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In-vitro screening of amylase producing halophilic bacteria isolated from seawater

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In this study, eight halobacterial strains were isolated from seawater. Among the eight isolates, four strains grew well in nutrient medium with 40 g/100 mL of sodium chloride concentration. Amongst all, HA01 was found to show highest amylase activity than others. Highest amylase producing bacteria, *i.e.* HA01 was subjected for 16S rRNA sequence based identification and it was found to be *Aquabacterium* sp. strain AS02. The organism showed optimal amylase production at pH 7 and temperature of 42 °C and NaCl concentration of 3 M. The two substrates (banana peel and potato peel) were used for the amylase production among which banana peel showed that highest amylase activity of 6.12 U/mL than potato peel (4.27 U/mL).

[**Keywords**: Amylase, Halophilic bacteria, Optimization]

Introduction

World's surface has nearly 40 % of salinity, a subset of extremophiles, Halophiles have the special ability to survive in salt-rich conditions[1]. It is reported that halophiles are graded as mild, moderate, and severe halophiles, based on this varying concentration of NaCl[2,3]. Halophiles can live in a variety of environments, including marshy lagoons, alkaline lakes, deep sea brines, salt mines, solar salts, and hypersalines. Halophiles are reported to secrete certain enzymes such as amylases, nucleases, proteases, lipases, chitosanase etc.[4,5]. Enzymes are ideal catalyst which allows the occurrence of various biochemical reaction to take place without undergoing permanent changes[6,7]. It is believed that amylases hydrolyze starch molecules to produce a variety of compounds, including dextrins and ever-smaller glucose-composed polymers[8,9].

There is a huge demand for amylase enzymes which has been obtained from various sources, including microbes, animals and plants due to its application in various sectors like bioethanol production, food industry, textile industry, leather industries[10]. Amylases are produced by yeast, bacteria, fungus, and actinomycetes. Halophiles are a valuable source of stable enzymes since their enzymes are salt- and temperature-tolerant, allowing them to catalyze reactions even under challenging circumstances[11]. Microbial amylases have potential applications in pharmaceutics, chemical industries, food manufacturing industries, direct fermentation of starch to ethanol, wastewater treatment for starch refining, and medicinal and clinical chemistry research[12-14]. Halophilic enzymes are very promising for industrial applications requiring hypersaline environments. The primary advantages of employing microorganisms for amylase enzyme production are primarily for effective and affordable bulk production and for the manufacture of amylase with specified properties[15,16]. The goal of the current study is to identify halophilic bacteria that produces amylase enzyme by screening, isolating, and identifying them in the saltwater of Harbor beach in Thoothukudi.

Materials and Methods

Collection of samples

Samples were taken at a depth of 15 to 30 cm from sea water at the harbour beach of Thoothukudi, Tamil Nadu, India. They were gathered in sterile containers, and further processing took place in the laboratory.

Screening of halophilic bacteria

To isolate the halophilic bacteria, 1 mL of seawater was streaked onto the plates containing Halophilic agar medium (HiMedia). The plates were then

incubated for one week at 37 °C after which colonies were observed in it. The isolated bacteria from the halophilic agar medium were further determined under the growth of hypersaline condition *i.e.* the isolated bacteria were streaked on nutrient agar medium with sodium chloride concentration of 25 g, 30 g, 35 g, 40 g in 100 mL of nutrient agar medium. Following the isolation of viable colonies, the plates were incubated at 37 °C, and the isolated colonies were kept for future use.

Screening of amylase producing halophilic bacteria

Using starch agar plates containing 25 % sodium chloride (NaCl), amylase generating strains of halophilic bacteria were tested. After two weeks, the plates were inundated with iodine solution and the halo zone around the colony was examined. The organism that created such zones was studied further.

