DETERMINATION AND OPTIMIZATION OF CRUDE EXTRACELLULAR AMYLASE EXTRACTED FROM DIFFERENT *BACILLUS* SPECIES

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ABSTRACT: Amylases are starch hydrolyzing enzymes and have commercial applications in different industries, such as food, fermentation, detergent and pharmaceuticals. In the current study amylases were extracted from bacteria found in kitchen wastes and rice fields. Isolates with maximum starch hydrolysis determined by the clear zones formation against blue background were selected and examined for further extracellular amylase activity. 16S rRNA gene sequencing confirmed these isolates as *Bacillus subtilis*, *Bacillus* sp. and *Lysinibacillus* sp. Highest dextrizing activity (83%)f and starch hydrolysis ratio (2.8) were shown by *Bacillus subtilis G3*. Furthermore optimum amylase activity was also checked at different temperatures, pH, media composition and substrate concentration. Optimum reaction temperature and pH for crude amylase activity from *Bacillus subtilis G3* was 60˚C -75˚C and 9pH in minimal media supplemented with 0.1% soluble starch. Thin layer chromatography and high performance liquid chromatography were carried out to investigate the starch hydrolyzed products (glucose, maltose and maltosaccharides) produced by amylolytic action of amylase. In addition to it molecular weight of amylase was found to be about 68kDa as revealed by SDS PAGE analysis after acetone precipitation. These bacteria could be genetically manipulated for large scale enzyme production which can be ultimately used for commercial purposes.

Key words: Crude extracellular amylase, *Bacillus*, Thermostable, Alkaliphilic, Starch hydrolysis, Enzyme assay.

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INTRODUCTION

Enzymes are so important in a manner that every creature on this earth produces enzyme for its survival and without enzymes there is no life existence on this planet. Enzymes have an extraordinary significance due to their catalytic ability and called biocatalyst protein. Enzymes are those globular proteins that made up of amino acids. These are imperative for a chemical process; otherwise process cannot be finished and even cannot begin. Enzymes are the widely produced substances and amongst the most fascinating and imperative substances found in nature. They have incredible significance in various biochemical reactions (Abd-Elhalem et al., 2015a) taking place in the cells that are involved in joining and breaking apart of some molecules. The production of all commercially important industrial enzymes is done through cultivation of bacteria or fungi because of their plasticity, rapid growth and accessibility in extensive amount. They release the enzyme extracellular and cause the assimilation of starch. Amylases are commercially utilized as a part of the distinctive procedures for example in brewing industry as a food, sugar induction by production of sugar syrups from starch which consist of glucose, maltose and higher oligosaccharides, pharmaceuticals and in preparing cold water dispersible laundry starches (Abd-Elhalem *et al.*, 2015b).

Amylases are important hydrolase enzymes which account for 25-30% of total industrial enzyme market (Pinjari and Kotari, 2018). Among several sources of amylases such as plants, animals and microorganisms, the bacteria and fungi are most preferable and significant due to their extensive utility. Additionally the production of amylases from indigenous bacteria could be optimized and genetically manipulated in biotechnological purposes for large scale amylase production (Alariya *et al.*, 2013). The most common amaylase producing bacteria are *Bacillus* species, most notably, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B.*

stearothermophilus which collectively make up virtually 60% of commercially available enzyme. Furthermore these species produce highly thermo-stable amylolytic enzyme thus have high significance in many industrial processes namely as starch liquefaction and saccharification (Drauz *et al.*, 2012).

It has been reported that several bacterial species tend to secrete extracellular amylases (*α*-amylase, *β*-amylase and glucoamylase) which hydrolyze the starch in variable manner and generate distinct products including dextrins, oligosaccharide and glucose (Hasan *et al.*, 2017). Among these amylases α-amylases (EC 3.2.1.1) are extracellular and endoacting enzymes belong to glycosyl hydrolase family 13 (GH-13). Members of this family have the ability of α-glycosidic linkage hydrolysis and transglycosylatic of starch thus yield α-limit dextrins and oligosaccharide with variable length. Both β-amylases (EC 3.2.1.2) and glucoamylases (EC 3.2.1.3) are exoacting enzymes belong to glycosyl hydrolase family 14 and 15 respectively that yield maltose, β -limit dextrins and glucose (Sundarram and Murthy, 2014). The modern and efficient food production and distribution system can best contribute for maintaining food security and livelihoods of the people ultimately leading towards foreign exchange earnings (Anonymous, 2019-2020).

The present study aimed to report the optimization and characterization of soluble starch digesting crude extracellular, thermostable and alkaliphilic amylases extracted from thermophilic *bacillus* species. Thermostability provides resistance against denaturing agents, proteolytic enzymes and solvents thus shows great importance in starch liquefaction and detergent industries.

MATERIALS AND METHODS

Sampling: Soil samples were taken from kitchen/domestic waste disposal sites and rice fields. Top soil was removed and samples were taken at a depth of 3-4 cm with sterile spatula. Physical parameters such as pH, temperature, color and texture of the soil samples were noted and the samples were brought to the laboratory in sterile containers.

Isolation, screening and characterization: The samples were serially diluted and 25ul of 10^{-4} and 10^{-6} dilutions were plated on Luria-Bertani (LB) agar medium aseptically and incubated at 37°C for 24 hours. The colonies were purified by quadrant streaking method on LB agar plates. The purity of the isolates was confirmed by Gram staining followed by microscopic analysis. Purified isolates were then screened for starch hydrolysis by streaking them on starch agar medium [(gL^{-1}) peptone 5.0; beef extract 3.0; starch (soluble) 2.0; agar 15.0] followed by incubation at 37°C for 72 hours. The plates were flooded with Lugol's iodine reagent [(gL^{-1}) iodine 1.5 and potassium iodide 15] and were observed for clear zones around the bacterial growth, an indication of starch hydrolysis. Isolates that gave positive results were then phenotypically and biochemically characterized following the scheme given in Bergey's Manual of Determinative Bacteriology (Sneath *et al.*, 1986). Genomic DNA of the bacterial isolates was isolated by using Favorprep^{TM}Tissue Genomic DNA Extraction Minikit (Favorgen Biotech Corp, Taiwan) following manufacturer's instructions. DNA yield was confirmed through agarose gel electrophoresis by using 1.0% agarose gel. DNA samples were stored at -20°C till further processing. Afterwards SSUrRNA gene amplification was done by using polymerase chain reaction (PCR). The primers used were 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (AAGGAGGTGTGCCARCCGCA) (Frank *et al.*, 2008). Final volume of each PCR reaction was 25ul and contained 2ul DNA template, 1ul each of forward and reverse primers, 12.5ul PCR Master Mix (2X, Thermo Fisher Scientific, Waltham, MA, USA) and 8.5ul deionized water. Results of the PCR were analyzed using agarose gel electrophoresis and the required amplicons were identified using DNA ladder (Thermo Scientific) and purified by using Gel Purification Kit (Favorgen Biotech Corp.,Taiwan). Purified amplicons were sequenced from MacrogenInc (South Korea) through dideoxy sequencing technique. Furthermore sequences were analyzed in FinchTV (van Dyk *et al.*, 2009) and were classified through NCBI BLAST tool. Top nearest homologs were downloaded and neighbour joining phylogenetic trees were made in Mega 5.0 using 100 bootstrap value (Tamura *et al.*, 2011).

