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EFFECT OF pH AND METAL IONS ON α-AMYLASE, PRODUCED FROM PROBIOTIC LACTIC ACID BACTERIA

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ABSTRACT

Amylases are starch degrading enzymes which can be obtained from different sources such as plants, animals and microorganisms. Among microbial sources bacteria and fungi are mostly used for the production of α-amylase both at small and industrial scale. Lactic acid bacteria are the preferred for the production of α-amylase because the microbial strain can be used as probiotics that possess antimicrobial activity by producing antimicrobial substances such as bacteriocin and also are the producers of industrially important enzymes such as amylases, and can also be used for the preservation of fermented food. The four probiotic bacterial strains isolated from milk samples were screened for amylolytic activity and two strains MM1 and GD were producing comparatively more amylase qualitatively with clearance zone of 5mm and 6mm respectively. The enzyme produced by both MM1 and GD strains showed maximum catalytic activity at pH 5. Metal ions such as Na_2SO_4, $MgSO_4$, $CuSO_4$, also enhanced the amylolytic activity. Both MM1 and GD bacterial strains produced maximum amylase units when refined starch was used as a substrate. Amylase found potential applications in food, feed, textile, detergent, paper, pulp, textile and biofuels industries.

1. INTRODUCTION

All living organisms produce enzymes that are proteins in nature. Amino acids linked together by peptide bonds and form high molecular weight compounds, the enzyme. According to Robinson who was written in his research paper in 2015, that enzymes are biological, sustainable natural catalyst, that enormously speed up the biochemical reactions, that accelerate the rate of chemical reaction by reducing the activation energy, necessary to initiate the reaction, thus dramatically increasing the rate of biochemical reactions. The existence of enzymes has been known for well over a century.

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In early 1800 the biological catalyst was first recognized and described, in studies of digestion of meat by secretion of stomach and the conversion of starch into sugar by saliva and various plants extracts. (Gaikaiwari *et al.*,) [1]. In 1961 according to the report of first enzyme commission (EC) of international union of pure and applied chemistry (IUPAC) enzymes were classified into six types on the basis of reaction mechanism; including; oxidoreductases, transferase, hydrolases, lyases, isomerases, ligases. Enzymes are present in all living organisms, and produce by animals, plants and microorganisms. In industrial sector the enzymes are produce by many microorganisms such as bacteria and fungi. Microbial enzymes are preferred over other alternative sources because of the following reasons. (Hasan *et al.*,) [2]; they are generally

cheaper to produce, their enzyme contents are more predictable and controllable, rapid growth of microorganisms on cheapest media, their product is more convenient and safer, plants and animals tissues contain more harmful materials than microbes, including phenolic compounds endogenous enzymes and proteases. The process of extraction and purification of enzymes from microbial source is easier in comparison with plants and animal sources. (Singh *et al.*,) [3]. Microbial enzymes are used in different industries such as food, leather, textile and pharmaceuticals and increasing rapidly over conventional method due to less harm to environment, greater efficiency, and the higher quality product. (Kamini *et al.*,) [4]. Microbes produce different types of enzymes such as xylanase, protease, gluco-amylase and alpha-amylase.

Amylase is the enzyme among the various enzymes that are widely used in industry. Amylase are abundantly found in human saliva and is also produce by pancreas. Amylase are the glycosidic hydrolysis that catalysis the breakdown of starch into sugars (monosaccharide and disaccharide). Diastase amylase is the first amylase to be discover and isolated by Anselme Payan in 1833. Today in starch processing industry the amylase replaces the chemical hydrolysis of starch, in biotechnological sector the amylolytic enzymes have great importance range from food, paper to textile industries. (Lin *et al.*,) [5]. Amylases are classified into Endoamylase and Exoamylase on the basis of manner in which they attack on glycosidic bond. The Endo-amylase hydrolyze starch molecule in random manner causing the formation of various chains of branched and linear oligosaccharide. Exoamylase hydrolyze the starch causing the formation of short end products. There are three types of amylases on the basis of their structure, α-amylase, β-amylase and γ-amylase that are quite different from each other. They do not differ only in their primary and tertiary structures, but also in their catalytic machineries and reaction mechanisms employed. (Sharifi-Rad *et al.*,) [6].

