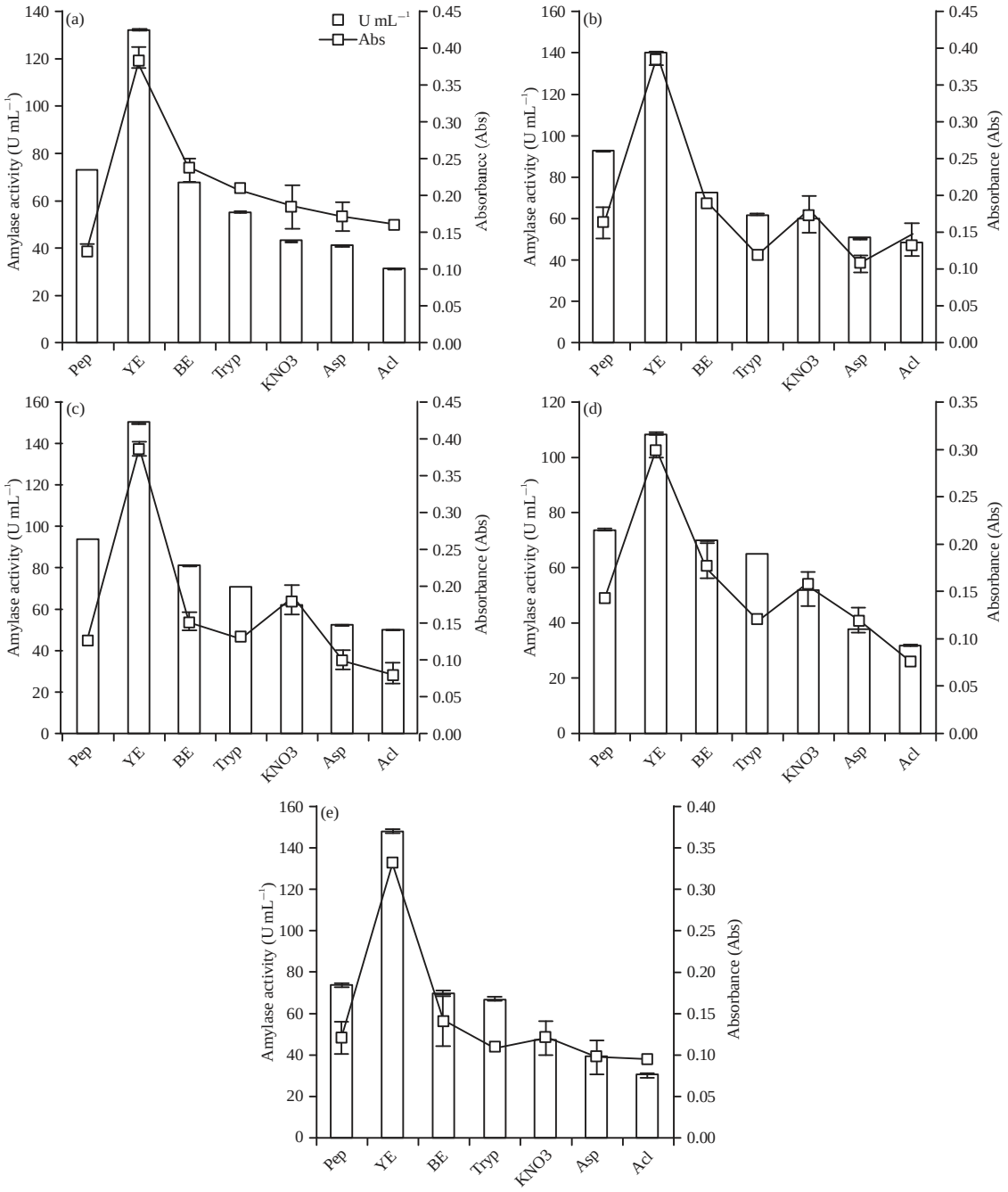

Fig. 6(a-e): Effect of nitrogen sources on amylase production. The isolates showed enhanced production of amylase in the presence of yeast extract, (a) Strain KPA, (b) Strain 018, (c) Strain AK, (d) Strain ARMATI and (e) Strain BIHPUR, Pep: Peptone, YE: Yeast extract, BE: Beef extract, Tryp: Tryptone, KNO_3: Potassium nitrate, Asp: Ammonium sulphate, Acl: Ammonium chloride

starch and yeast extract. Maximum amylase activity was obtained by strain AK followed by strain BIHPUR 0104, strain 018, strain KPA and strain ARMATI.

Biomass estimation: Dry Cell Weight (DCW, mg mL^{-1}) was analyzed for each strain at different parameters (pH, temperature, agitation speed and incubation time). The results indicate good biomass production for amylase production. The biomass production was more for all the five strains at optimum condition of selected parameters.

Effect of temperature and pH on the stability of crude amylase: Stability is one of the most important factors in studying characteristics of enzyme. Crude amylase obtained from strain KPA, strain 018, strain AK, strain ARMATI and strain BIHPUR 0104 was more stable till 60°C for 4 h of incubation and retained upto 58.7, 48.13, 52.15, 51 and 42.03% of the activity, respectively. The enzyme stability was reduced significantly above 60°C (Fig. 7).

The effect of pH on stability of amylase from novel strains is shown in Fig. 8. The crude amylase obtained from strain KPA, strain 018, strain AK, strain ARMATI and strain BIHPUR 0104 was stable at a wide range of pH from 5.0-9.0. The strains were found stable upto pH 9.0 for 4 h of