Optimization of bacterial growth conditions: Temperature and pH optima for the growth of the bacterial isolates were determined. For optimum temperature determination LB broth medium was inoculated with 100ul of overnight cultures of the bacterial isolates and incubated at different temperatures (25°C, 30°C, 37°C and 45°C) for 24 hours with shaking at 200rpm. Optical density was determined at 600nm afterwards using a spectrophotometer (Cecil Aquarius CE 7200). For the determination of optimum pH, LB broth medium was prepared and the pH of the media was set at 3, 5, 7, 9 and 11 by using 1N HCL or 1N NaOH. The flasks were inoculated with overnight cultures of the bacterial isolates and incubated at 30°C. OD_{600nm} was recorded using spectrophotometer (Cecil Aquarius CE 7200).

Extraction of crude extracellular amylase: Amylase production by bacterial isolates was done in RPP minimal medium (Bianchi *et al.*, 2010) supplemented with 1% starch. The medium was inoculated with overnight bacterial cultures and incubated at 30°C for 72 hours with shaking at 200 rpm. Following the incubation the

supernatant was separated by centrifugation (4000 rpm for 15 minutes) at 4°C (Sigma 3K30). All reactions were performed in triplicates and the supernatants were used as a source of crude extracellular amylase enzyme.

Starch hydrolysis ratio determination/measurement of amylase hydrolysis zones: Starch agar medium was stabbed with bacterial isolates followed by incubation at 30±2°C for 48 hours. Following incubation the plates were flooded with Lugol's iodine solution for 3-5 minutes to observe starch iodine complex and clear zones surrounding the bacterial growth. Starch hydrolysis ratio (SHR) was calculated by using following equation (Abd-Elhalem *et al.*, 2015):

SHR= clear halo zone diameter (mm)/ colony growth diameter (mm)

Crude extracellular amylase assay: Amylase activity was estimated by measuring the decrease in starch iodine complex due to the enzymatic action following the method reported by (Abd-Elhalem *et al.*, 2015) the bacterial amylase activity was estimated by adding 1.0ml of crude enzyme to 0.5ml of 0.1% starch gelatinized in water (15 minutes at 100°C with continuous mixing) and saturated in 0.5ml of 0.1M phosphate buffer (pH7.0). The mixture was incubated at 60°C for 30 minutes. After the incubation 1.0ml of 1.0M HCl was added to the mixture to stop the reaction followed by the addition of 1.0ml of iodine reagent (5.0mM I_2 and 5.0mM KI). Following color development absorbance was measured at 620nm by using UV spectrophotometer (Cecil Aquarius CE 7200). All the experiments were performed in triplicates and results were presented of the mean values. U/mL is defined as the amount of enzyme able to hydrolyze 1mg of starch in one minute under the assay conditions.

U/mL= (A_{620} control – A_{620}sample)/ (A_{620}/mg starch)/ 30 min/ml

A_{620} control is the absorbance of starch without enzyme. A_{620} sample is the absorbance of starch digested with amylase enzyme and A_{620}/mg starch is the absorbance 1mg of starch as determined from the standard curve.

Dextrizing activity was also determined by using Fuwa method (Abd-Elhalem *et al.*, 2015) as follows:

Dextrizing activity = (A_{620} control – A_{620}sample)/A_{620}controlx 100

One unit of dextrizing activity is defined as the amount of enzyme required to cause the reduction of 1% color intensity under the conditions described above.

Standard curve was prepared by using different starch concentrations (0.0, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2, 3.5, and 5.0 $mgmL^{-1}$) in distilled water without addition of any crude amylase enzyme. The standards were proceeded same as described above. The graph was plotted to achieve a standard curve of starch concentration against optical density at 620nm.

Optimization of Amylase Activity: Amylase assay was performed at varying temperatures (35°C, 45°C, 60°C and 75°C), pH (5, 7 and 9) and increasing substrate concentration (0.05%, 0.1% and 0.5%) in order to identify the optimal amylase activity by the bacterial isolates. The assay was performed and the absorbance was recorded as described earlier.

Thin layer chromatography (TLC) analysis of starch hydrolysis: Thin layer chromatography was done to determine the starch hydrolyzed products produced by the enzymatic action of amylases. Starch products can be separated on the basis of interaction with stationary phase (Wang *et al.*, 2009). Crude extracellular enzyme (collected as described earlier) was spotted on the TLC silica gel plate F254. The plates were air-dried and placed in the chromate-tank containing the mobile phase (n-butanol, acetic acid, diethyl ether and water) in 9:6:3:1(v/v/v/v) (Kim *et al.*, 2004). Development was completed within 3-4 hours; the plate was removed from the solution, dried and sprayed with staining reagent containing (0.15g orcinol, 8.2mL H_2SO_4 and 42mL dist.H_20) (Wittgens *et al.*, 2017). Air dried the plate and incubated at 110°C for 15 minutes to observe the products. TLC plate was also observed under UV light (254nm) before spraying the staining reagent to visualize the products.

High performance liquid chromatography (HPLC): High performance liquid chromatography was done by using the Aminex HPX-42A column. RPP culture supernatant was run on HPLC machine to determine the starch hydrolysis products.

SDS PAGE analysis: SDS PAGE analysis was done for the determination of molecular weight of amylase. Crude amylase was prepared as described above and concentrated through acetone precipitation (Simpson and Beynon, 2009). The preparation was electrophoresed in 12% resolving gel and 5% stacking gel with a known molecular weight protein marker (Thermo Scientific ™ Page ruler plus prestained protein ladder) at 200V for 1.0 hour using WEALTEC vertical electrophoresis system (or V-GES). After electrophoresis the gel was stained with coomassie blue R-250 dye in methanol-acetic acid-water solution (5:1:4 by volume) for 1.0 hour and destained overnight in the same solution without dye.

RESULTS

Physical features of sampling site: The garbage soil texture of waste disposal site was moist and clumped whereas rice field soil texture was soft. The temperature and pH of the samples were in the range of 26-32^{0}C and 7.4-8.8 respectively. Total 24 morphologically different bacterial colonies were selected and purified form these soil samples.