α-amylase are the endo-amylase that are present in the animals, plants and microorganisms including bacteria and archaea, the α-1-4 glycosidic bond in the anterior of amylopectin and amylose were hydrolyze by the α-amylase. (Pandey *et al.*,) [7]. The α-amylase are calcium metallo-enzyme and in the absence of calcium the α-amylase do not carry out the catalytic reaction. Among all types of amylases, α-amylase are usually used in industrial applications because these are produced extracellular, therefore, downstream processing and purification is less costly reduce the need of disruption. Amylases are wide spread in animals, plants and fungi and are also found in the unicellular eukaryotes and also in prokaryotes, bacteria and archaea. Reports have shown that mostly mesophilic fungi are the good producers of α-amylase. (Gupta *et al.*,) [8]. Though animals and plants produce amylase but in industries the enzymes used are generally from microbial source. The use of microbial enzymes in industries is due to number of benefits of microbial enzymes including less energy consumption in cultivation of microorganisms, specificity of reactions, thermostability, productivity and the cultivation of microorganisms are easy.

(Divakaran *et al.*,) [9] the production of microbial amylase from bacteria is dependent on the composition of medium, type of strain, incubation period and methods of cultivation, cell growth, nutrient requirement, temperature, pH, thermostability and metal ions. (Tiwari *et al.*,) [10]. Different species of bacteria produce α-amylase but in industries the *Bacillus* is mainly used. From thermophilic organisms' thermostable enzymes were isolated and these enzymes have numerous applications in industries because of its thermostability. The *Bacillus* strain is mostly preferred over the other bacterial sources for the production of thermophilic α-amylase because it has short generation time and its environmental and genetic manipulation is easy. Gupta *et al.,* [8]. Among the wide range of microbial species that secrets amylase its production from bacteria is cheaper and faster than from another microorganism. Also, the genetic engineering is easy in bacteria and is more susceptible for production of recombinant enzyme. Various bacterial strains such as *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are found to good producer of thermostable α-amylase and widely used for commercial production of the enzymes and other numerous applications. (Gurung *et al.*,) [11].

There are mainly two methods which are used for production of α-amylase on commercial scale including; Submerged fermentation (SmF), Solid state fermentation (SSF). The submerged fermentation is a traditional method of enzymes production from microbes which has been in use for a longer period of time and employs free flowing liquid substrate, such as molasses and broth. This fermentation technique is suitable for microorganisms such as bacteria that require high moisture content for their growth. This method has several advantages, SmF allows the utilization of genetically modified organisms to a greater extent than SSF, the sterilization of medium and purification

process of the end products can be done easily. (Sundarram and Murthy) [12].

The new method for production of enzymes is the solid-state fermentation which is used for those microorganisms that require less moisture for their growth. Bagasse, bran and paper pulp are the solid substrate used in this method. In this method the nutrient rich wastes are used as substrate and these wastes are recycle by this method, this is the main advantage of this method. In this fermentation technique, the substrate is utilized very slowly and steadily. The changes in different parameters such as salts, pH, incubation time, temperature change the production of amylase. (Sudharhsan *et al.*), [13]. Amylases are used in textile industry, paper industry, and detergent industry, food industry, analysis in clinical and medicinal chemistry.
The significance of present study is to produce the alpha amylase enzyme from lactic acid bacteria which can be used both as probiotic that possess antimicrobial activity and also are producers of industrially important amylase enzyme. Lactic acid bacterial strain can be used for the preservation of fermented food and the amylases produced by them can be employed in many industrial applications.

2. MATERIALS AND METHODS

Chemicals Used in Research Work
Various chemicals used during research work were $MgSO_4$. $7H_2O$, K_2HPO_4, NaCl, DNS Reagent, Rochelle's salt, phenol, sodium sulphate, Sodium hydroxide, Sodium acetate, HCl, Crystal violet, Safranin, Iodine solution, Ethanol, Oxidase reagent, H_2O_2, Malachite green and different sugars supplied by Microbiology Research Laboratory, Women University Mardan and Enzyme Engineering and Biofuel group, IBD, NIBGE, Faisalabad.

Collection of Sample
The samples (raw and processed milk and yogurt) were collected from different areas of Mardan.

Isolation of Bacterial Strains for α-amylase Production
For the isolation of bacterial strains, the raw and processed milk and yogurt samples were inoculated on Luria Bertani (LB) agar plates and incubated at 37 ºC for 24 hours. The isolated bacterial colonies were named as R1, R2, R3, S1, S2, S3, S4, S5, S6, MM1, MM2, MM3, NES, GD and OLP. The isolated bacterial colonies were further sub-cultured on LB agar plates through streaking and incubated at 37ºC for 24 in order to get pure bacterial isolates for further studies (Ho and Sze) [14].

