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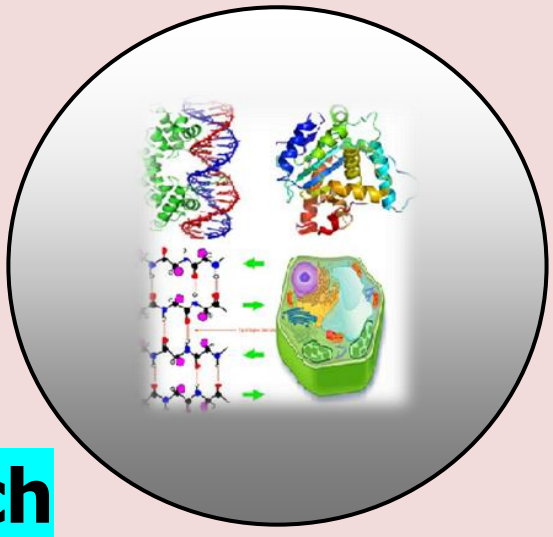
ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

UGC Approved Journal No. 62923
MCI Validated Journal
Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66

J. Biol. Chem. Research
Volume 36 (1) 2019 Pages No. 144-150



Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited in various International and National Scientific Databases

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 36, No. 1: 144-150, 2019
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 36/01/95/2019

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

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RESEARCH PAPER

Received: 03/01/2019 **Revised: 21/01/2019** **Accepted: 23/01/2019**

Amylase Enzyme Activity of Bacteria from Ginger cropsoils in Hunsur Taluk, Karnataka

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ABSTRACT

Amylases are one of the enzymes which can be produced from several sources such as plants, animals, bacteria and fungi. Microbial production of amylase is more fertile and cheaper than that of other sources. Starch degrading bacteria are most important for industries like food, fermentation, textile and paper. Amylase is a hydrolytic enzyme and in recent years, interest in its microbial production has increased vividly due to its wide spread use in food, textile, baking and detergent industries. Soil bacteria were isolated to determine their potential to produce amylase enzyme. 32 samples were collected from ginger crop soils in HunsurTaluk. The soil samples were isolated, then the isolates were screened for catalase test. The catalase positive isolates were then screened for starch hydrolysis test. The 9 amylase positive isolates were taken to estimate the amount of amylase enzyme activity by DNS method. The amylase positive isolates were identified as Bacillus spp., Staphylococcus spp., Clostridium spp., Lactobacillus spp., Pseudomonas spp., Micrococcus spp., The 3 isolates which showed high amylase activity is taken for pH optimization studies at different pH and at the incubation period of 24 hours. The results obtained reveals that these isolatesare good producers of amylase and could be used for production of amylase in food, brewery, textile and detergent industries.

Keywords: Bacteria Soil, Isolate, Amylase, Amylase activity and pH effect.

INTRODUCTION

Many microorganisms that live in the soil play essential role in maintaining life of this planet debasing or chemically amending molecules. Substantial human interest in soil organism stems from their ability to synthesize a variety of beneficial chemicals. Industrial microbiology concern itself with the isolation and explanation of microorganisms from natural environment such as soil and water (Pandey et al., 2000). Amylase is a group of vital enzymes which is mainly working in starch processing industries for hydrolysis of polysaccharides such as starch into simple sugar (Aiyer, 2005). Enzymes from microbial sources commonly meet industrial requests (Jayashankar and Geethanjali, 2018), due to their high production and heat stability.