Screening and characterization of starch hydrolyzing bacteria: All the isolates were streaked on 1% starch agar plates to screen them for starch hydrolysis. Five isolates R3, G4, GB, G3 and R2 were chosen on the basis of largest halozones around their growth on starch agar plates. All the isolates were gram positive rods, spore formers, catalase and oxidase positive. Isolates G3, G4 and R2 were further identified as *Bacillus subtilis*, *Bacillus* sp. G4 and *Lysinibacillus* sp. R2 respectively by SSU rRNA gene sequence classification through NCBI BLAST. *Bacillus* sp. G4, *Lysinibacillus* sp. R2, R3 and GB showed optimum growth at 30°C and pH7 whereas *Bacillus subtilis* G3 showed best growth at 45°C and pH9.

Starch hydrolysis ratio: Qualitative assay was done on starch agar plates at 30°C. Isolates *Bacillus subtilis* G3, *Bacillus* sp. G4 and *Lysinibacillus* sp. R2, R3 and GB showed starch hydrolysis ratio as 2.8, 1.81, 2.4, 1.53 and 2 respectively.

Dextrizing activity estimation: Dextrizing activity in terms of percentage reduction in starch iodine color intensity due to action of the amylase was determined. Best dextrizing activity was observed by isolates *Bacillus subtilis* G3, *Bacillus* sp. G4 and *Lysinibacillus* sp. R2 as 84%, 44% and 63% respectively. Maximum enzyme units (U/mL) in the hydrolysis of 0.5% starch was observed by isolates *Bacillus subtilis* G3, *Bacillus* sp. G4, and *Lysinibacillus* sp. R2 as 1, 0.52 and 0.763 respectively.

Optimization of Enzyme Activity:

- **Temperature:** Crude enzyme assay was performed at different temperatures to determine the maximum enzyme activity. *Lysinibacillus* sp. R2 showed optimum activity at 60°C and 75°C whereas all the other isolates showed maximum activity at 75°C.
- **pH:** The isolates R3, *Bacillus* sp. G4, GB and *Lysinibacillus* sp. R2 showed maximum activity at pH7. However *Bacillus subtilis* G3 showed best activity at 9pH. Decrease in enzyme activity was observed with decline in pH of the reaction mixture.
- **Substrate concentration:** Enzyme activity with different starch concentrations 0.05%, 0.1% and 0.5% was analyzed. Best activity was observed in presence of 0.1% starch.

Media composition: Crude amylase produced in different media composition was used for determinantion of amylase activity by three isolates *Bacillus subtilis G3*, *Bacillus* sp. G4 and *Lysinibacillus* sp. R2. Maximum activity was observed in the crude extract produced in the RPP minimal medium that was grown at 37°C for 72 hours.

Thin layer chromatography (TLC) analysis: TLC analysis of starch hydrolysis was done by running samples with mobile phase (n-butanol, acetic acid, diethyl ether and water) in 9:6:3:1(v/v/v/v). Isolates *Bacillus subtilis* G3 and *Bacillus* sp. G4 showed hydrolyzed products due to enzymatic action as glucose, maltose and maltosaccharides confirmed by running standards (glucose and maltose) with it. After spraying the staining reagents the carbohydrates were observed as brown color spots on the plate.

Molecular weight determination: SDS PAGE analysis of crude enzyme after acetone precipitation indicated the molecular weight of amylase as 68kDa confirmed by running a protein marker with it.

High performance liquid chromatography (HPLC) analysis of starch hydrolyzates: HPLC analysis showed glucose, maltose, maltotriose and maltotetraose as the starch hydrolysis products by running the crude extract samples on HPX-42A column.

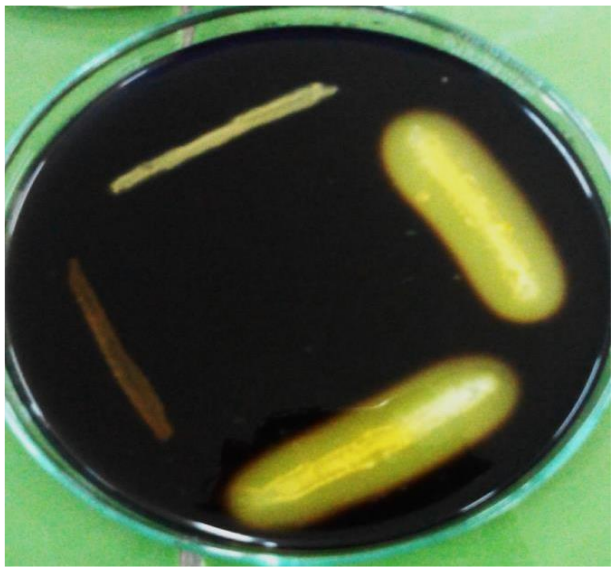

Fig 1. Screening of starch hydrolyzing bacteria, appearance of zones indicated the production amylases.

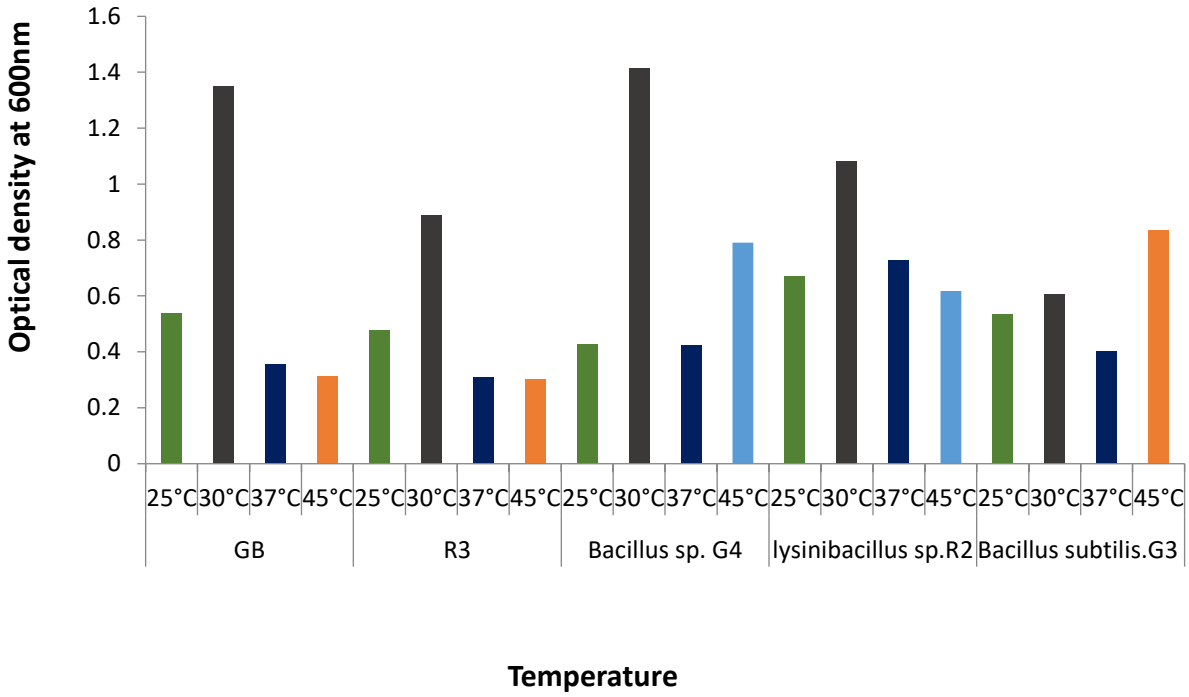

Fig 2. Growth of bacterial strains at different temperatures.