Qualitative Test for α-amylase
Tests isolates R1, R2, R3, S1, S2, S3, S4, S5, S6, MM1, MM2, MM3, GD, NES AND OLP were inoculated in the middle of LB agar plates supplemented with the 1% starch and incubated at 37ºC for 24 hours. After incubation the plates were flooded with iodine solution and were observed for the clearance zones around the amylase producer bacterial strains, indicating the hydrolysis of starch by extracellular α-amylase (Aleem *et al.*,) [15].

Qualitative Test for α-amylase
The four selected α-amylase producer strains R1, MM1, GD and NES, were screened for α-amylase production as described by (Yassin *et al.*,) [16] with some modifications.

Characterization of Bacterial Strains
Morphological and biochemical tests were done for the characterization of bacterial strains.

Morphological Characterization
For the morphological characterization, the appearance of bacterial colonies was checked. Also, the shape and motility of bacterial cells were observed under microscope. (Irfanullah *et al.*,) [17].

Biochemical Characterization
Different biochemical tests such as such as such as Gram staining, oxidase test, catalase test, spore forming test, milk coagulase test, indole test and glucose fermentation test oxidase test, catalase test, spore forming test, milk coagulase test, indole test and glucose fermentation test were done for characterization of bacterial isolates. (Irfanullah *et al.*,) [17].

Optimization of Conditions for the α-amylase Production
Effect of pH on α-amylase Production
For the optimization of pH, α-amylase production broth media of different pH (4, 5, 7, 8 and 9) was prepared supplemented with 1% starch (w/v) and after sterilization, inoculation and incubation at 37°C for 24 hours, the sample was taken and crude enzyme was extracted and then assayed for α-amylase. Alonazi *et al.*, [18].

Effect of Metal Ions on α-amylase Production
The effect of metal ions on α-amylase production was evaluated by inoculation and incubation of α-amylase producing strains in the broth media containing sodium sulphate, copper sulphate and magnesium sulphate in varying concentration [1%, 2% and 3% (w/v)] and the extracted sample was then evaluated for α-amylase (Gómez-Villegas *et al.*,) [19].

Effect of different substrate on amylase production

In order to check the effect of substrate on α-amylase production, different qualities of 1% starch such as starch-121(Raw), starch 034010 (Processed) and starch-356 (Refined) were added in the production media and the after inoculation and incubation of bacterial strains, the extracted sample was then evaluated for α-amylase (Gebreyohannes *et al.*,) [20].

3. RESULTS

Selection of α-amylase producing bacteria

Among 13 bacterial isolates obtained from raw and processed milk and yogurt, only 4 isolates showed the amylolytic activity, therefore, selected for further studies such as morphological and biochemical characterization. The four positive producers of α-amylase are shown in (Table 1).

Table 1. α-amylase Producing Lactic acid Bacterial Strains

S. No.	Source of sample	Isolates
1	Raw milk	R1
2	Milk	MM1
3	Processed Milk (Good Milk)	GD
4	Processed milk (Nestle)	NES

Characterization

On the basis of gram staining, biochemical and morphological characterization the bacterial isolates showed characteristics of *Lactobacillus*.

Morphological Characterization

The morphology of bacterial colonies were observed and the colonies were yellow or off-white in color, rounded shaped, elevated, smooth in appearance and possess soft or hard surface as shown in (Table 2) and (Figure 1). The motility of Bacterial isolates were also examined under the microscope as shown in (Table 3).

Table 2. Morphological Characteristic of Bacterial Isolates

S. No.	Bacterial Isolates	Shape	Color	Elevation	Appearance	Surface
1	R1	Rounded	Off white	Elevated	Smooth	Hard
2	MM1	Rounded	Off white	Elevated	Smooth	Soft
3	GD	Rounded	Yellowish	Elevated	Smooth	Soft
4	NES	Rounded	Yellowish	Elevated	Smooth	Soft

Figure 1. Morphology of Bacterial Isolate MM-1

Table 3. Evaluation of Motility of Bacterial Isolates

S. No.	Bacterial Isolates	Result
1	R1	Motile
2	MM1	Motile
3	GD	Motile
4	NES	Motile

Biochemical Characterization

Gram Staining

Gram staining results showed that among four bacterial isolates, three isolates (R1, MM1 and GD) were Gram positive while bacterial isolate NES was Gram negative. Moreover, three isolates (R1, MM1 and NES) were cocci while one (GD) was rod shaped as shown in (Table 4) and (Figure 2 & 3).