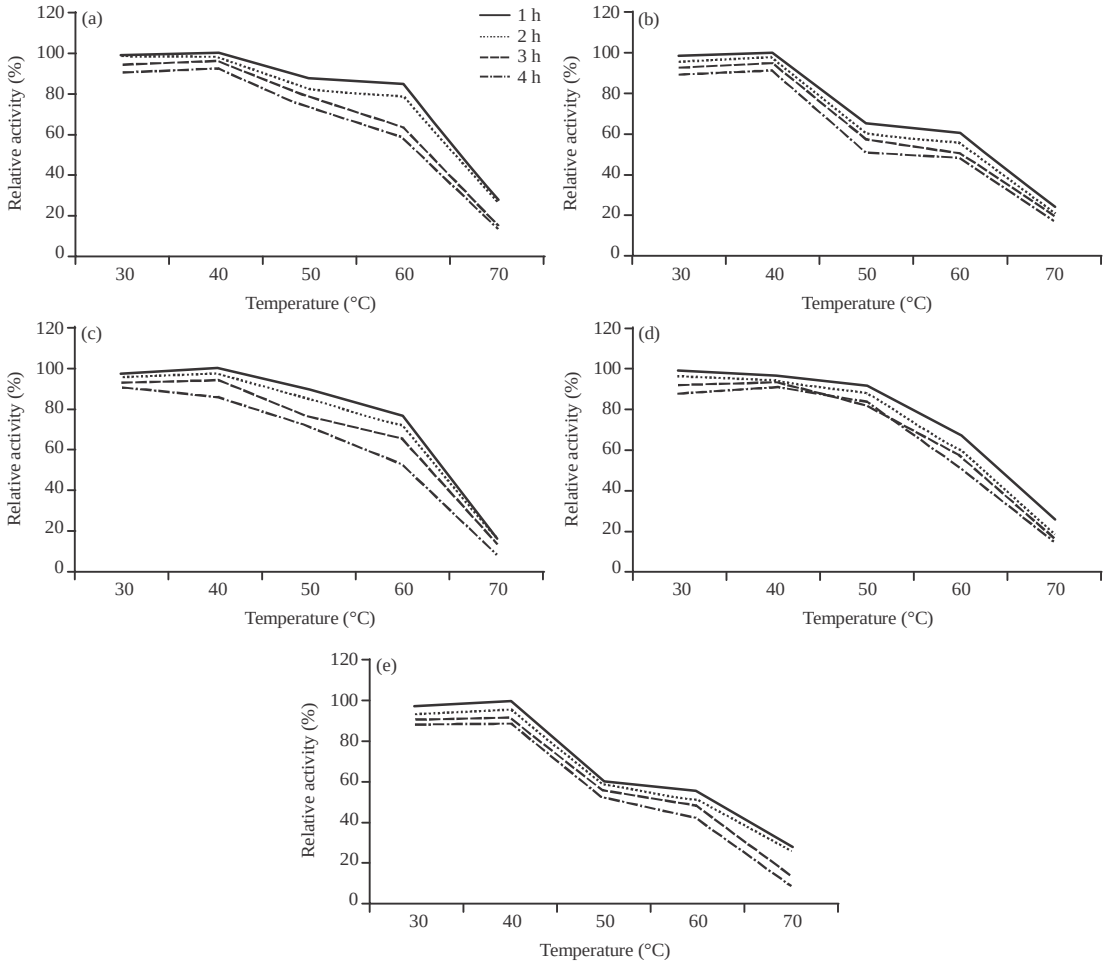

Fig. 7(a-e): Effect of temperature on stability of crude amylase. The crude amylase obtained from all the isolates was found to be thermostable upto 60°C for 4 h of incubation. Further, there was drastic decrease in the stability of the enzyme as the temperature rises up to 70°C, (a) Strain KPA, (b) Strain 018, (c) Strain AK, (d) Strain ARMATI and (e) Strain BIHPUR

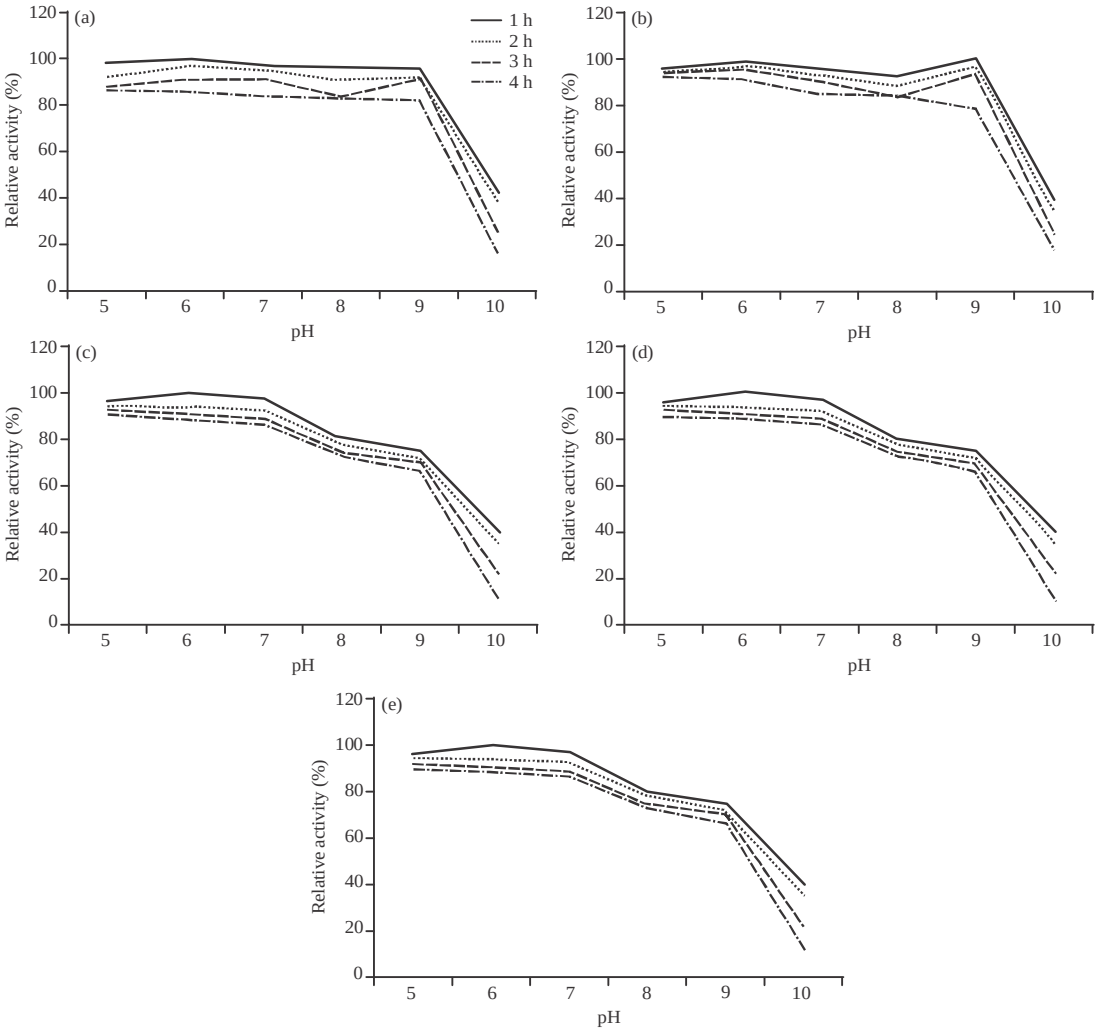

Fig. 8(a-e): Effect of pH on stability of crude amylase. The crude enzyme obtained from all the isolates was showing maximum stability up to pH 9 for 4 h of incubation. As the pH was increased further, the stability was lost drastically, (a) Strain KPA, (b) Strain 018, (c) Strain AK, (d) Strain ARMATI and (e) Strain BIHPUR

incubation. About 81.99, 78.56, 66.13, 59.12 and 86.13% of activity were retained by strain KPA, strain 018, strain AK, strain ARMATI and strain BIHPUR 0104, respectively at pH 9.0.

MAR Index determination and RNA secondary structure prediction: The MAR index value is a ratio of the number of ineffective antibiotics to the number of antibiotics exposed. The MAR value of the test organism was found to be zero (Data not shown). Results for the Minimum Free Energy (MFE) prediction in the form of secondary structure in dot bracket notation and the free energy were revealed in novel *Bacillus* isolates (Supplementary file; Fig. 9). The optimal secondary structure with a Minimum Free Energy (MFE) of -192.10, -276.80, -281.40, -279.40 and -247.40 kcal mol^{-1} were observed for strain KPA, strain BIHPUR 0104, strain ARMATI, strain AK and strain 018, respectively. Mountain plot representation and entropy for each position of the isolated strain's sequences were also determined (Supplementary file, Fig. 10). A mountain plot