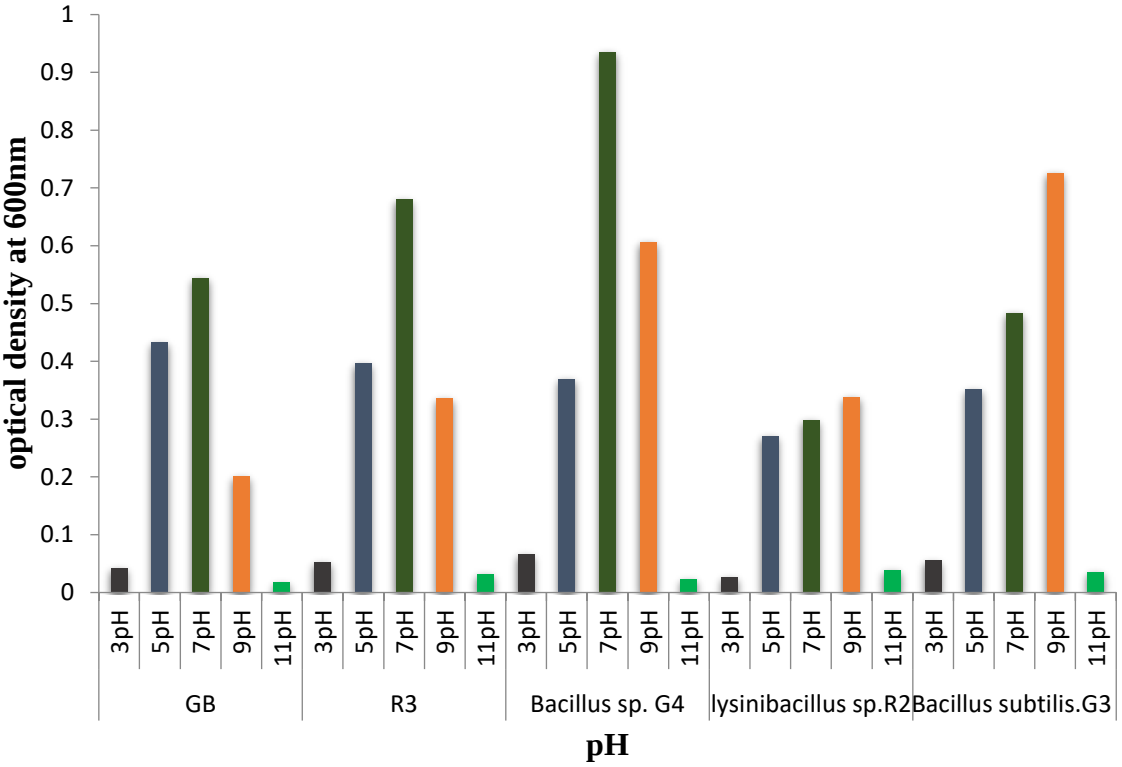

Fig 3. Growth of bacterial strains at different pH.

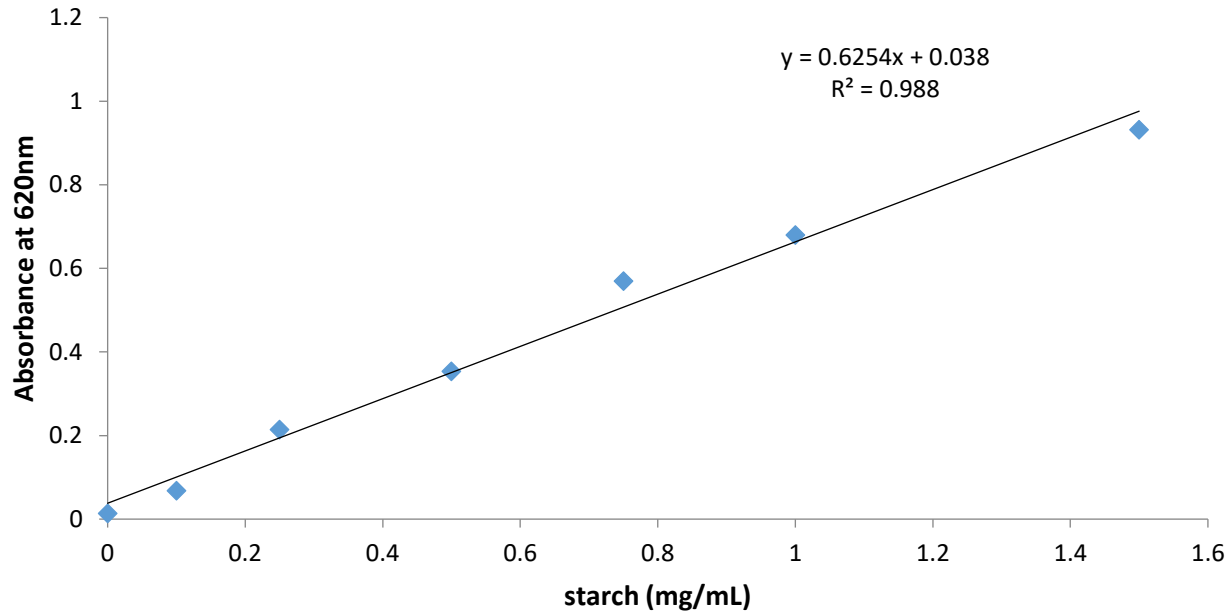

Fig 4. Standard curve of different starch concentration.