In addition, the amylases that are extracted require optimum conditions to show greatest activity. This includes parameters such as temperature and pH. Starch degrading amylase enzymes are most significant in biotechnology industries with vast applications in food, fermentation, textile and paper (Bahadure et al., 2010). They are degrading enzymes with degradation of starch into sugars by acting on α- 1,4-glycosidic bonds. (Daniel et al., 2010). Amylases arefound from several origins like plant, animal, bacterial and fungal sources. Microorganisms are usedfor the industrial production due to the benefits such as cost efficiency, constancy, less timeand space required for production (Oyeleke and Oduwole, 2009; Raom et al., 1998). Many

microorganisms are able to produce amylases including *Bacillus spp*., *Lactobacillus*, *Escherichia*, *Proteus*, *Strepotmyces sp*., *Pseudomonas sp.*etc. For production of amylase for industrial usages, isolation and characterization of new novel strains is anincessant process. In the present study, we report the isolation, screening and characterization of amylase producing bacteria from the soil samples collected from agricultural fields. Production conditions were optimized such as pH to achieve high enzyme production and better enzyme activity.

MATERIALS AND METHODS

Sample Collection

The soil samples were collected from 32 ginger crop soils in from Hunsur Taluk, Mysore District, Karnataka State India located on the way towards Gonikoppa, Kodagu District.

Isolation of Bacteria

One gram of the above collected soil sample was weighed and mixed to 9 ml of sterile distilled water. Serial dilution was done up to 10^{-5} and 10^{-6}, from each dilution, 1ml of the sample was poured into already prepared nutrient agar plates fortified with 1% starch. The inoculate were spread properly by using an L-rod. Control plates were also maintained along with inoculated plates for testing the sterility of medium. The plates were incubated for 24h- 48h at 37°C. After incubation, number of colonies in each plate was counted using a colony counter. Using colony morphology, shape, size and colour, major dominant flora was selected and streaked onto already prepared nutrient agar plates. Then the plates were incubated for 24h at 37°C. After incubation, the plates were observed and ideal colonies were isolated and maintained as stock culture in slants agar at 4°C for further use.

Screening of Catalase Test

To test the catalase, loop of test bacterial cultures were placed on glass slides individually. The cells were mixed in a drop of 3% hydrogen peroxideand an immediate bubbling indicated a positive catalase test.

Screening of Amylase Activity

Bacterial cultures were screened for amylolytic activity by starch hydrolysis test on starch agarplate (Shaw et al., 1995). The pure isolated colonies were streaked on starch agar plates with starch as the onlycarbon source. After incubation at 37°C for 24-48 hrs, the individual plates were flooded with Gram's iodine (Gram's iodine- 250 mg iodine crystals added to 2.5gm potassium iodide solution,and 125ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. In the zone of degradation, no blue colour forms, which is the basis of the detection andscreening of an amylolytic strain. The amylase producers displaying maximum diameter of zone of clearance, were further investigated (Gupta et al., 2003). The pure cultures were sub cultured at regular intervals and starch- nutrient agar slants were maintained at 4° C.

Amylase production medium

A loop full of bacterial culture was transferred from starch-nutrient agar slants to starch- nutrientbroth at pH 7 for activation and incubated in a shaker at 40° C at 120 rpm for 24 h. Fermentationmedium contained soluble starch (10 g/L) peptone (5 g/L), $(NH4)_2$ SO4 (2 g/L), KH2PO4, (1 g/L),K2HPO4 , (2g/L), MgCl2, (0.01 g/L) at pH 7.The fermentation medium was inoculated with the activated culture (20% v/v) and incubated in shaker at 37° C for 24 hrs. At the end of thefermentation period, the culture medium was centrifuged at 10000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

Amylase Enzyme Essay

Amylase activity was determined according to (Bernfeld, 1955) by pipetting 1ml of the culture extract enzyme into test tubes and 1ml of 1% starch soluble in citrate phosphate buffer having a pH of 6.5. The reducing sugar liberated is estimated by 3,5 Dinitrosalycyclic acid (DNSA) method (Fisher, and Stein, 1961).The reaction mixture is incubated in waterbath at 40° C for 30 minutes. A blank consisting of 1ml of soluble starch in citrate phosphate buffer is also incubated in a water bath at same temperature and time with the other test tubes. The reaction was terminated by adding 2ml of DNSA reagent in test tube and then immersing the tube in boiling water bath for 5 minutes to develop brown color, after with they were allowed to cool and 5ml of distilled water was added. The absorbance for all the test tubes was measured at 540 nm with spectrophotometer. Enzyme activity was defined as the amount of soluble starch hydrolyzed by 1ml of enzyme extract in one minute. In other words, one amylase unit of beta amylase is expressed as milligram of maltose produced during one minutes of incubation with 1% starch.