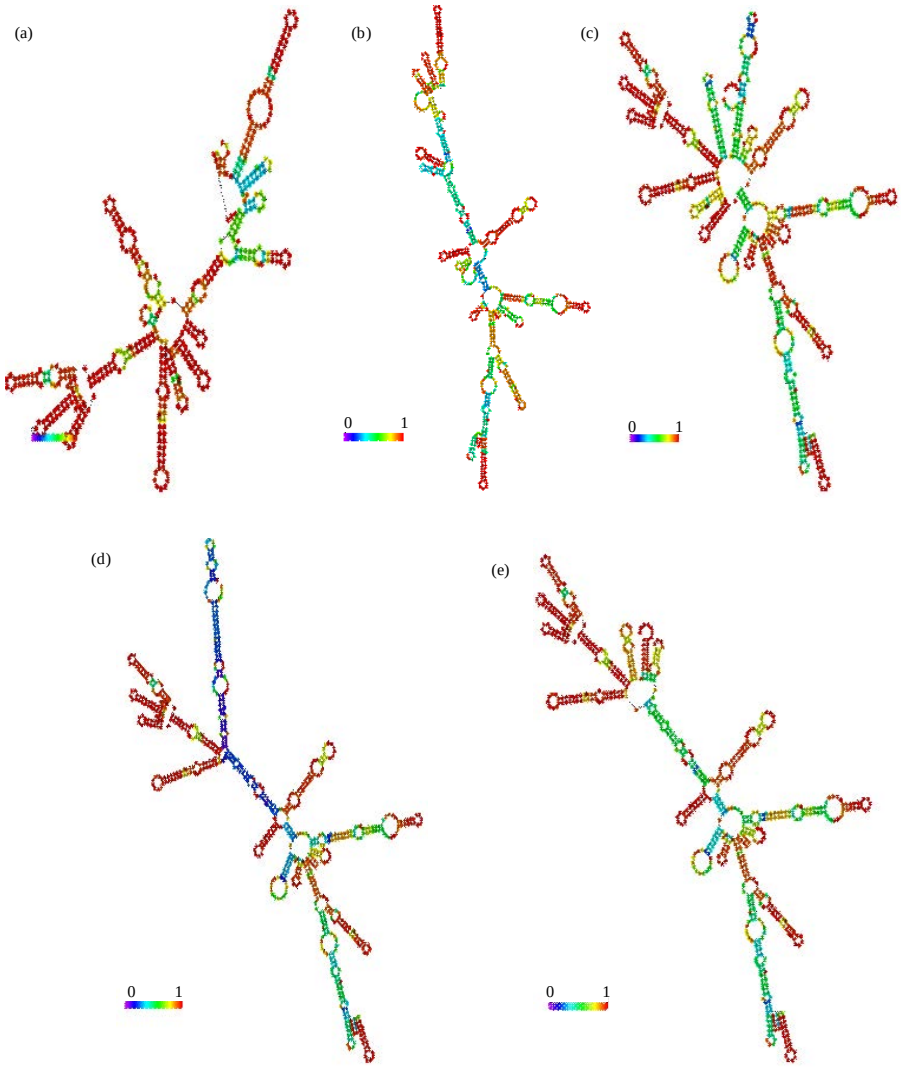

Fig. 9(a-e): RNA secondary structure prediction, (a) The structure above is coloured by base-pairing probabilities. For unpaired regions the colour denotes the probabilities of being unpaired. RNA secondary structure of strain KPA with minimum free energy value is -192.10 kcal mol^{-1}. The frequency of the MFE structure in the ensemble is 0.01%. The ensemble diversity is 50.06. The ensemble diversity is the average base-pair distance between all structures in the thermodynamic ensemble, (b) RNA secondary structure of strain 018 with minimum free energy value is -247.40 kcal mol^{-1}. The frequency of the MFE structure in the ensemble is 0%. The ensemble diversity is 140.86, (c) RNA secondary structure of strain AK with minimum free energy value is -279.40 kcal mol^{-1}. The frequency of the minimum free energy structure in the ensemble is 0%. The ensemble diversity is 128.8, (d) RNA secondary structure of strain ARMATI with minimum free energy value is -281.40 kcal mol^{-1}. The frequency of the MFE structure in the ensemble is 0%. The ensemble diversity is 152.14 and (e) RNA secondary structure of strain BIHPUR 0104 with minimum free energy value is -276.8 kcal mol^{-1}. The frequency of the MFE structure in the ensemble is 0%. The ensemble diversity is 122.25

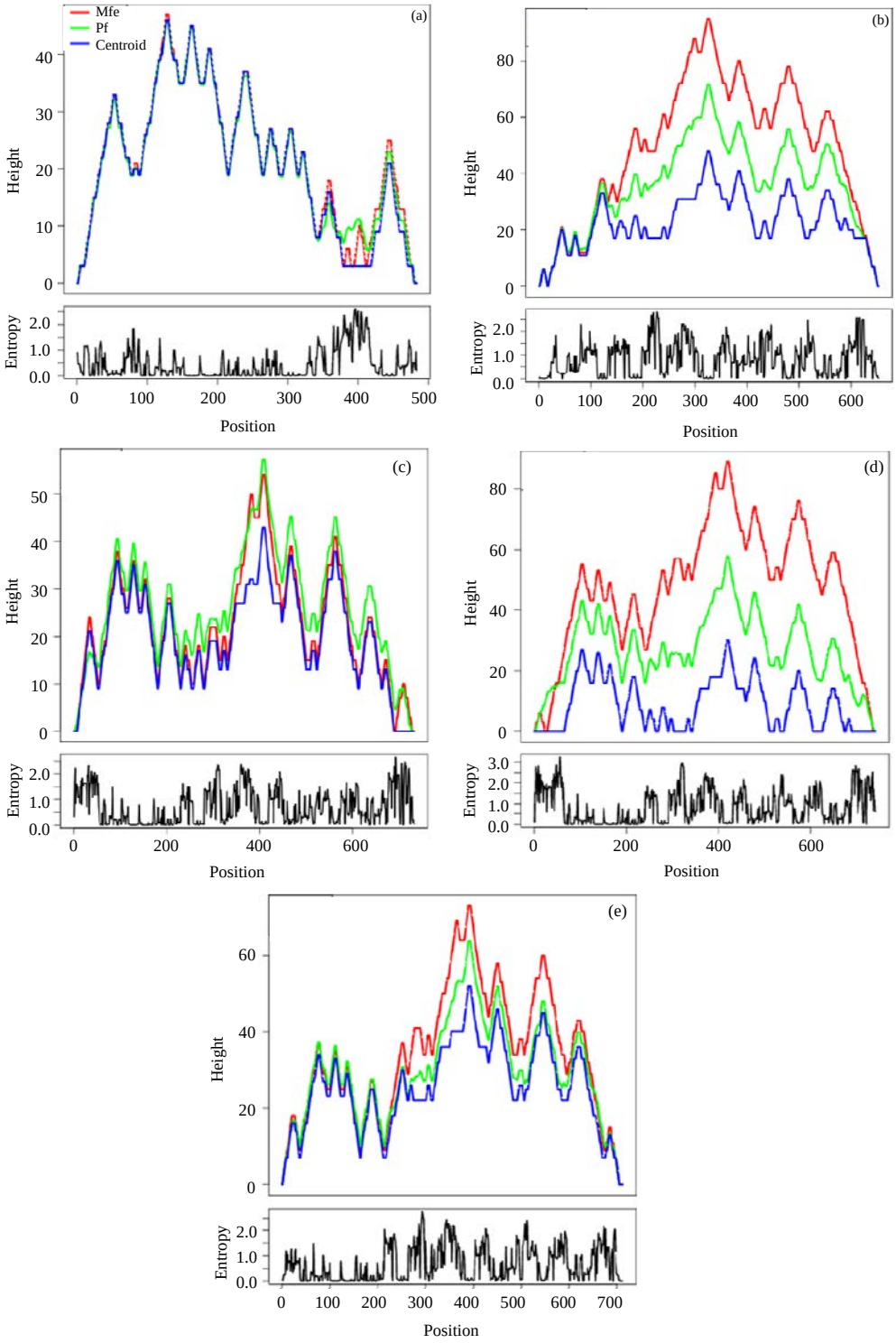

Fig. 10(a-e): Mountain plot and entropy prediction. Mountain plot and sequence entropy for each position of (a) Strain KPA, (b) Strain 018, (c) Strain AK, (d) Strain ARMATI and (e) Strain BIHPUR 0104

Table 2: GC-content (% G+C) in 16S rRNA of each *Bacillus* isolate

Strains	GC-content (%)
Strain KPA	55.1
Strain 018	54.7
Strain AK	55.4
Strain ARMATI	55.7
Strain BIHPUR 0104	55.3

represents a secondary structure in a plot of height versus position, where the height is given by the number of base pairs enclosing the base at given position i.e., loops correspond to plateaus (hairpin loops are peaks). Table 2 represents percentage GC (% G+C) content in the sequences of each isolate.

Phylogenetic tree of isolates: A neighbor- joining tree of isolates 16S rRNA sequences, including different strains of *Bacillus* species, clustered all the isolates belonging to the previously identified species to the corresponding species (Fig. 11).

DISCUSSION

In this study novel bacterial strains had been isolated from poultry feces soil samples on the basis of cultural, morphological and biochemical properties as well as portion of 16S rRNA gene. Members of the genus *Bacillus* produce large variety of extracellular enzymes, of which amylases play an important role at industrial scale. In the primary step of this investigation, the amylase producing *Bacillus* sp. were isolated from the collected samples by screening procedure as halo zone on starch agar plate through agar well diffusion method.