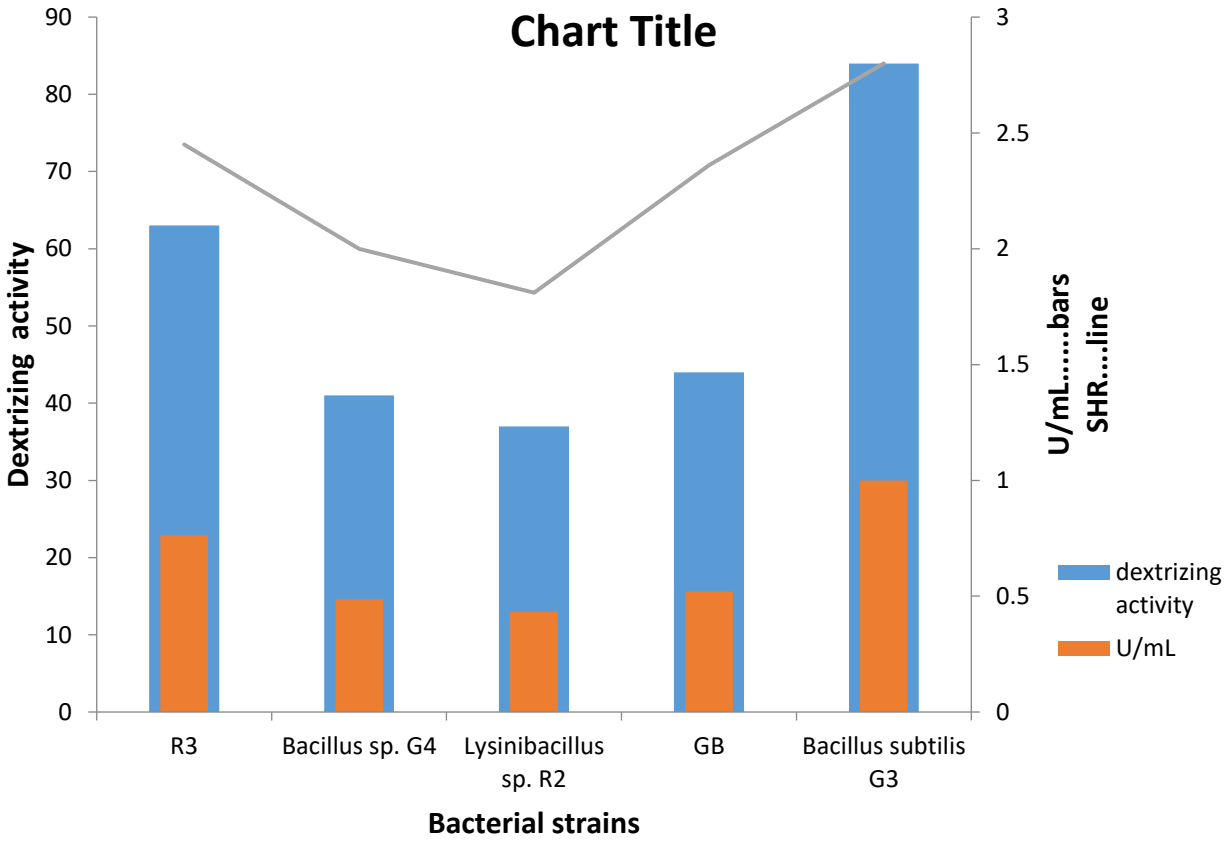

Fig 5. Dextrizing activity, enzyme activity in U/mL and starch hydrolysis ratio obtained from different bacterial isolates.

Effect of temperature and pH on amylase activity:

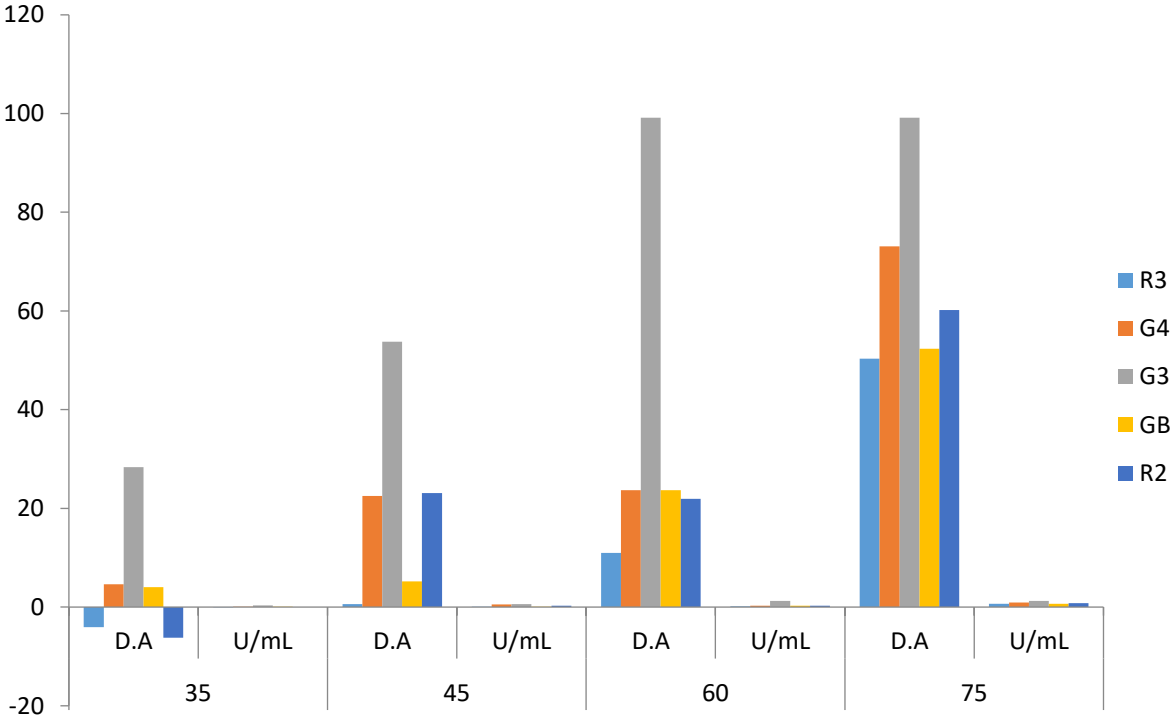

Fig 6. Effect of different temperature on amylase activity.

