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Optimization and Production of α -Amylase from Halophilic *Bacillus* Species Isolated from Mangrove Soil Sources

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Abstract In the present study, four bacterial isolates from a mangrove soil were screened for their ability to produce α-amylase using submerged fermentation. *Bacillus* MJK1, MJK2, MJK6 and MJK10 which were assigned to be *Bacillus* species proved to be the best α- amylase producer. Various effects of pH, temperature, incubation time, carbon source and salinity were checked. Different carbon supplements were used to enhance the enzyme production and the highest yield was obtained with 2% soluble starch as supplements. The presence of fructose, maltose, sucrose, glucose reduced the production of amylase. The optimum pH, temperature, and incubation period for amylase production by the isolate was found to be 8.0, 50°C and 72 hrs respectively. The production medium with increase in addition of NaCl, diminished the production of amylase. The presence of NaCl in the culture media promoted extracellular amylase even in the presence of 4% NaCl.

Keywords: *α- amylases, Bacillus, mangrove soil, rhizosphere*

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1. Introduction

Alpha amylase (α-1, 4 glucan-glucanohydrolase *EC 3. 2.1. 1*), the starch degrading enzyme is widely distributed in nature which accounts for about 30% of the world's enzyme production. This extracellular enzyme hydrolyses α-1,4 glucosidic linkages randomly throughout the starch molecule in an endo-fashion producing oligosaccharides and monosaccharides including maltose, glucose and alpha limit dextrin [1,2,3]. The microbial amylases are the most produced and used in industry, due to their productivity, thermo stability, suitability over wide pH range and biocompatibility. α- amylase can be produced by different species of microorganisms, but for commercial applications, α- amylase is mainly derived from the genus *Bacillus* [4,5]. Alpha amylase is a hydrolytic enzyme and in recent years, interest in its microbial production has increased dramatically due to its wide spread use in food, textile, baking and detergent industries [6]. Optimization of various parameters and manipulation of media are one of the most important techniques used for the overproduction of enzymes in large quantities to meet industrial demands [7]. Significant application of α- amylase requires particular properties with respect to specificity, stability, temperature and pH dependence. Interactions of these parameters are reported to have a profitable influence on the production of the enzyme.

Mangroves provide shelter and nurturing sites for many marine microorganisms. Due to the presence of rich source of nutrients mangroves are called the homeland of microbes. Mangroves inhabit intertidal zone with high salinity and can tolerate wide range salinities under natural conditions [8]. The gray mangrove *Avicennia marina* has the ability to adapt its pneumatophores to micro-topographical irregulations regularly sloping intertidal zone [9]. *Avicennia marina* is the most common species planted for mangrove restoration and is highly salt tolerant [10]. In the present study, four *Bacillus* sp. strains were isolated from the soil of coastal mangrove. The aim was to evaluate extracellular alpha amylase by *Bacillus* sp. under various culture parameters in an attempt to establish an industrially applicable *Bacillus* sp. as a source of alpha amylase and optimize its extracellular amylase secretion conditions.

2. Materials and Methods

2.1. Study Area

The Muthupettai mangroves located 10^{o} 25'N and 79^{o} 39'E situated along the south east coast of Thiruvarur district, TamilNadu, India. Many tributaries of the river Cauvery such as Paminiyar, Koraiyar, Kilaithangiyar, Kandankurichanar and Marakkakoraiyar are flows through Muthupettai lagoon. *Avicennia marina* is the dominant

mangrove species in Muthupettai and accounts for nearly 95 % of the vegetative cover. The samples were collected from rhizhosphere, sediment and sludge soil during the period of November, 2011.

2.2. Microorganism and Screening Conditions

The heterotrophic bacteria were enumerated and isolated by total viable count method. The sample was processed using serial dilution and spread plate technique. Amylolytic activity was determined as a zone of hydrolysis around the colonies on starch agar plates containing a media composed with (gL^{-1}), soluble starch, 20 g; Peptone, 10 g; Yeast extract, 4 g; NaCl, 0.5 g; $MgSO_4$, 0.5 g; $CaCl_2$, 0.2 g; Agar : 15 g, after 24 hrs of incubation at 37°C. Plates were incubated at 37°C for 42 hrs. After incubation period, plates were checked for amylolytic activity by flooding with 1 % iodine in 2 % potassium iodide [11].