The culture conditions were found to have profound influence on extracellular amylase production (Table 3). In our study the changes in pH from 5-10 caused change in the amylase activity for each isolate. *B. subtilis* strain KPA, *B. subtilis* strain AK and *B. tequilensis* strain ARMATI were showing maximum enzyme activity at pH 6. The study strongly favours the finding of Mishra and Behera (2008), who demonstrated that *Bacillus* sp. had shown maximum amylase production at pH 6. Similar results were also obtained by Bakri *et al*. (2012) and Ramesh and Lonsane (1991). Krishnan and Chandra (1989) and Asgher *et al.* (2007) examined that *Bacillus* sp. had shown maximum amylase activity at pH 7. In our study *B. licheniformis* strain BIHPUR 0104 showed maximum amylase activity at neutral pH. Similar, results were observed by Nusrat and Rahman (2007), who demonstrated maximum production of amylase by *Bacillus* sp. at pH 7. *B. licheniformis* strain 018 in the present context was showing maximum enzyme activity at pH 9. Our results agree partially with the finding of Ul-Haq *et al*. (2002), who demonstrated that *Bacillus subtilis* had maximum amylase activity at alkaline pH. It is clear from the present investigation that strain 018 can be used at large scale in starch processing and baking industries because of its alkaline tolerant nature. pH played an important role in the production of extracellular amylase by each isolate. This is because substrate binding and catalysis are often dependent on charge distribution on both substrate and in particular enzyme molecules. pH markedly affects the metabolic pathways of microorganisms. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the microorganisms thus lowering their overall metabolic activity (Willey *et al*., 2008).

Temperature had profound effect on amylase production. All the isolates except *B. tequilensis* strain ARMATI showed maximum amylase activity at 35°C. The finding of this present study is more or less similar to the reports of Vijayalakshmi *et al*. (2012), who demonstrated that optimum

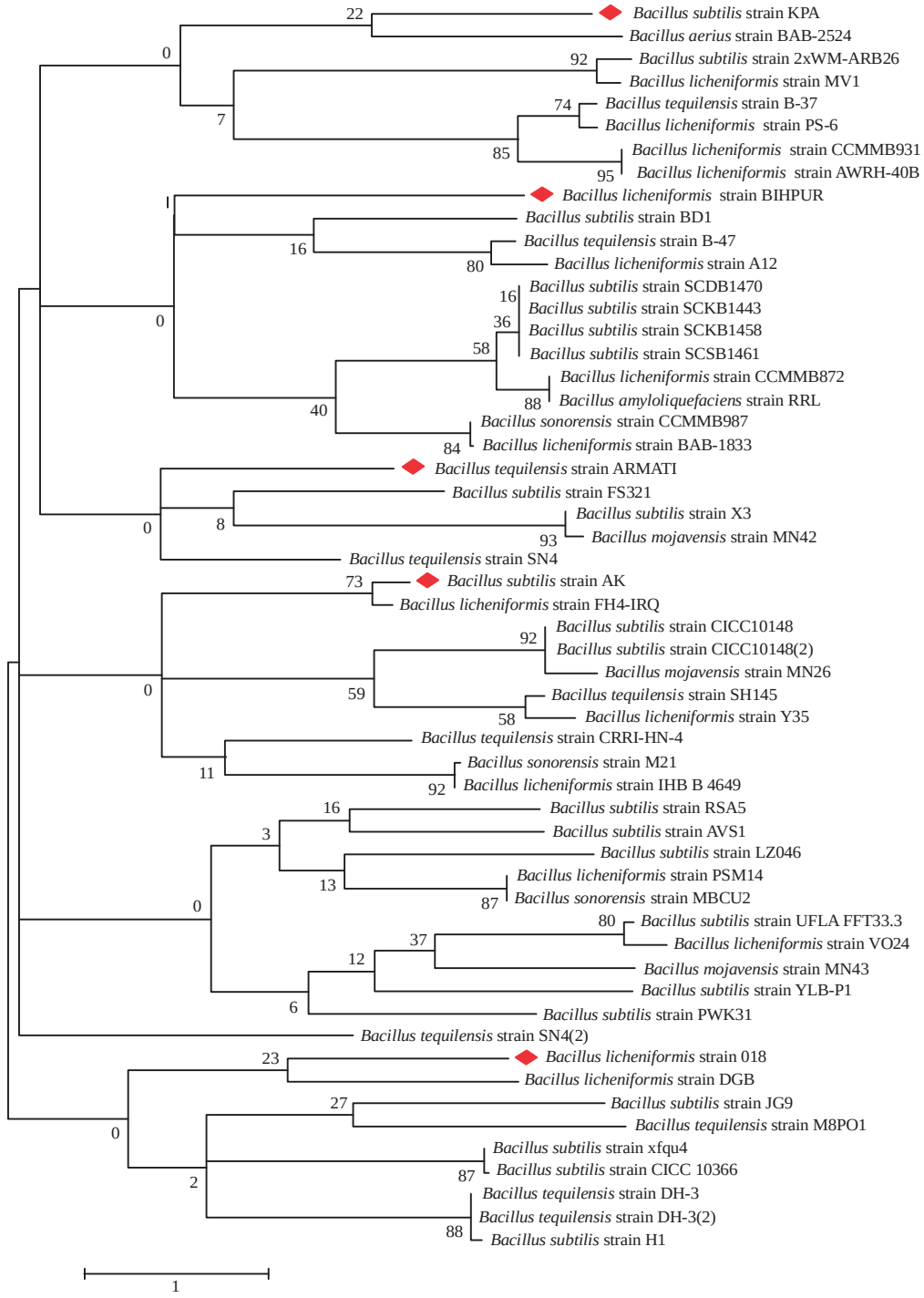

Fig. 11: Phylogenetic tree based on a comparison of the 16S ribosomal RNA sequences of *Bacillus* isolates and some of their closest phylogenetic relatives. The phylogenetic tree was constructed on the aligned datasets by Input order method using neighbor joining method using the program MEGA 4.0. The numbers on the tree indicates the percentages of bootstrap sampling derived from 1000 random samples. Novel isolates characterized in the present study are indicated in red bullets

Table 3: Effect of culture conditions for extracellular amylase activity and biomass production from novel *Bacillus* isolates in shake-flask cultivations