Identification of isolated organism

The highest zone produced organism was subjected for morphological and biochemical studies. Molecular identification by 16S rRNA sequence was done by subjecting the culture for bacterial DNA isolation[17], further purified by isoamyl alcohol: chloroform and amplified by PCR with forward and reverse primers. The Primer of 27F 5' AGAGTTTGATCMT GGCTCAG 3', another primer of 1492R of 5' TACGGYTACCTTGTTACGACTT 3' was used in this study. PCR was performed under the standard thermal cycling conditions. Sequence obtained (ABI 3730xl sequencer, Applied Biosystems) was filed in GenBank.

Optimization of amylase production

Optimization of amylase was done using various conditions such as: NaCl concentration, incubation time, temperature, pH and agitation. The absorbance of 24 h grown culture was set at 0.2 O.D and was used as inoculum. Amylase activity was determined as follows - 2 mL crude enzyme extract (supernatant of grown culture) was added with starch solution (1 mL of 1 % soluble starch dissolved in a buffer containing 20 mM of Tris-HCl, 10 mM of $CaCl_2$ and 2 M of NaCl at pH 7.4) and incubated at 50 °C for 15 min. The 3, 5 dinitrosalicylic acid (DNS) reagent was diluted 5 times with water and vortexed after each dilution after being added and heated in a boiling water bath for 15 min, the DNS reagent was then removed. The quantity of sugar reduction was measured using a conventional sugar-reduction graph (glucose). The amount of enzyme that releases one μmol of glucose reduction per minute under the assay conditions was referred to as one unit of α-amylase activity.

Results and Discussion

Isolation of extreme halophilic bacteria

Eight halobacterial were isolated from seawater using halophilic isolation agar and four strains (HA01, HA02, HA03, HA04) grew well on nutrient medium with 20, 30 and 40 g/100 mL sodium chloride concentration. Thus, extreme halophilic organism which could live on 40 % NaCl was isolated. Abd Samad *et al.*[18] reported that 20 – 30 % NaCl concentration was needed for the isolation of extreme halophilic bacteria. Ibekwe *et al.*[19] reported that normal bacterial colonies did not appear at high salt concentration from 15 % NaCl (w/v). The extreme halophilic bacteria were further selected for screening process of amylase production.

Screening of amylase producing halophilic bacteria

High salt resistant amylase has much attention in industries[16]. Thus, present study concentrated to screen amylase producing halophilic bacteria from sea water. Figure 1 displays the zone of clearance on starch agar plates by various halophilic isolates. The best amylase producers among them were reported to be HA01 and HA03. While the presence of blue color around the growth indicated negative findings, the presence of a robust zone of starch hydrolysis around the colony was proven to be amylase positive strains[20].

Among the two (HA01, HA03), HA01 was found to have highest (32 U/mL) amylase activity than

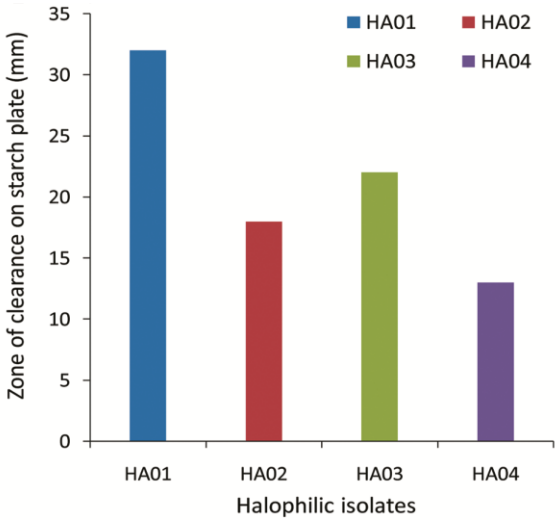

Fig. 1 — Zone of clearance on starch plates by different halophilic isolates

HA03 (Table 1). The enzymatic profile variability depended on the bacterial species and the substrate nature[21]. Bajpai *et al*.[22] reported that *Haloferax* sp. HA10 having a maximum output of α amylase at 3M NaCl, suggests good adaptation and enzyme stability under very saline conditions. Thus, HA01 was subject for identification by 16S rRNA sequencing and identified to be *Aquabacterium* sp. strain AS02 (accession number MT421776) (Fig. 2).