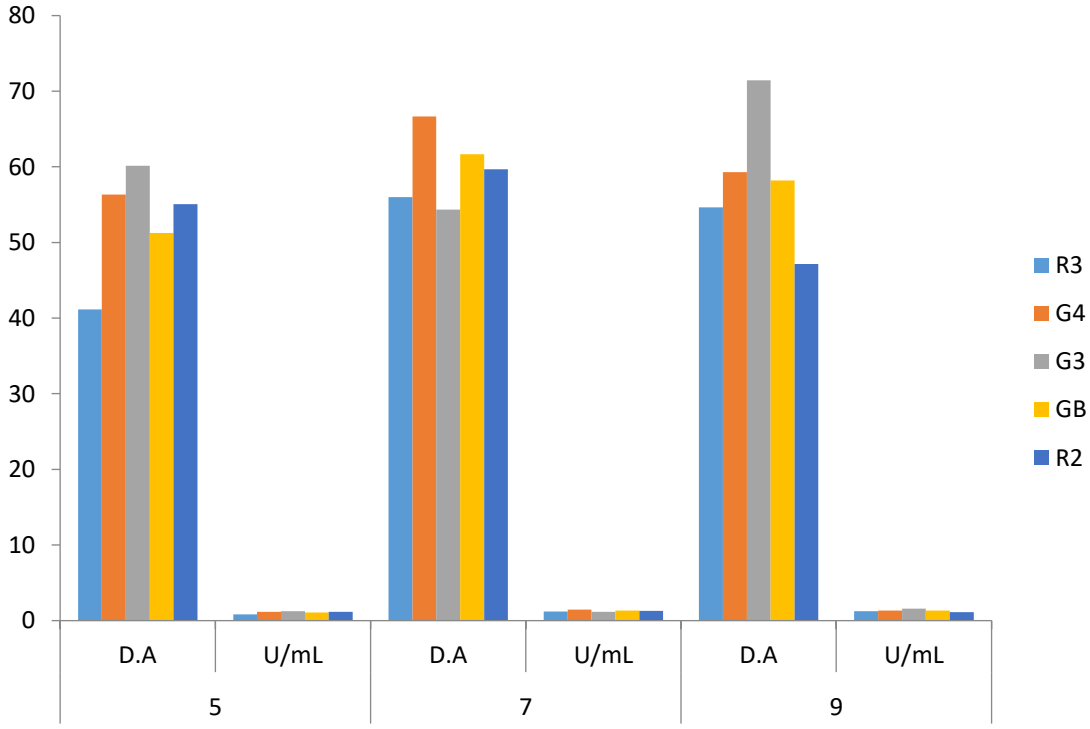

Fig 7. Effect of different pH on amylase activity.

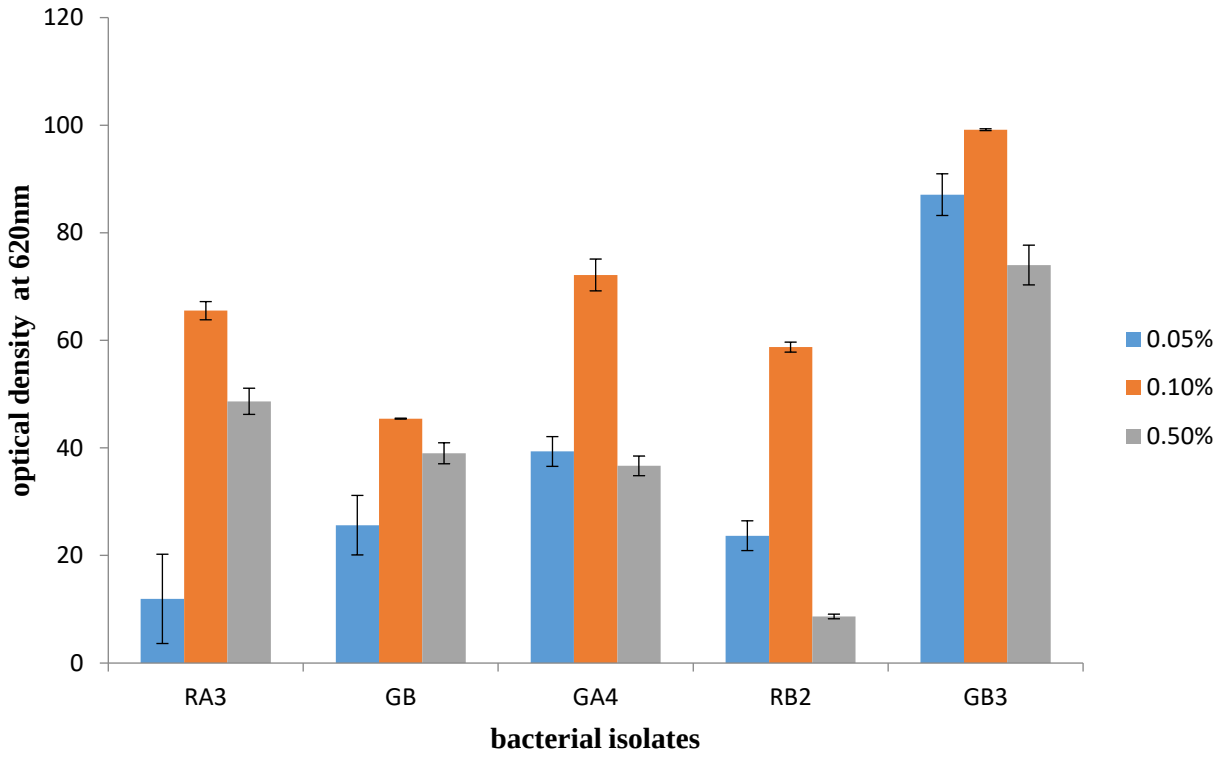

Fig 8. Effect of different starch concentration on amylase activity.