Culture conditions	Amylase activity (U mL^{-1}) (Mean±SD)	Relative activity (%)	Total soluble protein (mg mL^{-1}) (Mean±SD)	Dry weight of biomass (mg mL^{-1}) (Mean±SD)
***Bacillus subtilis* strain KPA**				
pH				
5	21.012±0.51	33.94	0.321±0.02	0.4±0.02
6	61.893±0.35	100.00	0.467±0.01	2.0±0.01
7	22.036±0.51	35.60	0.359±0.02	0.63±0.01
8	25.191±0.42	40.70	0.413±0.01	0.65±0.03
9	35.031±0.35	56.59	0.458±0.03	1.2±0.01
10	50.781±0.26	82.04	0.296±0.021	1.3±0.01
Temperature (°C)				
30	50.782±0.12	83.86	0.150±0.01	1.2±0.02
35	60.551±0.33	100.00	0.304±0.02	1.8±0.02
40	45.862±0.21	75.74	0.589±0.04	1.6±0.01
45	40.001±0.35	66.06	0.411±0.01	1.5±0.02
50	35.782±0.21	59.09	0.40±0.01	1.5±0.01
Agitation speed (rpm)				
120	31.021±0.22	49.31	0.216±0.03	1.3±0.03
130	62.910±0.35	100.00	0.477±0.04	2.0±0.01
140	35.001±0.13	55.63	0.311±0.01	1.3±0.01
150	30.10±0.24	47.84	0.211±0.01	0.6±0.02
160	30.08±0.11	47.63	0.210±0.02	0.7±0.01
Incubation time (h)				
12	30.97±0.23	47.02	0.1120±0.03	0.8±0.02
24	65.86±0.12	100.00	0.4520±0.02	2.2±0.01
48	40.605±0.21	61.66	0.3810±0.01	1.8±0.01
72	26.896±0.31	40.83	0.2110±0.01	1.9±0.03
96	22.811±0.33	34.63	0.0677±0.02	1.6±0.02
Carbon source				
Glucose	60.523±0.31	47.71	0.321±0.02	1.4±0.01
Starch	125.752±0.35	100.00	0.432±0.01	2.5±0.01
Xylose	75.123±0.25	59.73	0.311±0.02	1.9±0.02
Sucrose	52.321±0.28	41.60	0.221±0.01	1.6±0.02
Lactose	50.023±0.11	39.77	0.178±0.01	1.8±0.01
Mannose	45.541±0.21	36.21	0.213±0.03	1.1±0.02
CMC	41.021±0.14	32.62	0.021±0.01	1.4±0.02
Nitrogen source				
Peptone	72.976±0.22	55.15	0.115±0.02	0.8±0.02
Yeast extract	132.321±0.11	100.00	0.338±0.03	2.7±0.02
Beef extract	68.00±0.17	51.39	0.221±0.01	2.0±0.01
Tryptone	55.231±0.16	41.74	0.117±0.01	1.3±0.03
KNO_3	43.001±0.12	32.49	0.222±0.03	1.6±0.01
Ammonium sulphate	40.875±0.15	30.89	0.117±0.01	1.1±0.02
Ammonium chloride	31.310±0.1	23.66	0.224±0.02	0.6±0.035
***Bacillus licheniformis* strain 018**				
pH				
5	22.212±0.12	39.92	0.325±0.03	1.5±0.02
6	32.886±0.25	59.11	0.451±0.01	1.6±0.02
7	30.739±0.38	55.25	0.675±0.02	1.6±0.01
8	50.124±0.24	90.10	0.668±0.02	2.0±0.02
9	55.631±0.26	100.00	0.905±0.02	2.5±0.01
10	40.874±0.12	73.47	0.634±0.03	2.2±0.03
Temperature (°C)				
30	35.262±0.49	67.09	0.435±0.03	0.6±0.03
35	52.555±0.35	100.00	0.908±0.02	2.0±0.02
40	30.578±0.35	58.18	0.248±0.02	0.8±0.01
45	30.523±0.21	58.07	0.148±0.05	0.6±0.01
50	25.110±0.22	47.77	0.112±0.04	0.4±0.01
Agitation speed (rpm)				
120	50.025±0.37	66.23	0.352±0.05	2.0±0.01
130	52.548±0.28	69.58	0.902±0.04	1.7±0.02

Table 3: Continue

Culture conditions	Amylase activity (U mL^{-1}) (Mean±SD)	Relative activity (%)	Total soluble protein (mg mL^{-1}) (Mean±SD)	Dry weight of biomass (mg mL^{-1}) (Mean±SD)
140	42.735±0.39	56.58	0.687±0.01	1.0±0.02
150	75.521±0.22	100.00	0.461±0.02	2.8±0.01
160	51.321±0.12	67.95	0.405±0.01	2.0±0.01
Incubation time (h)				
12	45.212±0.48	59.96	0.212±0.03	0.63±0.01
24	75.399±0.25	100.00	0.665±0.04	2.8±0.02
48	40.307±0.13	53.45	0.585±0.05	1.0±0.02
72	31.006±0.32	41.12	0.297±0.04	1.0±0.01
96	21.101±0.26	27.98	0.138±0.01	0.5±0.02
Carbon source				
Glucose	81.523±0.31	57.91	0.321±0.02	1.4±0.01
Starch	140.752±0.35	100.00	0.432±0.01	2.5±0.01
Xylose	73.123±0.25	51.95	0.311±0.02	1.9±0.02
Sucrose	60.321±0.28	42.85	0.221±0.01	1.6±0.02
Lactose	50.023±0.11	35.533	0.178±0.01	1.8±0.01
Mannose	43.541±0.21	30.93	0.213±0.03	1.1±0.02
CMC	40.021±0.14	28.43	0.021±0.01	1.4±0.02
Nitrogen source				
Peptone	92.976±0.22	63.97	0.115±0.02	0.8±0.02
Yeast extract	145.321±0.11	100.00	0.338±0.03	2.7±0.02
Beef extract	81.00±0.17	55.73	0.221±0.01	2.0±0.01
Tryptone	62.231±0.16	42.82	0.117±0.01	1.3±0.03
KNO_3	60.001±0.12	42.82	0.222±0.03	1.6±0.01
Ammonium sulphate	50.875±0.15	35.00	0.117±0.01	1.1±0.02
Ammonium chloride	48.310±0.1	33.24	0.224±0.02	0.6±0.03
***Bacillus subtilis* strain AK**				
pH				
5	41.512±0.43	60.47	0.485±0.02	1.0±0.01
6	68.641±0.33	100.00	0.891±0.01	1.5±0.01
7	38.662±0.24	56.32	0.473±0.02	1.0±0.02
8	35.119±0.32	51.16	0.192±0.02	1.0±0.01
9	30.516±0.16	44.45	1.08±0.03	0.8±0.01
10	34.114±0.18	49.69	1.09±0.02	0.9±0.01
Temperature (°C)				
30	45.634±0.57	68.33	0.441±0.02	1.2±0.02
35	66.782±0.56	100.00	0.624±0.03	2.2±0.01
40	50.091±0.48	75.00	0.295±0.01	2.0±0.01
45	40.769±0.59	61.04	0.245±0.03	1.5±0.02
50	30.101±0.51	45.07	0.165±0.01	1.4±0.01
Agitation speed (rpm)				
120	40.121±0.35	57.81	0.111±0.01	1.0±0.02
130	69.399±0.45	100.00	0.339±0.04	2.0±0.01
140	20.813±0.39	29.99	0.209±0.02	0.5±0.01
150	20.803±0.29	29.81	0.201±0.01	0.5±0.01
160	18.81±0.31	27.10	0.21±0.01	0.6±0.03
Incubation time (h)				
12	45.22±0.59	64.02	0.121±0.04	0.8±0.03
24	70.628±0.58	100.00	1.027±0.03	2.5±0.02
48	48.909±0.43	69.24	0.668±0.03	1.5±0.01
72	33.736±0.26	47.76	0.345±0.02	1.0±0.01
96	29.98±0.12	42.44	0.221±0.02	1.2±0.03
Carbon source				
Glucose	88.523±0.31	60.73	0.321±0.02	1.4±0.01
Starch	145.752±0.35	100.00	0.432±0.01	2.5±0.01
Xylose	81.123±0.25	55.65	0.311±0.02	1.9±0.02
Sucrose	67.321±0.28	46.18	0.221±0.01	1.6±0.02
Lactose	62.023±0.11	42.55	0.178±0.01	1.8±0.01
Mannose	53.541±0.21	36.73	0.213±0.03	1.1±0.02
CMC	50.021±0.14	34.31	0.021±0.01	1.4±0.02
Nitrogen source				
Peptone	93.976±0.22	62.51	0.115±0.02	0.8±0.02
Yeast extract	150.321±0.11	100.00	0.338±0.03	2.7±0.02
Beef extract	82.00±0.17	54.54	0.221±0.01	2.0±0.01