2.3. Presumptive Identification of Bacterial Isolates

Later the phenotypic identification of isolated bacterial strains was determined according to Bergey's manual of systematic bacteriology.

2.4. Production Medium

All the isolates of *Bacillus* were cultured in starch agar plates at 37°C to obtain better colony of each isolate. Single colonies from each plate were subcultured on 25 mL of liquid medium containing (gL^{-1}): starch, 20; peptone, 10; yeast extract, 4; NaCl, 0.5; $MgSO_4$, 0.5; $CaCl_2$, 0.2. The pH of the medium was adjusted to 7 before autoclaving. The 10 mL of inoculums was transferred into 90 mL of sterile starch broth medium and incubated for 37°C for 24 hrs. Then the culture broth was centrifuged at 13,000 rpm for 10 min at 4°C to remove the cell. The supernatant was collected and labeled as crude enzyme and stored at −20°C for further studies.

2.5. Enzyme Assay

Alpha amylase activity was measured using a method of Bernfeld [12]. The sample was heated up to 68°C to denature beta amylase present in it. 100 µL α- amylase and 250 µL of 2% starch solution in 20 mM sodium phosphate buffer were incubated at 50°C for 10 min. The reaction was terminated by adding 250 µL of 3, 5-di nitrosalicylic acid reagent, (3, 5 di nitrosalicyclic acid, 1.0 g; Sodium hydroxide, 20 g; Sodium potassium tartarate, 30 g; Distilled water, 100 mL) followed by boiling in water bath for 5 min. The reaction mixture was cooled and diluted with 2 ml of water and the absorbance was measured at 540 nm using a UV-Vis Spectrophotometer (Cyberlab, UV-100, USA). One unit of amylase activity was defined as the amount of enzyme which released 1 µM glucose under the assay conditions.

2.6. Optimization of Culture Conditions for the Production of Enzyme

The factors such as temperature, pH, incubation time, salinity, sources of carbon affecting production of amylase were optimized by varying conditions. The experiments were conducted in 250 mL Erlenmeyer flask containing production medium. After sterilization by autoclaving, the flasks were cooled with loop full of growing culture. Inoculated and maintained under various operational parameter separately such as pH (6.0, 6.5, 7.0, 7.5, 8), temperature (30, 35, 40, 45, 50°C), incubation period (24, 48, 72, 96, 120 h), carbon source (glucose, starch, maltose, lactose and sucrose each at 1 %), and salinity (0.5, 1,2,3,4 %). After 48 h (except for incubation period effect), the culture filtrate was assayed in triplicate for amylase activity.

3. Result and Discussion

Samples were collected from rhizosphere, sediment and sludge soil from mangrove swamps in southeast coast of India. A total of 192 microbial strains isolated from soils were screened for the amylase production on starch agar plates and followed by incubation at 37°C for 24 hrs. The medium containing 1 % soluble starch (gL^{-1}), 0.2 % yeast extract, 0.5 % peptone, 0.1 % $MgSO_4$, 0.1% NaCl and 0.02% $CaCl_2$ (pH 7.0). Amylolytic isolates were selected by flooding the agar plates with 1% Iodine solution. From 192 different bacterial isolates obtained from soil, 11 isolates were selected. Typical cultural and morphological characteristics were observed and nine isolates were screened for *Bacillus* species. Amylase activity of the culture supernatants from these nine isolates was determined. Out of these nine isolates, the best potent isolates were finally chosen and maintained on nutrient agar slant at 4°C for further studies.

Table 3.1. Description of the test strains and their sources

S. No	Isolate No.	Source	Zone of clearance on starch medium (diameter, mm)
1.	MJK1	Rhizosphere	34
2.	MJK2	Rhizosphere	26
3.	MJK6	Sediment	32
4.	MJK10	Rhizosphere	32

3.1. Identification of *Bacillus*

All the isolates which exhibited clear zone of hydrolysis on starch agar medium was further inoculated into nutrient broth for enrichment and subjected to conventional method of identification. The four isolates were found to be *Bacillus* sp. from the biochemical test results. Biochemical profiling of the positive isolates were listed in table.

Table 3.2. Biochemical tests for *Bacillus* sp.