Optimization of amylase production

Effect of temperature on amylase activity

Aquabacterium sp. strain AS02 showed maximum production at 42 °C (Fig. 3). A few of the halophilic microorganism at a higher temperature displayed improved activity and stability[23]. For instance, *Micrococcus halobius* amylase showed optimal activity at 50 – 55 °C[24].

Table 1 — Amylolytic activity of halophilic isolates

S. No	Isolates amylase activity	U/ml
1.	HA01	32
2.	HA03	22

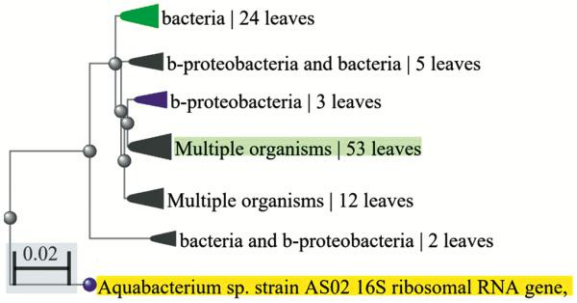

Fig. 2 — Phylogeny tree of isolated *Aquabacterium* sp. strain AS02

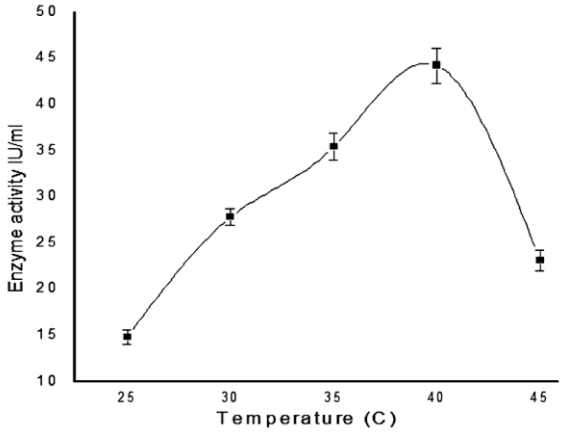

Fig. 3 — The effect of temperature on the activity of amylase produced by halophilic bacterial strains

Effect of pH on amylase activity

Aquabacterium sp. strain AS02 exhibited an optimal amylase enzyme activity at pH 8 after which a gradual decrease in enzyme activity was observed (Fig. 4). Similar to the results, *Micrococcus halobius* exhibited a stable amylase enzyme at pH of 6.5[(ref. 24)]. The pH of the growth medium has a significant impact on the bacteria's ability to produce enzymes and change their morphology[25]. Furthermore, saccharification for the biological generation of starch and other uses of thermophilic amylase in the creation of starch sweeteners are both required[26].

Effect of agitation on amylase activity

Only because of agitation, the bacteria in the fermentation medium receives oxygen. Zafar *et al.*[27] reported that the optimized agitation speed for the production of amylase enzyme is 150 - 300 rpm. Simair *et al.*[28] reported that agitation is essential for higher enzyme titre and growth. In the current study, *Aquabacterium* sp. strain AS02 was producing more amylase at 100 rpm at 37 °C (Fig. 5).

Effect of incubation

In the current study, *Aquabacterium* sp. strain AS02 was producing more amylase at 48 h (Fig. 6).

Experimental design and statistical analysis

Response Surface Methodology (RSM) was used for the optimization of the culture conditions for the amylase enzyme production by *Aquabacterium* sp. strain AS02. Central Composite Design (CCD) was used consisting of four factors at two level pattern[4]. Different combination of pH, temperature, incubation time, agitation were used in this study (Table 1;