Table 3: Continue

Culture conditions	Amylase activity (U mL^{-1}) (Mean±SD)	Relative activity (%)	Total soluble protein (mg mL^{-1}) (Mean±SD)	Dry weight of biomass (mg mL^{-1}) (Mean±SD)
Tryptone	71.231±0.16	47.38	0.117±0.01	1.3±0.03
KNO_3	68.001±0.12	45.23	0.222±0.03	1.6±0.01
Ammonium sulphate	52.875±0.15	35.17	0.117±0.01	1.1±0.02
Ammonium chloride	50.31±0.1	33.46	0.224±0.02	0.6±0.03
***Bacillus tequilensis* strain ARMATI**				
pH				
5	40.011±0.63	79.95	0.332±0.05	1.0±0.01
6	50.045±0.61	100.00	0.662±0.03	2.0±0.02
7	40.117±0.59	80.16	0.374±0.02	1.2±0.01
8	34.438±0.34	68.81	0.349±0.01	0.6±0.03
9	30.98±0.41	61.90	0.828±0.02	0.5±0.02
10	25.96±0.42	51.87	0.477±0.03	0.4±0.01
Temperature (°C)				
30	35.971±0.48	76.22	0.381±0.04	1.2±0.02
35	37.501±0.37	79.46	0.665±0.02	1.0±0.01
40	47.189±0.41	100.00	0.669±0.01	2.3±0.03
45	35.813±0.36	75.89	0.402±0.02	2.0±0.02
50	25.091±0.37	53.16	0.311±0.01	1.0±0.01
Agitation speed (rpm)				
120	32.004±0.67	67.8	0.113±0.03	1.0±0.02
130	47.197±0.59	100.00	0.445±0.04	2.5±0.03
140	36.083±0.13	76.45	0.312±0.01	2.0±0.01
150	39.013±0.23	82.65	0.311±0.02	2.0±0.01
160	31.01±0.34	65.70	0.302±0.02	1.5±0.01
Incubation time (h)				
12	28.212±0.61	56.05	0.213±0.02	1.2±0.03
24	50.327±0.36	100.00	0.678±0.07	2.5±0.02
48	29.325±0.69	58.26	0.803±0.05	0.8±0.01
72	19.019±0.59	37.77	0.377±0.04	0.5±0.02
96	16.01±0.38	31.81	0.202±0.04	0.5±0.02
Carbon source				
Glucose	74.523±0.31	74.95	0.321±0.02	1.4±0.01
Starch	100.752±0.35	100.00	0.432±0.01	2.5±0.01
Xylose	61.123±0.25	60.66	0.311±0.02	1.9±0.02
Sucrose	52.321±0.28	51.93	0.221±0.01	1.6±0.02
Lactose	50.023±0.11	49.64	0.178±0.01	1.8±0.01
Mannose	32.541±0.21	32.29	0.213±0.03	1.1±0.02
CMC	31.021±0.14	30.78	0.021±0.01	1.4±0.02
Nitrogen source				
Peptone	73.976±0.22	68.29	0.115±0.02	0.8±0.02
Yeast extract	108.321±0.11	100.00	0.338±0.03	2.7±0.02
Beef extract	70.00±0.17	64.62	0.221±0.01	2.0±0.01
Tryptone	65.231±0.16	60.27	0.117±0.01	1.3±0.03
KNO_3	51.001±0.12	47.08	0.222±0.03	1.6±0.01
Ammonium sulphate	42.875±0.15	39.58	0.117±0.01	1.1±0.02
Ammonium chloride	32.31±0.1	29.82	0.224±0.02	0.6±0.03
***Bacillus licheniformis* strain BIHPUR 0104**				
pH				
5	45.562±0.51	72.19	0.113±0.01	1.2±0.01
6	49.736±0.38	78.80	0.118±0.05	1.4±0.02
7	63.114±0.42	100.00	1.05±0.02	2.2±0.01
8	40.262±0.47	63.79	0.384±0.06	1.8±0.01
9	33.525±0.58	53.11	0.345±0.04	0.5±0.02
10	27.7±0.59	43.88	0.509±0.04	1.2±0.01
Temperature (°C)				
30	42.955±0.13	71.46	0.892±0.03	1.2±0.01
35	60.103±0.33	100.00	1.02±0.01	2.5±0.01
40	40.81±0.51	67.90	0.352±0.02	1.0±0.01
45	42.191±0.43	70.19	0.32±0.04	0.8±0.02
50	40.098±0.55	67.34	0.201±0.05	0.8±0.02

Table 3: Continue

Culture conditions	Amylase activity (U mL^{-1}) (Mean±SD)	Relative activity (%)	Total soluble protein (mg mL^{-1}) (Mean±SD)	Dry weight of biomass (mg mL^{-1}) (Mean±SD)
Agitation speed (rpm)				
120	60.782±0.22	83.31	1.18±0.01	2.0±0.02
130	53.026±0.21	72.68	0.563±0.02	1.5±0.01
140	61.751±0.12	84.63	1.2±0.01	2.0±0.01
150	72.957±0.33	100.00	0.382±0.03	2.5±0.03
160	45.212±0.53	61.97	0.276±0.02	1.8±0.01
Incubation time (h)				
12	58.012±0.52	77.25	0.421±0.02	1.5±0.03
24	74.09±0.35	100.00	0.958±0.01	2.6±0.01
48	55.172±0.12	73.47	0.873±0.01	1.8±0.01
72	40.289±0.25	53.64	0.451±0.03	1.0±0.02
96	31.02±0.11	41.31	0.317±0.02	0.4±0.01
Carbon source				
Glucose	98.523±0.31	69.90	0.321±0.02	1.4±0.01
Starch	140.752±0.35	100.00	0.432±0.01	2.5±0.01
Xylose	84.123±0.25	59.76	0.311±0.02	1.9±0.02
Sucrose	70.321±0.28	49.96	0.221±0.01	1.6±0.02
Lactose	65.023±0.11	46.19	0.178±0.01	1.8±0.01
Mannose	52.541±0.21	37.32	0.213±0.03	1.1±0.02
CMC	31.021±0.14	22.03	0.021±0.01	1.4±0.02
Nitrogen source				
Peptone	73.976±0.22	49.87	0.115±0.02	0.8±0.02
Yeast extract	148.321±0.11	100.00	0.338±0.03	2.7±0.02
Beef extract	70.00±0.17	47.19	0.221±0.01	2.0±0.01
Tryptone	67.231±0.16	45.32	0.117±0.01	1.3±0.03
KNO_3	51.001±0.12	34.38	0.222±0.03	1.6±0.01
Ammonium sulphate	40.875±0.15	27.55	0.117±0.01	1.1±0.02
Ammonium chloride	30.31±0.1	20.43	0.224±0.02	0.6±0.03

amylase production by *Bacillus* sp. was obtained at 32°C. Enzyme activity was 100% at 40°C for strain ARMATI. The finding totally agree with the study of Raul *et al*. (2014) and Deb *et al*. (2013), who examined the effect of temperature on amylase activity from *Bacillus* strains and observed maximum enzyme activity at 40°C. However, our study was against the finding of Teodoro and Martins (2000), who found that amylase produced by *Bacillus* sp. gave the best activity at 50°C. On the other hand, amylase reported by other workers had temperature optima of 37-70°C (Swain *et al*., 2006; Saxena *et al*., 2007). The variations among our findings and the previous reports may be because of the source of the isolation and types of bacterial strains. In order to convert starch substrate into product, amylase must collide with and bind to the substrate at the active site. Increasing or decreasing the temperature of a system will increase or decrease the number of collisions of enzyme and substrate per unit time. Thus, within limits, the rate of the reaction will change. For the mesophilic bacteria, as the temperature of the system increases, it causes thermal denaturation of the protein and enzymes. Thus too much heat can cause the rate of an enzyme catalyzed reaction to decrease because the enzyme or substrate becomes denatured and inactive.

Aeration is one of the effective factors for the improvement of aerobic fermentation. The present experiments were carried out under the variation of shaking speed ranging from 120-160 rpm in order to optimize the aerobic condition in shake flask cultivation. According to the present context results, maximum amylase activity by strain KPA, strain AK and strain ARMATI was observed at 130 rpm. On the other hand, strain 018 and strain BIHPUR 0104 showed maximum enzyme activity at 150 rpm. Strain 018 and strain BIHPUR 0104 showed maximum enzyme activity at higher agitation rate. The reason behind this may be the uniform distribution of nutrient and

supplied oxygen for the cultivation of these particular strains in the fermentation medium. Agitation rates in the range of 100-250 rpm have also been reported for the production of alpha amylases (Vijayalakshmi *et al*., 2012; Santos and Martins, 2003).