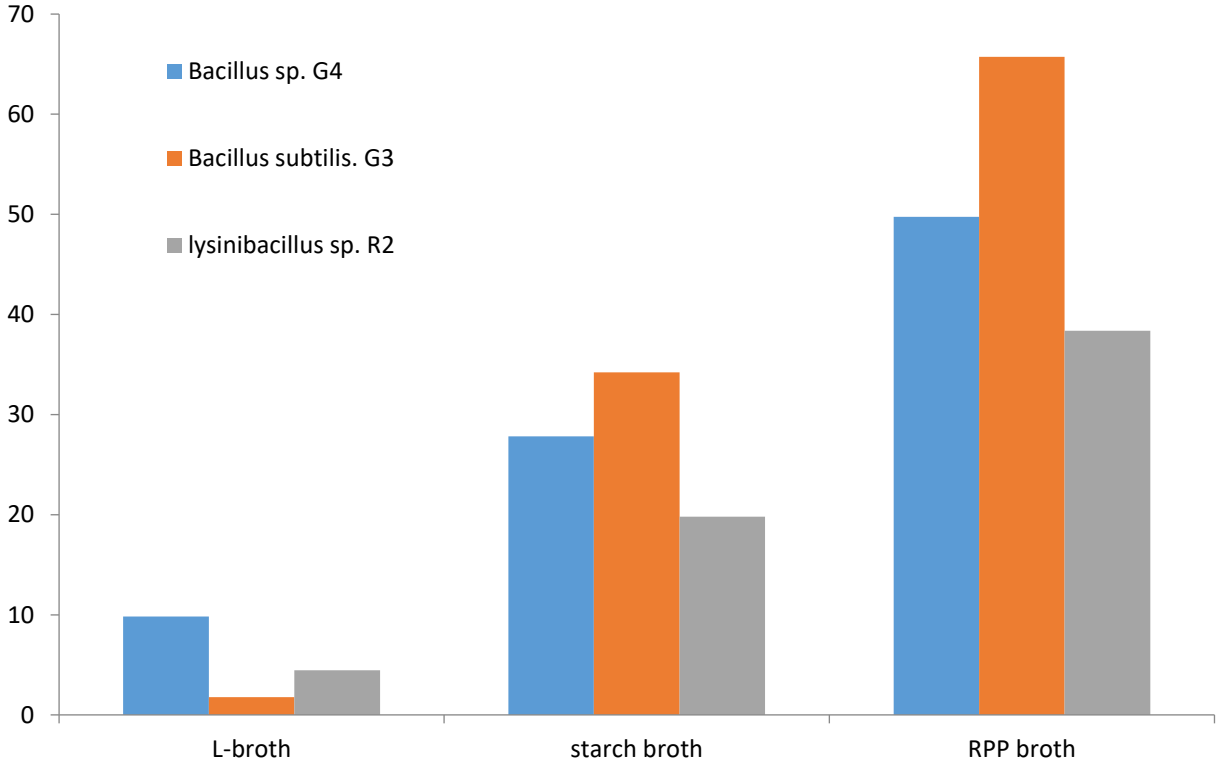

Fig 9. Effect of different media composition on amylase activity.

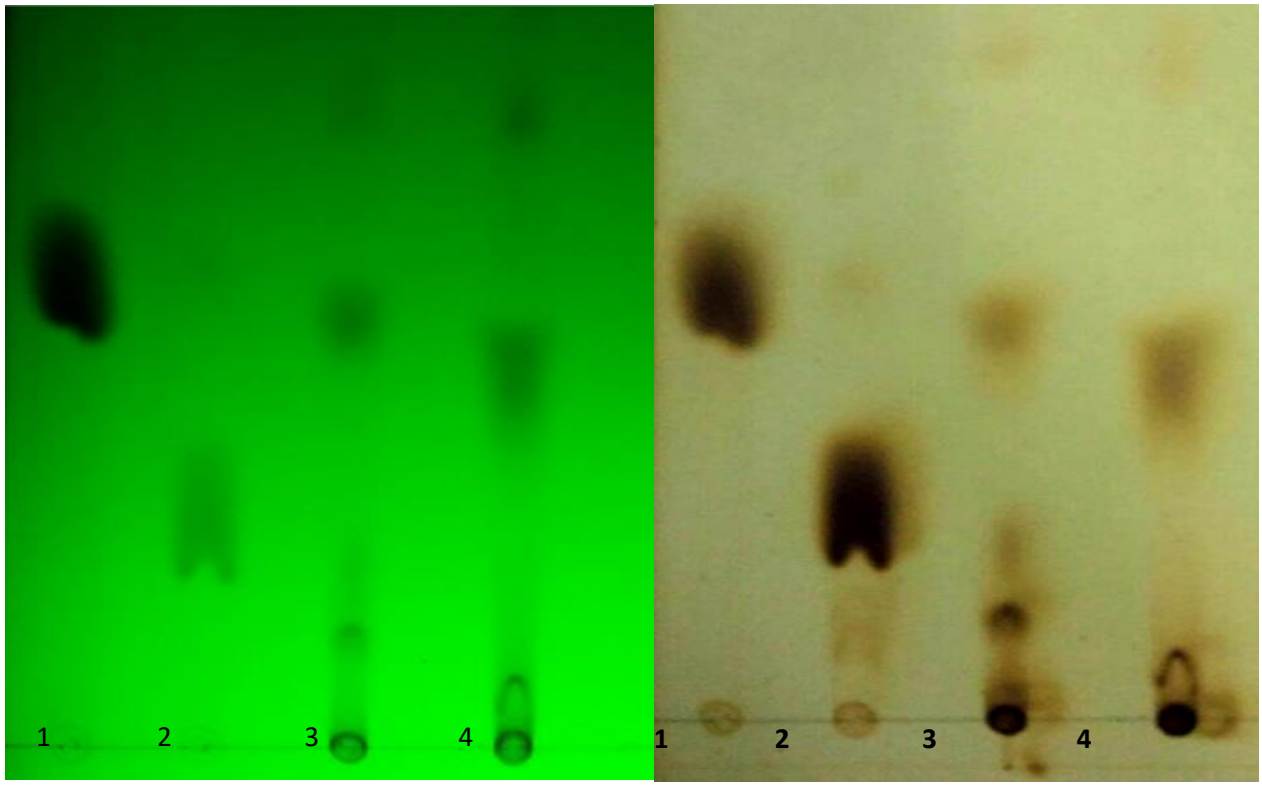

Fig 10. Analysis of starch hydrolysis products (a) TLC plate image under UV light (254nm) **(b)** TLC plate after spraying the staining reagent showed the hydrolyzed products in brown color. Lane 1: Glucose (0.4%); Lane 2: maltose (0.4%); Lane 3-4: duplicates of *Bacillus subtilis*. G3

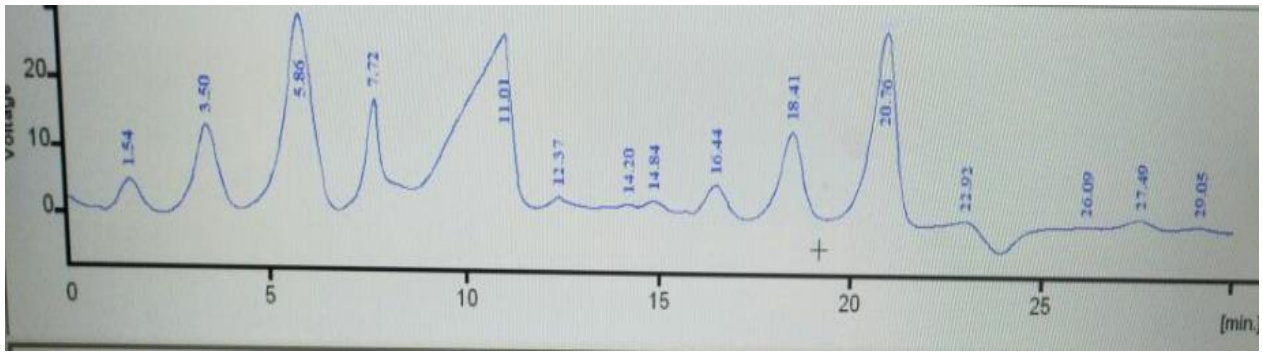

Fig 11. HPLC analysis of crude extract indicates the starch hydrolyzed products as peak of glucose (20.76), maltose (18) and maltotriose (16) was observed.