Identification of Amylase Positive Isolates

Gram staining was performed to know whether the isolate was Gram positive or negative. Theisolates were observed under the microscope to obtain the colony morphology i.e. color, shape,size, nature of colony and pigmentation (Parmar and Pandya., 2012). The tests for biochemical characterization wereperformed such asoxidase test, carbohydrate fermentation test, IMViC, Gelatin hydrolysis, urease test, Hydrogen sulphide production test, and nitrate production test.

RESULTS AND DISCUSSION

SCREENING FOR CATALASE ACTIVITY

For the selected 46 isolates catalase test was performed. 25 isolates showed positive result for the catalase test (Table 1).

SCREENING FOR STARCHHYDROLYSIS

The catalase positive isolates were further screened for starch hydrolysis.

Table 1. Bacterial isolates showing positive and negative for starch hydrolysis.

Isolates	Result
AF1	Negative
AF2	Positive
AF3	Negative
AF4	Positive
AF5	Negative
AF6	Positive
AF7	Negative
AF8	Negative
AF9	Negative
AF10	Negative
AF11	Positive
AF12	Negative
AF13	Positive
AF14	Negative
AF15	Negative
AF16	Positive
AF17	Negative
AF18	Negative
AF19	Negative
AF20	Negative
AF21	Positive
AF22	Positive
AF23	Negative
AF24	Positive
AF25	Negative

The 25 isolates were screened for amylolytic activity by streaking individual isolates on starch agar medium. The agar plates were incubated at 37° C for 24 to 48 hours. Culture plates were flooded with iodine to identify zone of clearing around culture. 9 isolates showed positive for starch hydrolysis., AF2, AF4, AF6, AF11, AF13, AF16, AF21, AF22 and AF24. (Bala et al., 2013) isolated bacteria from ginger soil to determine their potential to produce amylase enzyme by streaking individual isolates on 1% starch agar and the plates were incubated at 37°C for 24 to 48 hours.

The amylase activity was assayed for the 9 Amylase positive isolates (Table 2) incubated for 24 hours. The results obtained for the isolates AF2, AF4, AF6, AF11, AF13, AF16, AF21, AF22 and AF24 showed 65, 55, 55,72.5, 52.5, 75, 42.5, 55, and 80µg/ml respectively. The isolates AF2, AF11 and AF24 showed maximum activity, all the three isolates were identified as *Bacillus sp*. The results are similar to the study conducted by (Padma and Pallavi, 2016) performed amylase enzyme activity for *Bacillus* isolated from different agricultural soil showed that *Bacillus sp.* had its maximum activity of 0.998U/ml at 72 hours of incubation period.

AMYLASE ENZYME ASSAY

Table 2. Amylase enzyme assay (OD at 540 nm).

SI.NO.	Volume of std glucose (ml)	Conc. of glucose (ml)	1% starch solution (ml)		DNS reagent (ml)			Optical density 540nm
1	0.0	0.0	-	Incubate @ 40^0c for 70mins	2	Boil for 5mins and cool	Add 5ml of distilled water	0.000
2	0.2	50	-		2			0.136
3	0.4	100	-		2			0.306
4	0.6	150	-		2			0.434
5	0.8	200	-		2			0.563
6	1.0	250	-		2			0.709
Samples	-	-	-		-			-
AF2	1	-	1		2			**0.170**
AF4	1	-	1		2			0.155
AF6	1	-	1		2			0.158
AF11	1	-	1		2			**0.193**
AF13	1	-	1		2			0.146
AF16	1	-	1		2			0.146
AF21	1	-	1		2			0.118
AF22	1	-	1		2			0.156
AF24	1	-	1		2			**0.219**

OPTIMIMUM pH OF AMYLASE ENZYME ACTIVITY

Table 3. Optimization of amylase enzyme activity.