Table 4. Gram Staining of Bacterial Isolates

S.NO	Bacterial Isolates	Shape of Cells	Gram Staining
1	R1	Cocci	Positive
2	MM1	Rod shape	Positive
3	GD	Cocci	Positive
4	NES	Cocci	Negative

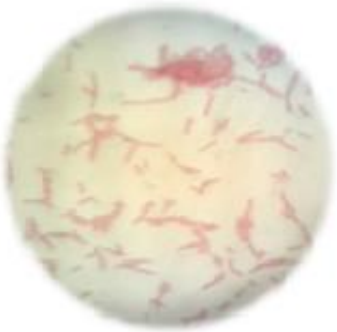

Figure 2. Gram Positive Bacterial Isolate

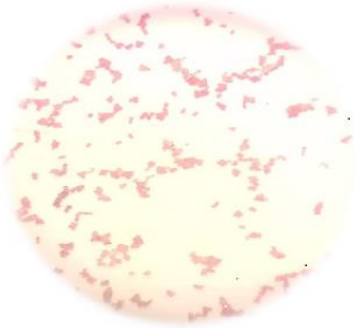

Figure 3. Gram Negative Bacterial Isolate

Oxidase Test

Oxidase test was done to check that either bacterial isolates can produce *cytochrome* C enzymes or not, and all bacterial isolates were oxidase positive by observing change in color (appearance of bluish color (Table 5) and (Figure 4).

Table 5. Oxidase Test of Bacterial Isolates

S.NO	Bacterial Isolates	Results
1	R1	Positive
2	MM1	Positive
3	GD	Positive
4	NES	Positive

Figure 4. Oxidase Positive Bacterial Isolate GD

Catalase Test

The catalase test was done to check that either the bacterial isolates can breakdown the hydrogen peroxide into water and oxygen or not. Among four bacterial strains, MM1 did not form the bubbles and is catalase negative, while all other strains are catalase positive as shown in (Table 6) and (Figure 5 & 6).

Table 6. Catalase Test of Bacterial Isolates

S.NO	Bacterial Isolates	Bubble Formation
1	R1	Positive
2	MM1	Negative
3	GD	Positive
4	NES	Positive

Figure 5. Catalase Positive Bacterial Isolate R1

Figure 6. Catalase Negative Bacterial Isolate GD

Spore Staining

All the bacterial isolates appeared pink under microscope, indicated that all the strains are non-sporing bacteria (Table 7) and (Figure 7).

Table 7. Spore Staining of Bacterial Isolates

S.NO	Bacterial Isolates	Color	Spore Formation
1	R1	Pink	Negative
2	MM1	Pink	Negative
3	GD	Pink	Negative
4	NES	Pink	Negative

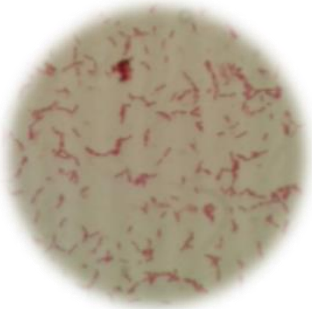

Figure 7. Spore Staining of Bacterial Isolates

Milk Coagulase Test

Two bacterial isolates R1 and GD showed the coagulation of milk while other two bacterial isolates (MM1 and NES) were negative for coagulase test as shown in (Table 8) and (Figure 8)

Table 8. Milk Coagulase Test for Bacterial Isolates

S.NO	Bacterial Isolates	Milk Coagulation
1	R1	Positive
2	MM1	Negative
3	GD	Positive
4	NES	Negative

Figure 8. Milk Coagulation of Bacterial Isolates

Indole Test

All bacterial isolates were positive for indole test and all bacterial strains were observed to form rings (Table 9) and (Figure 9)

Table 9. Indole Test of Bacterial Isolates

S.NO	Bacterial Isolates	Ring formation
1	R1	Positive
2	MM1	Positive
3	GD	Positive
4	NES	Positive