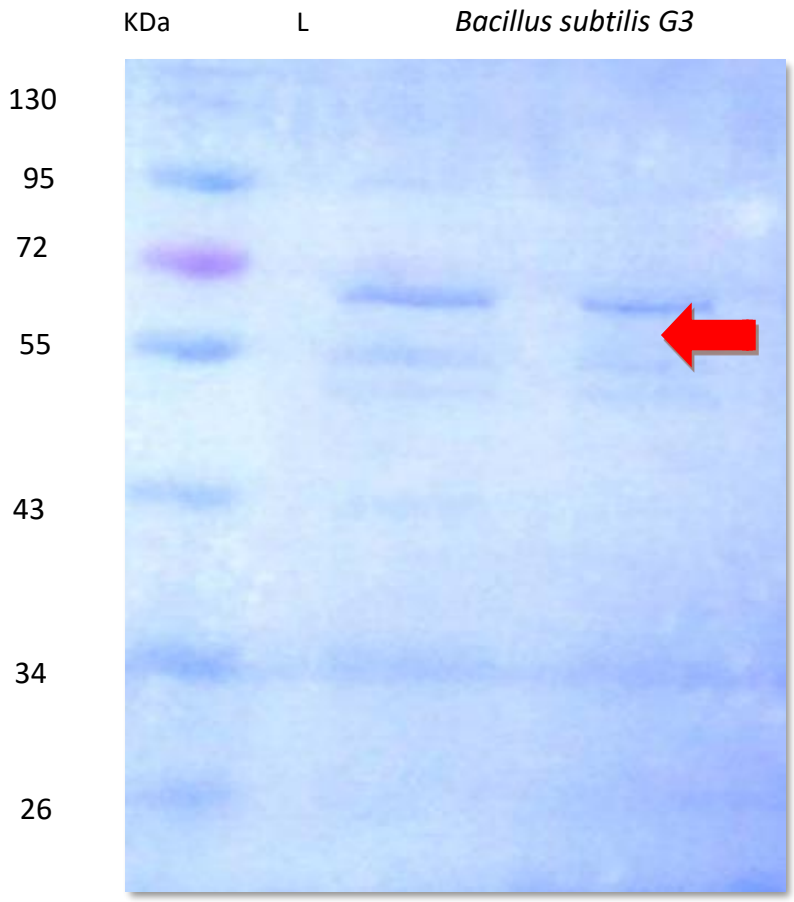

Fig 12. SDS PAGE analysis of crude enzyme after acetone precipitation on 12% homogenoized polyacrylamide gel. Lane 1 (page ruler prestained protein ladder), lane 2-3 are duplicates of *Bacillus subtilis G3*. Red arrow indicates molecular weight of crude amylase as 65kDa.

DISCUSSION

Amylases from microorganisms have replaced the acidic hydrolysis in many starch processing industries. In the present study, amylase producing bacteria were isolated from kitchen/domestic waste disposal sites and rice field due to the richness of starchy materials in these sites. The bacteria showing largest halo zones of starch hydrolysis were identified through 16S rRNA sequencing as *Bacillus subtilis* G3, *Bacillus* sp. G4 and *lysinibacillus* sp. R2. The genus *Bacillus* has a great importance in industrial applications due to extracellular enzymes production (Schallmey *et al.*, 2004).

Bacterial culture optimization is the foremost important step to get a maximum number of bacterial cells in order to get maximum amylase production. In the present study amylase producing bacterial isolates showed maximum growth at 30°C and pH7 indicating their mesophilic and neutrophilic nature. However *Bacillus subtilis* G3 showed best growth at 45°C and pH9 indicating its thermophilic and alkaliphilic nature. Thermostability is very important from industrial point of view for starch liquefaction processes (Vieille and Zeikus, 2001).

The crude extracts of selected isolates showed dextrizing activity in terms of percentage reduction of color intensity and starch hydrolysis ratio (SHR) in the range 37%-84% and 1.81-2.8 respectively. Vaseekaran *et al.* (2010) reported SHR of 3.4-4 by isolates GS1, FS1 and BS1 respectively whereas (Alk and Ibrahim, 2011) reported SHR of 1.5 by *B. licheniformis*. Maximum SHR and dextrizing activity in our study was found in strain *Bacillus subtilis* G3 as of 2.8 and 84% respectively.

Amylases are inducible enzymes and no amylase production was detected in LB-broth cultures. (Manonmani and Kunhi, 1999) reported the interference of constituents of LB medium supplemented with starch in the enzyme production assay. Suflhydryl groups and thiol compounds compete with starch to form complex

with iodine and thus decrease the staining properties of starch iodine complex in LB-broth cultures.

However while using minimal medium with starch the bacteria do not have any other carbon source to get energy for their growth and metabolic activities. In the present study minimal media consisting of trace elements, vitamin solution and starch were used for enzyme production. Vitamin supplementation can stimulate as well as increase the production of amylases by bacteria (Tripathi *et al.*, 2017). K_2HPO_4 enhances the amylase production as reported by (Aygan *et al.*, 2014). The enzyme activity is enhanced by the presence of divalent ions as calcium (Ca^{+2}) and manganese (Mn^{+2}) ions which play important role in the enhancement of amylase activity by interacting with active site domains of amylases (Burhan *et al.*, 2003).

Effect of different temperatures and pH on amylase activity was also determined in present study. Our study illustrates that isolates were showing maximum amylase activity at 75°C and 7 pH except *Bacillus subtilis* G3 whose optimum activity was observed at 9 pH indicating its alkaline nature. Alkaline amylases can be used in variety of industrial processes that operate at alkaline pH such as production of detergent (Aygan *et al.*, 2014).

Temperature optima of the bacterial growth as well as of enzyme activity were also determined in this study. The study revealed that isolates were growing best at 30°C but the amylase activity was higher at 75°C. (Sodhi *et al.*, 2005) also reported a great difference in growth optima (37°C) and optimal temperature for enzyme activity (60°C) by *Bacillus* sp. PS-7.

Formation of starch hydrolysis products using crude enzyme was analyzed by thin layer chromatography. The hydrolyzed products after crude enzyme treatments were glucose, maltose and maltosaccharides confirmed through running standards of glucose and maltose with the samples. The same products were also obtained after high performance liquid chromatography analysis. Our results are in accordance with (Das *et al.*, 2004) who reported *B.* Subtilis DM-03 that yields predominately glucose and maltose after starch hydrolysis.

SDS PAGE analysis indicated the molecular weight of amylase enzyme up to 68kDa determined by using protein marker. The more prominent band was considered as the amylase band as indicating the induction of enzyme due to starch as the only substrate. Molecular weight of α-amylases usually ranges between 50-60KDa (Coutinho and Reilly, 1997; Li *et al.*, 2016).

Conclusion: The crude extracellular amylase activity produced from different *Bacillus*. sp was observed from the samples collected from the soil. Various factors were applied to get the maximum amylase yield and maximum enzyme activity such as pH, temperature, substrate composition and concentration etc. The best activity was observed by the *B. subtilis* that was thermostable, alkaliphilc in nature and the ability of *B. subtilis* amylase to tolerate high temperature and its activity at alkaline pH enables it to be further used for starch liquefaction processes and also in detergent industry.

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