Isolates	pH	amylase enzyme activity (glucose µg/ml/minute)		
		8 hrs	**16 hrs**	**24 hrs**
AF2	6	15.21	64.99	78.20
AF11	6	11.34	66.26	78.81
AF24	6	13.30	57.54	76.65
AF2	7	10.80	60.65	82.10
AF11	7	10.92	54.81	80.70
AF24	7	17.26	55.06	79.08
AF2	8	20.90	59.75	80.33
AF11	8	18.59	62.61	83.01
AF24	8	16.66	60.35	81.65

The pH of the production medium plays a significant role in microbial growth and hence impacts onthe enzyme production. In the present study the enzyme activity for different pH ranging from 6.0 - 8.0 was determined by keeping the optimum time and temperature combination.The isolate AF11 had maximum amylase production was found at pH 8.0 after 24 hrs. As shown in (Table 3) the isolates were able to grow at the pH range of 6, 7 and 8. The amylolytic activity was maximum at 24 hours for all the 3 isolates. The isolate AF2 showed maximum activity in pH 7 and isolate AF 11 showed maximum activity at pH 8, while isolate AF24 showed maximum activity in pH 8. Therefore, the pH range of 7-8 was optimum for amylase activity. The optimum pH for *B. Lichen iformis*; *B. subtilis*, *A. niger* and *A. fumigatus* was pH 7 with amylase activity ranging from 0.000471- 0.00457 mg/ml/sec according to Bala et al., 2013. Bhaskara et al., 2011 reported that the pH of media is one of the regulatory parameters during fermentation. Highest amylase enzyme production was observed highest at pH 7.0. A gradual decrease in the enzyme yield was obtained towards pH below 5.0 and above 10. Similar pH optimum for amylase production was reported by Oyeleke et al., 2010; Shafaat et al., 2011; Varalakshmi et al., 2010. An increase in the enzyme production was observed from 4h to 24h. The effect ofincubation time on alpha amylase production was found to be statistically significant ($p < 0.05$).

The results are similar to the study conducted by Jogezai et al., 2011 on α-amylase production by *Bacillus subtilis* in shake flask for different intervals of time (0 to 72 h). The production of enzyme was reached maximum at 48 h after inoculation. Further increase in incubation period however, did not show any significant increase in enzyme production rather it was decreased. This is because the cells would have reached decline phase with lowered enzyme synthesis.

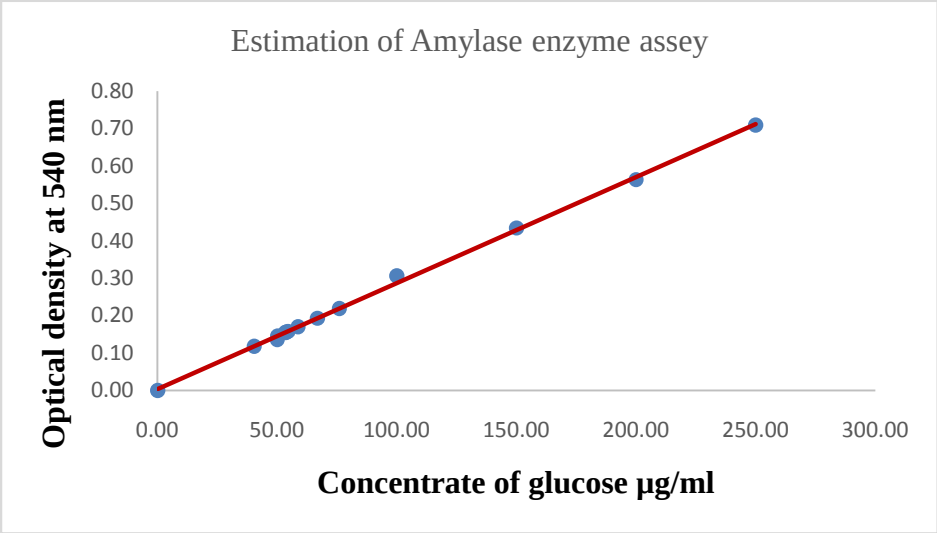

Figure 1. Estimation of Amylase enzyme assay.