Incubation period is one of the most important parameter in metabolic activity and growth of bacteria. The results obtained in this study indicated that highest yield of amylase by all the *Bacillus* strains were obtained at 24 h of incubation. A decline in amylase activity afterwards was probably due to depletion of nutrients in the fermentation medium of the microorganisms, causing stressed and unfavourable conditions for the bacteria resulting in reduction of enzyme activity. Our reports were against the findings of Vijayalakshmi *et al.* (2012) and Deb *et al.* (2013), who demonstrated maximum amylase activity by *Bacillus* sp. at 48 h of incubation. On the other hand the present study strongly favours the finding of Abate *et al.* (1999) and Burhan *et al.* (2003), who observed significant amylase production for *Bacillus* sp. at the end of 24 h. Findings of the corresponding experiments revealed that the time course of enzyme activity varies with the source of isolation, strains used, genetic makeup of strains and cultivation conditions. Incubation beyond the optimum time course was generally accompanied by a decrease in the growth rate and enzyme productivity, which gradually declined after 24 h of incubation. The decreased activity in the later phase of growth was probably due to catabolite repression by readily metabolizable substrate glucose (Lin *et al*., 1998).

Carbon is the essential element for the growth and metabolism of bacteria. The enzyme production is stimulated in the presence of various carbon sources. In the present context, optimal level of extracellular amylase from all the isolates was recorded when starch was used as a sole carbon source. The results totally agree with the finding of Samanta *et al.* (2013), who demonstrated maximum amylase production in the presence of starch. Deb *et al.* (2013) recorded maximum amylase production from *Bacillus* sp. in the presence of corn flour. Various organic and inorganic nitrogen sources were tested to estimate the maximum amylase production from the isolates. Yeast extract was found to be the best nitrogen source for all the isolates in order to achieve maximum production of amylase. The present investigation supports the finding of Ravindar and Elangovan (2013) and Dash *et al.* (2015) but do not correlate with the finding of Bozic *et al.* (2011), who demonstrated tryptone as a potential nitrogen source for enzyme production. Yeast extract has been reported to play an important role in enzyme production due to the presence of essential elements and growth factors (Porsuk *et al*., 2013).

In the present investigation crude amylase obtained from strain KPA, strain 018, strain AK, strain ARMATI and strain BIHPUR 0104 was more stable up to 60°C for 4 h of incubation and retained upto 58.7, 48.13, 52.15, 51 and 42.03% of the activity, respectively. Our study was against the finding of Samanta *et al*. (2013), who demonstrated that crude amylase obtained from bacteria was more stable at 40°C but supports more or less the finding of Guleria and Chatanta (2014) and Deb *et al.* (2013), who obtained amylase stability at 50°C. Similar observation was reported by Sharma *et al*. (2014). Thus, the results concluded that the crude enzyme is moderately temperature stable. Significant enzyme stability at higher temperatures would be important for several industrial processes. The industrial importance of an enzyme will be more when the effect of temperature input on its optimal activity is less.

The most desirable characteristic of novel isolates of present investigation was steady stability at alkaline pH. The crude amylase obtained from the isolates showed stability at a wide range of pH from 5.0-9.0. The outcome of the present study is more or less similar to the finding of Sharma *et al*. (2014) and Asgher *et al*. (2007), who demonstrated stability of amylase at a wide range of pH 3.5-12. Many of the microorganisms have been found to produce amylase with pH

optima near neutral region but with high stability in alkaline conditions. Stability at alkaline pH values may be due to charged amino acid residues. The enzymes stable in alkaline conditions were characterized by a decreased number of acidic residues and an increased number of arginines (Hakulinen *et al*., 2003). Strains isolated in the present context could be a good source for biotechnological applications at industrial scale because of the alkali stability nature of enzyme.

The prediction of RNA secondary structure is based on free energy minimization. The free energy minimization lowers the total Gibbs free energy giving stability to the sequence. RNA structure even helps to determine the evolutionary stability. Thermodynamic approaches use dynamic programming to compute the optimal secondary structure for a single RNA sequence with globally minimal free energy (Zuker and Stiegler, 1981). In the current study we discussed the five different strains of *Bacillus* sp. by RNA structure prediction. The results showed that the percent of energy structure among five strains was different. The colour of nucleotides indicates the rate of variation among strains. The variations include the shape of RNA secondary structure, the value of energy and different plot sequencing. RNA structure plays an important role in the life cycle of bacteria and provides the ability to understand evolution and stability.

Minimum free energy for the prediction of optimal secondary structure is the method for searching the structure with stable energies. Gibbs free energy of RNA secondary structure for each bacterial isolate provides evolutionary stability to the sequences and determines it as a stable model (Sankari and Khusro, 2014).

DNA with high GC- content is more stable than DNA with low GC- content. The GC base pairs are more stable than AU base pairs due to the fact that GC bonds have 3 hydrogen bonds and AU only has 2 hydrogen bonds, which makes high GC-content RNA structure more tolerant to high temperature. Previous studies have shown that GC-content of 16S rRNA is highly correlated with optimal growth in prokaryotes (Wang and Hickey, 2002; Nakashima *et al*., 2003). In the present investigation maximum GC- content was calculated for strain ARMATI. The strain was showing maximum amylase activity and stability at 40 and 60°C, respectively. As other strain's 16S rRNA GC-content was less than that of strain ARMATI, hence those strains were not able to produce more amylase at high temperature (40°C). Enzyme activity for the other strains declined above 35°C. The study established an assumption that higher GC- content favours the growth of bacteria at higher temperature resulting higher enzyme activity and stability. Further research in this field would be of great help in order to identify the co-relation between GC- content, the enzyme activity and stability among the prokaryotes.

16S rRNA gene sequences to study bacterial phylogeny and taxonomy have been by far the most common molecular marker. The 16S rRNA gene consists of highly conserved regions which describe the level and occurrence of phylogenetic relationships. The 16S ribosomal RNA-based molecular identification could achieve identification because of the presence of species-specific variable regions. This molecular approach has been extensively used for bacterial phylogeny, leading to the establishment of large public-domain databases and its application to bacterial identification, including that of environmental and clinical uncultured microorganisms, unique or unusual isolates and collections of phenotypically identified isolates (Drancourt *et al*., 2000).

CONCLUSION

The results obtained from the present investigation indicate enhanced production of extracellular amylase from novel strains of *Bacillus sp.* under optimized medium components and culture conditions by using traditional OFAT method. The most significant findings of this study

were the ability of these strains to produce maximum level of extracellular amylase after only 24 h of incubation and the stability of enzyme at 60°C and alkaline pH. The best process parameters were found to be starch as the sole carbon source and yeast extract as nitrogen source. Strains isolated in the present context could be a good alternative of the commercial strains in starch processing industries as biotechnological and physiological aspects. A further study with more focus on enhanced production of enzyme either by conventional mutation studies or antimicrobials stresses is therefore suggested. Another study should also be continued to characterize different enzymes from poultry farm bacteria for their industrial applications.

ACKNOWLEDGMENT

Authors thank Department of Plant Biology and Biotechnology, Loyola College for providing necessary facilities during this research activity.

REFERENCES

Abate, C.M., G.R. Castro, R. Sineriz and D.A.S. Callieri, 1999. Production of enzymes by *Bacillus amyloquefaciens*in pure culture and in co-culture with *Zymomonas mobilis*. Biotechnol. Lett., 21: 249-252.

Asgher, M., M.J. Asad, S.U. Rahman and R.L. Legge, 2007. A thermostable amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. J. Food Eng., 79: 950-955.

Bakri, Y., H. Ammouneh, S. El-Khouri, M. Harba and P. Thonart, 2012. Isolation and identification of a new *Bacillus*strain for amylase production. Res. Biotechnol., 3: 51-58.

Bozic, N., J. Ruiz, J. Lopez-Santin and Z. Vujcic, 2011. Optimization of the growth of and α-amylase production by*Bacillus subtilis* IP 5832 in shake flask and laboratory fermenter batch cultures. J. Serbian Chem. Soc., 76: 965-972.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Burhan, A., U. Nisa, C. Gokhan, C. Omer, A. Ashabil and G. Osman, 2003. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillu s*sp. Isolate ANT-6. Process Biochem., 38: 1397-1403.