Figure 9. Indole Positive Bacterial Isolates

Carbohydrate Fermentation

Tests for carbohydrates fermentation was done in order to check the type of sugar fermented by bacterial isolates and results clearly indicated that all bacterial isolates could not ferment glucose (Table 10) while can ferment sucrose observed by either change of color or by formation of gas bubbles (Table 11) and (Figure 10)

Table 10. Glucose Fermentation Test

S.NO	Bacterial Isolates	Glucose fermenter
1	R1	Negative
2	MM1	Negative
3	GD	Negative
4	NES	Negative

Table 11. Sucrose Fermentation Test

S.NO	Bacterial Isolates	Color Change	Bubble Formation
1	R1	Yellow	Negative
2	MM1	Orange	Negative
3	GD	Orange	Negative
4	NES	Orange	Negative

Figure 10. Sucrose Fermentation of Bacterial Isolates

Qualitative screening of α-amylase Producing Lactic acid Bacterial Strains

Among the four α-amylase producing lactic acid bacterial isolates (R1, MM1, GD and NES) only two isolates (MM1 and GD) were selected for further study because these two strains produced clearance zones of larger diameters such as 5mm and 6mm respectively (Table 12) and (Figure 11).

Table 12. Clearance Zone Diameter of α-amylase producing lactic acid bacteria

S.NO	Bacterial Isolates	Zone Diameter
1	R1	4mm
2	MM1	5mm
3	GD	6mm
4	NES	4.1mm

Figure 11. Qualitative Assay for α-amylase

Optimization of Optimum Conditions for α-amylase Production

Effect of pH on α-amylase Production

The pH has great effect on α-amylase production and amylolytic activity. Maximum α- amylase were produced by both MM1 (U/ml) and GD (U/ml) at pH 5 as shown (Figure 12) and (Figure 13)

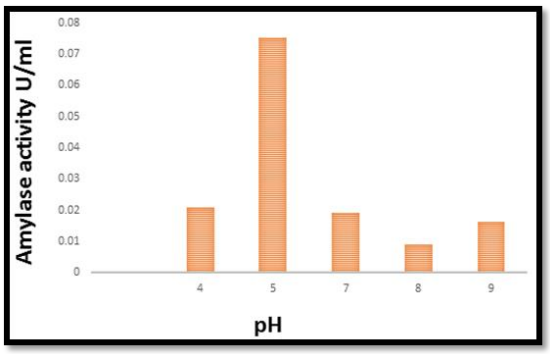

Figure 12. Effect of pH on Amylase production (U/ml) of *lactic acid* Bacteria MM1

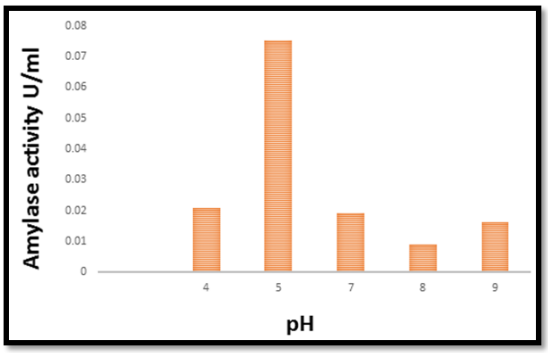

Figure 13. Effect of pH on Amylase production (U/ml) of lactic acid Bacteria GD

Effect of Metal Ions on α-amylase Production

Effect of Na_2SO_4 on α-amylase Production

The effect of Na_2SO_4 in different concentrations (0.1, 0.2, 0.3%) was evaluated on α-amylase production and maximum enzyme (U/ml) were produced in the presence of 0.1% (w/v) Na_2SO_4 (Figure 14 & 15).

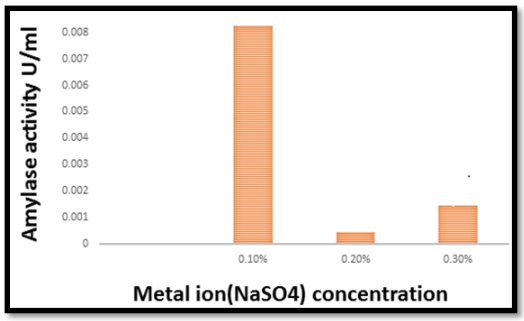

Figure 14. Effect of Na_2SO_4 in different concentrations on Amylase production (U/ml) of Lactic acid Bacteria MM1