RESULTS FOR AMYLASE POSITIVE ISOLATES

(A) Based on cultural characteristics

All the nine amylase isolates were subjected for the morphological characterization and biochemical test for their identification (table 4).

Table 4. Culture characteristics of isolates.

Isolates	Shape	Margin	Consistency	Elevation	Opacity	Pigment
AF2	Irregular	undulate	moist	umbonate	opaque	White, dull
AF4	Irregular	uneven	moist	raised	opaque	White
AF6	Irregular	even	moist	flat	opaque	White
AF11	Round	undulate	moist	umbonate	opaque	White, dull
AF13	Irregular	uneven	moist	raised	opaque	White
AF16	Irregular	uneven	moist	raised	opaque	White
AF21	Irregular	uneven	moist	raised	opaque	White
AF22	Irregular	even	moist	raised	opaque	Pale yellow
AF24	Round	undulate	moist	umbonate	opaque	White

(B) Based on Gram's staining

Table5. Gram staining results.

Isolates	shape	arrangement	Garm's reaction
AF2	Baccilli	Single/chain/cluster	Positive
AF4	Cocci	Single/chain/cluster	Positive
AF6	Cocci	Single/chain/cluster	Negative
AF11	Baccilli	Single/chain/cluster	Positive
AF13	Cocci	Single/chain/cluster	Negative
AF16	Cocci	Single/chain/cluster	Positive
AF21	Baccilli	Single/chain/cluster	Negative
AF22	Baccilli	Single/chain/cluster	Positive
AF24	Baccilli	Single/chain/cluster	Positive

(c) Based on Biochemical tests

Table 6. Biochemical tests for the isolates.

Isolates	Oxidase test	Carbohydrate test			IMViC				Gelatin hydrolysis	Urease test	H2S production
		glucose	sucrose	lactose	I[1]	M[2]	V[3]	C[4]			
AF2	+	-	+	+	_	-	+	+	+	_	-
AF4	+	-	+	+	_	-	-	+	-	+	-
AF6	+	+	+	+	+	-	-	+	-	+	-
AF11	+	+	+	_	_	+	+	+	+	_	-
AF13	+	+	-	+	_	+	-	+	-	+	-
AF16	+	-	-	+	_	+	-	+	-	+	-
AF21	+	+	+	+	+	-	-	+	-	+	-
AF22	+	-	-	+	_	+	-	+	+	_	-
AF24	+	+	+	+	_	-	+	+	-	_	-

1= Indole Production, 2= Methyl Red, 3= Vogues Proskauer's, 4=Citrate Utilization

Based on the cultural characteristics, Gram's staining and biochemical tests the amylase producing isolates were identified as *Bacillus spp., Staphylococcus spp., Clostridium sps, Lactobacillus spp., Pseudomonas spp., Micrococcus spp.* These results agreed with (Bhaskara et al., 2011), isolated the bacteria from agricultural soil and identified the amylase producing isolates as *Bacillus subtilis, Bacillus licheniformis, Micrococcus sps.,* and *staphylo coccus.*

CONCLUSION

The present study showed that the isolated bacteria from ginger crop soilsproduced appreciableamount of amylase enzyme. Further, the study also revealed that amylase enzyme produced by theisolated bacteria can be used for industrial application like starch modificationwith better efficiency with the increase in temperature.

ACKNOWLEDGEMENTS

The authors are thankful to Mangalore University for providing the fellowships and all the facilities required to carry out this work.

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