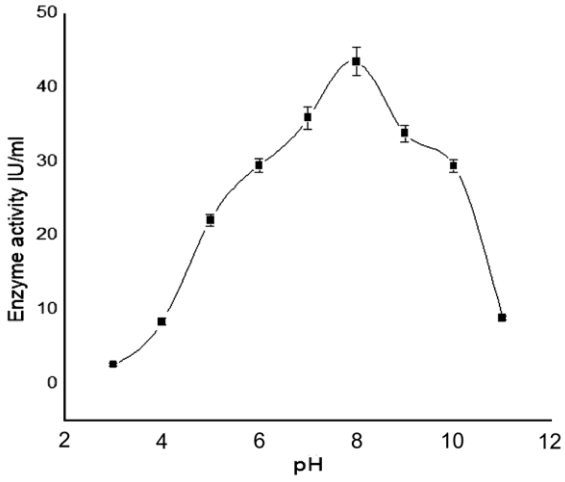

Fig. 4 — The effect of pH on the activity of amylase produced by halophilic bacterial strains

Figs. 7 – 9). The model was analyzed using Design Expert 7.0 software variable on the amylase production. Table 2 provides the experimental design, the actual response, and the projected response.

The quadratic equation

Enzyme activity = +54.23 - 0.27 A -2.21 B - 0.82 C + 1.46 D - 2.57 AB - 3.25 AC -2.71 AD + 1.15 BC - 1.31 BD - 3.09 CD - 8.52 A^2 - 8.58 B^2 - 8.68 C^2 - 8.80 D^2

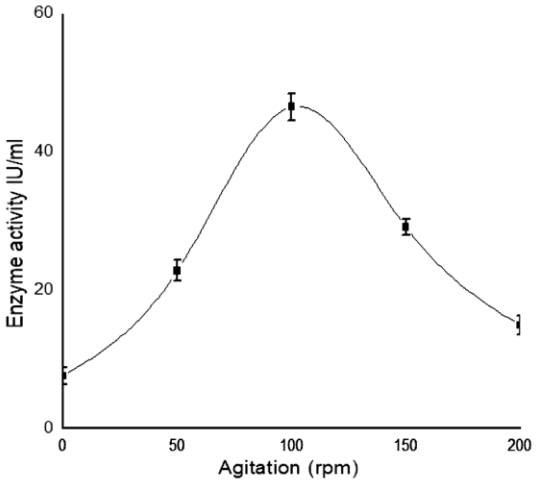

Fig. 5 — The effect of agitation on the activity of amylase produced by halophilic bacterial strains

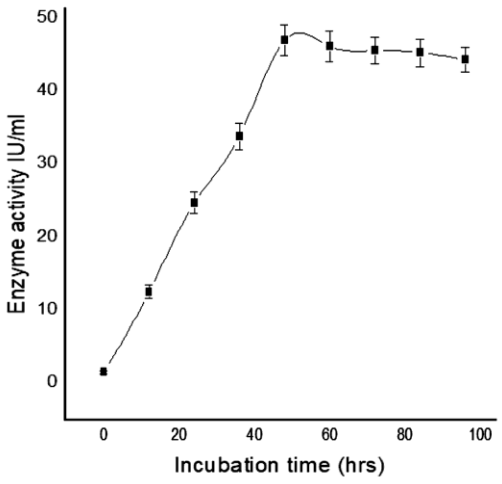

Fig. 6 — The effect of incubation time on the activity of amylase produced by halophilic bacterial strains

The response surface quadratic model's ANOVA yielded an F-value of 102.3942, and the model's P-standard was $p < 0.0001$, showing its significance. The model's discrepancy coefficient was CV = 8.760 %. The resolution coefficient, which implies that sample difference of more than 98 % was accredited to the variables and just 2.5 % variance could not be explained by the model, was used to evaluate the model's goodness of fit. Additionally, the corrected determination coefficient was adequate to support the model's importance[29].