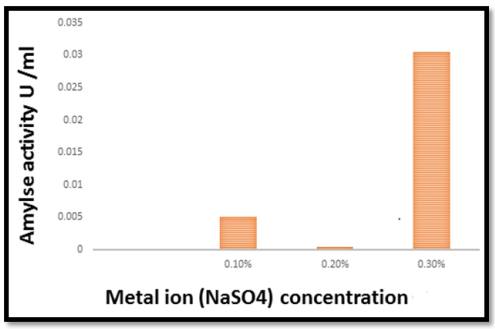

Figure 15. Effect of Na_2SO_4 in different concentrations on Amylase production (U/ml) of Lactic acid Bacteria GD

Effect of $MgSO_4$ on α-amylase Production

The effect of $MgSO_4$ in different concentrations (0.1, 0.2, 0.3%) was evaluated on α-amylase production and maximum enzyme (U/ml) were produced in the presence of 0.2% (w/v) $MgSO_4$ (Figure 16 & 17).

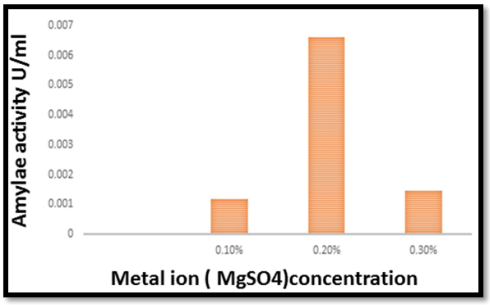

Figure 16. Effect of $MgSO_4$ in different concentrations on Amylase production (U/ml) of Lactic acid Bacteria MM1

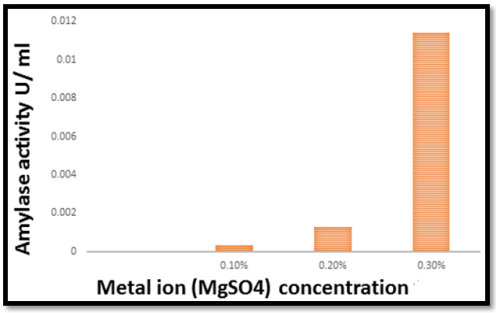

Figure 17. Effect of $MgSO_4$ in different concentrations on Amylase production (U/ml) of Lactic acid Bacteria GD

Effect of different starch on α-amylase Production

The effect of different starches starch-121(Raw), starch 034010 (Processed) and starch-356 (Refined) were determined on α-amyase production and maximum enzyme units were produced by both MM1 and GD in the presence of Refined starch-356 as shown in (Figure 20 and 21).

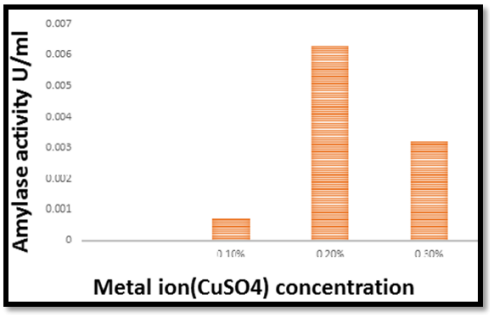

Figure 18. Effect of $CuSO_4$ in different concentrations on Amylase production (U/ml) of Lactic acid Bacteria MM1

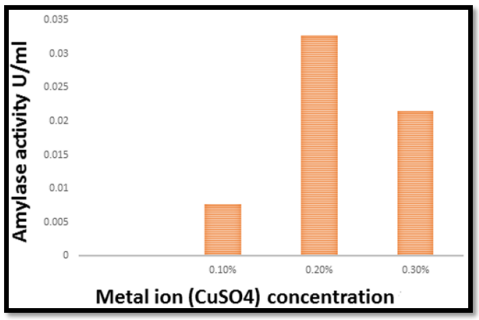

Figure 19. Effect of $CuSO_4$ in different concentrations on Amylase production (U/ml) of Lactic acid Bacteria GD

4. DISCUSSION

Amylase are enzymes that hydrolyze long chain of carbohydrate and due to this reason it is used in many industrial applications such as detergent, textile, paper and pulp industry. It is also used in food industries such as beverages and bakery.