Dash, B.K., M.M. Rahman and P.K. Sarker, 2015. Molecular identification of a newly isolated *Bacillus subtilis*BI19 and optimization of production conditions for enhanced production of extracellular amylase. BioMed Res. Int., Vol. 2015. 10.1155/2015/859805

De Souza, P.M. and P.O. Magalhaes, 2010. Application of microbial alpha amylases in industry: A review. Braz. J. Microbiol., 41: 850-861.

Deb, P., S.A. Talukdar, K. Mohsina, P.K. Sarker and S.A. Sayem, 2013. Production and partial characterization of extracellular amylase enzyme from *Bacillus amylolique faciens* P-001. SpringerPlus, Vol. 2. 10.1186/2193-1801-2-154

Drancourt, M., C. Bollet, A. Carlioz, R. Martelin, J.P. Gayral and D. Raoult, 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clin. Microbiol., 38: 3623-3630.

Guleria, A. and D.K. Chatanta, 2014. Isolation, screening and optimization of microorganism producing amylase. Global. J. Res. Anal., 3: 181-185.

Gupta, A., V.K. Gupta, D.R. Modi and L.P. Yadava, 2008. Production and characterization of α-amylase from *Aspergillus niger*. Biotechnology, 7: 551-556.

Hakulinen, N., O. Turunen, J. Janis, M. Leisola and J. Rouvinen, 2003. Three dimensional structures of thermophilic β 1, 4 xylanases from *Chaetomium thermophilum* and *Nonomuraea flexuosa*. Eur. J. Biochem., 270: 1399-1412.

Issac, B., H. Singh, H. Kaur and G.P.S. Raghava, 2002. Locating probable genes using Fourier transform approach. Bioinformatics, 18: 196-197.

Krishnan, T. and A.K. Chandra, 1989. Purification and characterization of *α*-amylase from *Bacillus Licheniformis* CUMC 305. Applied Environ. Microbiol., 46: 430-437.

Krumperman, P.H., 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to indentify high-risk sources of fecal contamination of foods. Applied Environ. Microbiol., 46: 165-170.

Li, H., Z. Chi, X. Wang, X. Duan, L. Ma and L. Gao, 2007. Purification and characterization of extracellular amylase from the marine yeast *Aureobasidium pullulans*N13d and its raw potato starch digestion. Enzyme Microb. Technol., 40: 1006-1012.

Lin, L.L., C.C. Chyau and W.H. Hsu, 1998. Production and properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. Biotechnol. Applied Biochem., 28: 61-68.

Liu, X.D. and Y. Xu, 2008. A novel raw starch digesting α-amylase from a newly isolated *Bacillus* sp. YX-1. Purification and characterization. Bioresour. Technol., 99: 4315-4320.

Mathews, D.H., M.D. Disney, J.L. Childs, S.J. Schroeder, M. Zuker and D.H. Turner, 2004. Incorporating chemical modification constraints into a dynamic programming algorithm for prediction of RNA secondary structure. Proc. Nat. Acad. Sci. USA., 101: 7287-7292.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Mishra, S. and N. Behera, 2008. Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. Afr. J. Biotechnol., 7: 3326-3331.

Nakashima, H., S. Fukuchi and K. Nishikawa, 2003. Compositional changes in RNA, DNA and proteins for bacterial adaptation to higher and lower temperatures. J. Biochem., 133: 507-513.

Nusrat, A. and S.R. Rahman, 2007. Comparative studies on the production of extracellular α-amylase by three mesophilic *Bacillus* isolates. Bangladesh J. Microbiol., 24: 129-132.

Pandey, A., P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh and R. Mohan, 2000. Advances in microbial amylases. Biotechnol. Applied Biochem., 31: 135-152.

Porsuk, I., S. Ozakin, B. Bali and E.I. Yilmaz, 2013. A cellulase-free, thermoactive and alkali xylanase production by terrestrial*Streptomyces*sp. CA24. Turk. J. Biol., 37: 370-375.

Ramesh, M.V. and B.K. Lonsane, 1991. Regulation of alpha-amylase production in *Bacillus licheniformis* M27 by enzyme end-products in submerged fermentation and its overcoming in solid state fermentation system. Biotechnol. Lett., 13: 355-360.

Raul, D., T. Biswas, S. Mukhopadhyay, S.K. Das and S. Gupta, 2014. Production and partial purification of *α* amylase from*Bacillus subtilis*(MTCC 121) using solid state fermentation. Biochem. Res. Int. 10.1155/2014/568141

Ravindar, D.J. and N. Elangovan, 2013. Molecular identification of amylase producing *Bacillus subtilis*and detection of optimal conditions. J. Pharm. Res., 6: 426-430.

Samanta, A., D. Mitra, S.N. Roy, C. Sinha and P. Pal, 2013. Characterization and optimization of amylase producing bacteria isolated from solid waste. J. Environ. Protec., 4: 647-652.

Sankari, D. and A. Khusro, 2014. Biochemical, molecular characterization and sequence analysis of keratinase producing novel strain of *Bacillus licheniformis* isolated from poultry farm. Int. J. Pharm. Pharm. Sci., 6: 457-461.

Santos, E.D.O. and M.L.L. Martins, 2003. Effect of the medium composition on formation of amylase by*Bacillu s*sp. Braz. Arch. Biol. Technol., 46: 129-134.

Saxena, R.K., K. Dutt, L. Agarwal and P. Nayyar, 2007. A highly thermostable and alkaline amylase from a *Bacillus*sp. PN5. Bioresour. Technol., 98: 260-265.

Shah, K.P., K.H. Chandok, P. Rathore, M.V. Sharma, M. Yadav and S.A. Nayarisseri, 2013. Screening, isolation and identification of polygalacturonase producing *Bacillus tequilensis* strain EMBS083 using 16S rRNA gene sequencing. Eur. J. Biol. Sci., 5: 9-13.

Sharma, K., S. Bhutty, S.P. Khurana and U.K. Kohli, 2014. Isolation, identification and optimization of culture conditions of *Bacillu s*sp. strain PM1 for alkalo-thermostable amylase production. Br. Microbiol. Res. J., 4: 369-380.

Sneath, P.H.A., 1994. Gram Positive Rods. In: Bergey's Manual of Determinative Bacteriology, Holt, J.G. (Ed.). 9th Edn., Lippincott Williams and Wilkins, Philadelphia, PA., pp: 2106-2111.

Swain, M.R., S. Kar, G. Padmaja and C.R. Ray, 2006. Partial characterization and optimization of production of extracellular *α*-amylase from Bacillus subtilis isolated from culturable cowdung microflora. Pol. J. Microbiol., 55: 289-296.

Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.

Teodoro, C.E.D.S. and M.L.L. Martins, 2000. Culture conditions for the production of thermostable amylase by *Bacillus*. Brazil J. Microb., 31: 298-302.

Ul-Haq, I., N. Riaz, H. Ashraf and M.A. Qadeer, 2002. Effect of inorganic salts on the production of α-amylase by *Bacillus subtilis*. Ind. J. Plant Sci., 2: 115-119.

Vijayalakshmi, K., S. Sushma, S. Abha and P. Chander, 2012. Isolation and characterization of *Bacillus subtilis*KC3 for amylolytic activity. Int. J. Biosci. Biochem. Bioinform., 2: 336-341.

Wang, H.C. and D.A. Hickey, 2002. Evidence for strong selective constraint acting on the nucleotide composition of 16S ribosomal RNA genes. Nucleic Acids Res., 30: 2501-2507.

Willey, J.M., L.M. Sherwood and C.J. Woolverton, 2008. Prescott, Harley and Kleins's Microbiology. 7th Edn., McGraw-Hill Higher Education, USA., ISBN: 9780071102315, Pages: 1088.

Zuker, M. and P. Stiegler, 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res., 9: 133-148.