S. No.	Biochemical tests	Results
1.	Gram's reactions	Gram positive, rod
2.	Indole test	Positive
3.	Methyl red test	Negative
4.	Voges-proskauer test	Positive
5.	Citrate utilization test	Positive
6.	Starch hydrolysis	Positive
7.	Catalase test	Negative
8.	Oxidase test	Positive
9.	Urease test	Positive
10.	Test for H_2S production	Positive
11.	Fermentation tests	
	Glucose	Acid
	Fructose	Acid
	Lactose	Acid
	Sucrose	Acid

3.2. Optimization Conditions of α- Amylases Enzyme

Though, different *Bacillus* species have similar growth patterns and enzyme profiles, but their optimized conditions vary, depending upon the strain. Optimization of the process parameters is needed for improved production of enzyme to make the process cost effective [13]. The *Bacillus* strains MJK1, MJK2, MJK6 and MJK10 were selected for the optimization of enzyme production based on the DNS assay.

3.2.1. Effect of pH

The effect of pH on the activity of α- amylase obtained from the selected four strains of *Bacillus* was analyzed by using DNS method. The enzyme was stable in the experimental range of pH 6 to 8. The strain MJK2 showed the optimum pH for amylase activity was determined as pH 8 (98±1.1 U mL^{-1}) after 48 hrs incubation.

Table 3.3. Optimization of amylase production in *Bacillus* sp. in various pH

S. No.	pH	Activity U mL^{-1}			
		MJK1	MKJ2	MJK6	MJK10
1.	6.0	62±1.1	63±2.8	59±1.9	60±0.9
2.	6.5	76±1.6	78±1.7	68±0.5	72±0.3
3.	7.0	83±1.2	87±1.7	75±1.6	78±0.1
4.	7.5	86±1.7	92±2.8	79±2.1	82±1.6
5.	8	89±2.1	98±1.1	81±1.1	85±0.9

In the present study the maximum enzyme activity was occurred at the optimum pH 8. So here it is noted that α-amylase was stable at 8 and it was determined as alkaline α- amylases from *Bacillus* sp. The result was similar to the work done by Kikani and Singh, [14] showed that the optimum pH for amylolytic activity of α- amylase was around 7.5 to 8 which was for *Bacillus* sp. Pancha *et al.* examined that the effect of pH on the activity showed that the optimum occurred at pH 8, which is in agreement with the results of other work [15].

3.2.2. Effect of Temperature

The effect of temperature on α- amylase was identified by altering the reaction temperatures ranges from 30 to 50°C. The effect of temperature on the amylolytic activity was examined at pH 8. The optimum temperature for the enzyme ranges between 50°C for strain MJK2 (94±1.6). Below this temperature, the enzyme activity was declined gradually.

Table 3.4. Effect of temperature on the amylase from *Bacillus* sp. strains

S. No.	Temperature (°C)	Activity U mL^{-1}			
		MJK1	MKJ2	MJK6	MJK10
1.	30	59±1.2	62±1.1	51±1.3	55±2.2
2.	35	71±1.8	75±2.3	62±1.4	67±1.6
3.	40	79±0.5	81±1.7	70±0.9	75±1.2
4.	45	83±1.0	88±1.6	79±1.5	81±1.0
5.	50	87±1.9	94±1.6	82±0.7	84±0.9

In the present study, the maximum enzyme activity was found at the optimum temperature of 50°C. These results are in accordance with the report of Vijayalakshmi *et al. [13]* who reported 50°C to be the optimal temperature for *B. subtilis* KC3. Lin *et al.* [16] and Yang *et al.* [17] who reported 55°C to be the optimal temperature for enzyme synthesis from *Bacillus* sp.TS-23 and *B. alcalophilus* respectively. This result was related to the work done by Asgher *et al* [6]. He observed that the maximum enzyme activity at optimum temperature of 60°C to α- amylase enzyme isolated from *Bacillus* sp. The reports showed that the activity at 100°C was about 2 times greater [18].

3.2.3. Effect of Carbon Source

The effect of carbon source on the activity of α-amylase was measured by incorporating 2 % of various carbon sources such as starch, maltose, glucose, and lactose and sucrose. The optimum source for amylase with increased enzymatic activity was obtained when starch is used as substrate. The strain MJK2 showed higher activity 143±3.4 for the enzyme.