Total of 15 isolates were isolated from raw and processed milk and yoghurt and cultured on LB agar plates. All strains were assayed qualitatively for α-amylase on 1% starch supplemented LB media agar pates and the basis of clearance zones only 4 isolates (R1, MM1, GD and NES) were selected as these were the producers of enzyme. The bacterial isolates produce α-amylase extracellularly, which hydrolyze starch and only end products glucose and small oligosaccharides or disaccharides will be present in the media. When the plates were flooded with brownish color iodine solution, starch and iodine complex produced bluish/purplish color and the clearance zones indicates absence of starch in the surrounding media, therefore, no bluish color were produced. Among four bacterial isolates, two strains

GD and MM1 produced zones of comparatively larger diameter than R1 and NES, therefore, selected for further studies.

For the identification of bacterial isolates various morphological and biochemical tests were performed, and results clearly indicates that isolated bacteria are Lactic acid bacteria (*Lactobacillus*). Among four bacterial isolates, three were gram positive while one was gram negative. Moreover, among the four bacterial isolates three were cocci, catalase positive and motile while one is rod shape, catalase negative and non-motile. Spore staining results have shown that all bacterial isolates were non-sporing. Aygan *et al.*, [21], also conducted the study on α-amylase. In their study, 247 isolates were tested for amylase production on agar plates containing soluble starch, among them 231 isolates were amylase positive isolates which were selected after application of iodine vapor. Among 231 isolates, the A10 strain was chosen for enzyme production, and biochemical and morphological studies have shown that the bacterial strain was aerobic, gram-positive rod shaped, catalase positive, spore forming and motile.

Moreover, the effect of pH on the α-amylase produced from lactic acid bacteria, MM1 and GD and result have shown that maximum enzyme units were produced at pH 5. These results have shown, that the optimum pH for the growth of bacterial isolates is pH 5 and *Lactobacillus* MM1 and GD cannot tolerate high and low pH. The same study was also conducted by (Amutha, and Priya) [22], in which the amylase production and amylolytic activities were evaluated at pH 4, 5, 6, 7, 8, 9, 10 and 11. The results showed that almost equivalent amount of amylase were produced at pH 5 and 6. No significant amount of enzyme and bacterial growth were obtained at acidic pH (4) and basic pH (10 and 11), which clearly indicates that bacteria was not extremophile (acidophilic and alkaliphilic) while maximum amount of amylase (11.94 U/ml) was produced at pH 6.

The results of the current research project were also supported by the study conducted by Sharma, & Vamil [23]. They have determined the effect of different pH (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) on α-amylase production by the *Bacillus amyloliquefaciens*. The result showed that the amylase activity was extremely low at pH 4 and activity was increasing continuously by increasing pH, but decrease in amylolytic activity was observed at extremely alkaline pH and maximum activity was obtained at pH 7.5, therefore, the pH 7.5 is optimum pH for α-amylase. The co-factors are required for enzymes because they can regulate the enzyme activity and modify the catalysis. Mostly metal ions act as regulators, and they can act as both inhibitors and activators. Some metal ions can enhance the enzyme activity thus act as activator while others can reduce the enzyme activity and act as inhibitors. In the current research project, the effects of three different metal ions ($MgSO_4$, $CuSO_4$, Na_2SO_4) in different concentrations (0.1, 0.2, 0.3%) were tested for amylase production and results revealed that all metal ions have increased the enzyme production by *Lactobacillus* MM1 and GD and amylolytic activity. Growth of *Lactobacillus* MM1 and GD were also measured and it was found that maximum growth occurred in the presence of Mg^{+2} followed by Na^{+} , while little growth was observed in the presence of Cu^{+2}. Jha *et al.*, [24] in their study tested the effect of different metal ions (Mg^{+2}, Cu^{+2}, Na^{+}, Ca^{+2} and K^{+}) on bacterial growth and they have observed that no growth was seen in presence of Mg^{+2} and K^{+2} but little or no growth was observed in the presence of Cu^{+2} and Ca^{+2}. Nominal growth was seen in the presence of Na^{+}, but maximum growth observed in the presence of Mg^{+2} and K^{+}. The results in the current project is also supported by the study conducted by (Lonsane and Ramesh) [25] who reported maximum growth and enzyme activity was observed in the presence of $Mg^{+}2$. (Gupta *et al.*,) [26] also reported that maximum activity of amylase was obtained in the presence of Mg^{+2} followed by Na^{+}.

Similarly, (Silpa *et al.*,) [27] evaluated the effect of varying concentration of Ca^{+2}, Cu^{+2}, Mg^{+2}, Fe^{+2}, and Mn^{+2} on enzyme activity and it was found that the Mg^{+2} show maximum activity at 2g/l, which decreased when the concentration was increases. Mg^{+2} and Ca^{+2} show better activity at 2g/l while other show less activity. In the current research project, Mg^{+2} enhance the enzyme activity by increasing the concentration of Mg^{+2} and maximum enzyme units were obtained in the presence of 0.3% (w/v) Mg^{+2} 2g/l. While in case of Na^{+} the amylase activity was reduced with increasing the concentration and maximum activity was obtained in the presence of 0.1% (w/v) Na^{+}. In case of Cu^{+2} the maximum enzyme activity was obtained in the presence of 0.2% (w/v), while further increase in the concentration have reduced the amylase activity.

Different carbon sources greatly affect the microbial growth and amylase production rate. In the current research project the effect of three different types of starches (Raw, Processed and Refined) were checked on the *Lactobacillus* MM1 and GD and maximum enzyme units were obtained in the presence of 1% (w/v) refined starch (356). (Sreekant *et al.*,) [28] also evaluated the effect of different carbon sources

(glucose, sucrose and corn starch) on the α-amylase produced by *Bacillus* sp. CFR 67 and results have shown that maximum biomass was obtained in the presence of corn starch while maximum α-amylase was obtained in the presence of glucose.

5. CONCLUSION

Amylase are enzymes that hydrolyze long chain of carbohydrate and due to this reason, it is used in many industrial applications such as detergent, textile, paper and pulp industry. It is also used in food industries such as beverages and bakery.
Total of 15 isolates were isolated from raw and processed milk and yoghurt and cultured on LB agar plates. All strains were assayed qualitatively for α-amylase on 1% starch supplemented LB media agar pates and the basis of clearance zones only 4 isolates (R1, MM1, GD and NES) were selected as these were the producers of enzyme. The bacterial isolates produce α-amylase extracellularly, which hydrolyze starch and only end products glucose and small oligosaccharides or disaccharides will be present in the media. When the plates were flooded with brownish color iodine solution, starch and iodine complex produced bluish/purplish color and the clearance zones indicates absence of starch in the surrounding media, therefore, no bluish color were produced. Among four bacterial isolates, two strains GD and MM1 produced zones of comparatively larger diameter than R1 and NES, therefore, selected for further studies.

For the identification of bacterial isolates various morphological and biochemical tests were performed, and results clearly indicates that isolated bacteria are Lactic acid bacteria (*Lactobacillus*). Among four bacterial isolates, three were gram positive while one was gram negative. Moreover, among the four bacterial isolates three were cocci, catalase positive and motile while one is rod shape, catalase negative and non-motile. Spore staining results have shown that all bacterial isolates were non-sporing (Aygan *et al.,*) [21], also conducted the study on α-amylase. In their study, 247 isolates were tested for amylase production on agar plates containing soluble starch, among them 231 isolates were amylase positive isolates which were selected after application of iodine vapor. Among 231 isolates, the A10 strain was chosen for enzyme production, and biochemical and morphological studies have shown that the bacterial strain was aerobic, gram-positive rod shaped, catalase positive, spore forming and motile.

Moreover, the effect of pH on the α-amylase produced from lactic acid bacteria, MM1 and GD and result have shown that maximum enzyme units were produced at pH 5. These results have shown, that the optimum pH for the growth of bacterial isolates is pH 5 and *Lactobacillus* MM1 and GD cannot tolerate high and low pH. The same study was also conducted by Amutha, & Priya [22], in which the amylase production and amylolytic activities were evaluated at pH 4, 5, 6, 7, 8, 9, 10 and 11. The results showed that almost equivalent amount of amylase were produced at pH 5 and 6. No significant amount of enzyme and bacterial growth were obtained at acidic pH (4) and basic pH (10 and 11), which clearly indicates that bacteria was not extremophile (acidophilic and alkaliphilic) while maximum amount of amylase (11.94 U/ml) was produced at pH 6.

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6. FUTURE PROSPECTS

The future prospects of the current research project can be;

- Further improvement in α-amylase production by mutagenesis
- Determination of α-amylase genes sequences in MM1 and GD by PCR and Sequencing
- Molecular weight determination of α-amylase by SDS gel electrophoresis.
- Determination of amino acid sequences of α-amylase by various bio-informatics tools

7. CONFLICT OF INTEREST

All authors have declared that there is no conflict of interests regarding the publication of this article.

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