Table 3.5. Effect of carbon source on the activity of enzyme amylase

S. No.	Substrate (2 %)	Activity U mL^{-1}			
		MJK1	MKJ2	MJK6	MJK10
1.	Starch	140±1.9	143±3.4	132±0.9	137±2.1
2.	Maltose	125±1.2	128±0.6	112±0.7	117±1.9
3.	Glucose	135±2.6	139±1.7	124±1.1	128±0.7
4.	Lactose	113±0.6	117±1.1	105±1.8	110±2.4
5.	Sucrose	127±2.3	130±2.6	121±0.7	124±2.1

In current investigation, the maximum enzyme activity was found at the optimum substrate of 2% soluble starch. This result was related to the work done by Srivastava and Baruah [19]. Maximum enzyme activity was found with 2% starch as the substrate when the crude enzyme was allowed to react with different substrate concentrations was reported by Vijayalakshmi *et al. [13]*. α- amylase production was induced by starch and maltose when supplemented to the medium as the only carbon source [20,21].

3.2.4. Effect of IncubationPeriod

The effect of incubation time on the activity of enzyme was analyzed by supplying 2 % soluble starch at pH 8 and incubate at 50°C for various incubation times (24 to 120 hrs) as in table. The significant activity for amylase was obtained from MJK2 (146±2.8) at the optimum incubation period of around 72 to 96 hrs.

Table 3.6. Effect of incubation period on the activity of enzyme amylase

S. No.	Incubation time (hrs)	Activity U mL^{-1}			
		MJK1	MKJ2	MJK6	MJK10
1.	24	91±0.1	94±0.7	85±2.1	89±1.5
2.	48	117±1.4	120±2.8	105±0.7	112±1.9
3.	72	143±1.7	146±2.8	138±0.4	140±2.4
4.	96	131±0.9	136±1.1	125±2.6	129±1.5
5.	120	98±1.5	105±2.3	96±1.2	98±1.1

In the present study the enzyme activity was optimized at 72 hrs of incubation period. The result was similar with Vijayalakshmi *et al.* [13] that highest activity obtained from 48 hrs of incubation period for *Bacillus* sp. from her studies and goes with another work Haq *et al.* [22] for *Bacillus* sp. Asgher *et al.*, [6] optimized *B. subtilis* incubation period at 24 hrs. In the case of α- amylase production by *B. flavothermus*, enzyme production and biomass peaked twice and highest activity was obtained after 24 hrs [23]. Similar findings have been reported on *B. amyloliquefaciens* [24] and *Bacillus* sp. ANT-6 [25].

3.2.5. Effect of Salinity

The effect of salinity on amylase activity was identified by altering the reaction with varying concentration of sodium chloride ranges from 5 to 40 mg L^{-1}. The effect of alkalinity on the amylolytic activity of *Bacillus* sp. was

examined at pH 8 with 50°C provided with soluble starch as substrate and incubated at 72 hrs. The optimum alkaline range for the enzyme activity ranges between 5 and 40. Above this range the enzyme activity was declined. The strain MJK2 showed 175±1.1 activity in 5 mgL^{-1} of NaCl can be noted from table.

Table 3.7. Effect of salinity on the activity of enzyme amylase

S. No.	Salinity (mgL^{-1})	Activity U mL^{-1}			
		MJK1	MKJ2	MJK6	MJK10
1.	5	172±0.8	175±1.1	162±1.8	168±0.5
2.	10	161±1.0	165±2.4	154±0.4	157±2.1
3.	20	133±1.5	138±0.8	127±0.7	131±1.5
4.	30	117±0.8	120±2.3	105±1.6	115±0.9
5.	40	104±1.1	108±0.1	95±2.4	99±0.8

In the present study α- amylase from *Bacillus* sp. had an optimal salinity 5 mgL^{-1} NaCl. Even though the bacteria were isolated from coastal soil, it produces low concentration of amylase. Hence, it can be a facultative halophilic in nature. Similar results were found in work of *B. subtilis* strain AS-S01a [26]. *Penicillium fellutanum* from mangrove soil had similar salinity effect [27]. Saxena *et al.* [20] isolated alkaline stable α- amylases from soil. Kikani and Singh [14] reported an extreme halophilic *Anoxybacillus beppuensis* from clay type soil collected from hot water reservoir.

4. Conclusion

In conclusion, the result has shown that the amylolytic enzyme yield can be increased by the optimized condition. Present study revealed that the mangrove halotolerant *Bacillus* sp. may have practical applications in the starch industry on account of the stability at alkaline pH, salt concentration and also in temperature. Further studies to purify and characterize the amylase produced by these strain will be investigated.

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