The quadratic regression equation was analyzed for obtaining the predicted response. The 30 design

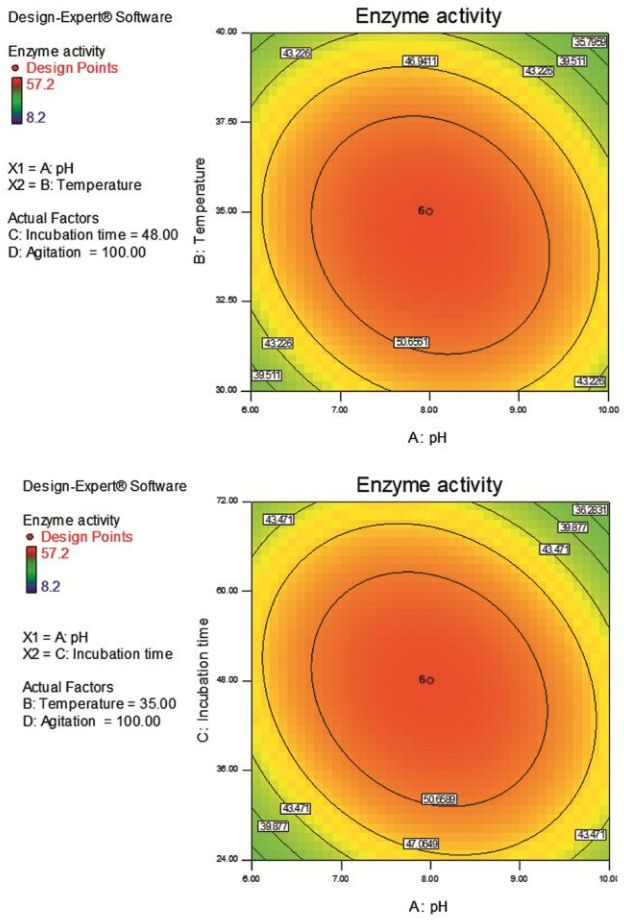

Fig. 7 — Contour plot for the effect of (pH and temperature); (pH and incubation time) on the activity of amylase enzyme

Table 2 — Actual and coded value of different variables

Factor	Name	Units	Actual		Coded		Mean
			Low	High	Low	High	
A	pH		6	10	-1	1	8
B	Temperature	C	30	40	-1	1	35
C	Incubation time	Hour	24	72	-1	1	48
D	Agitation	rpm	50	150	-1	1	100

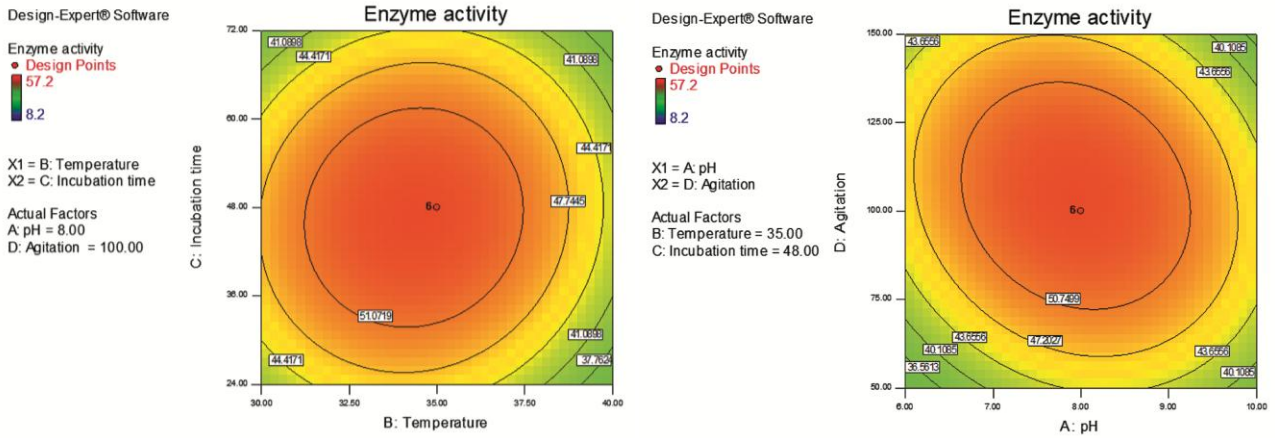

Fig. 8 — Contour plot for the effect of (pH and agitation; temperature and incubation time) on the activity of amylase enzyme

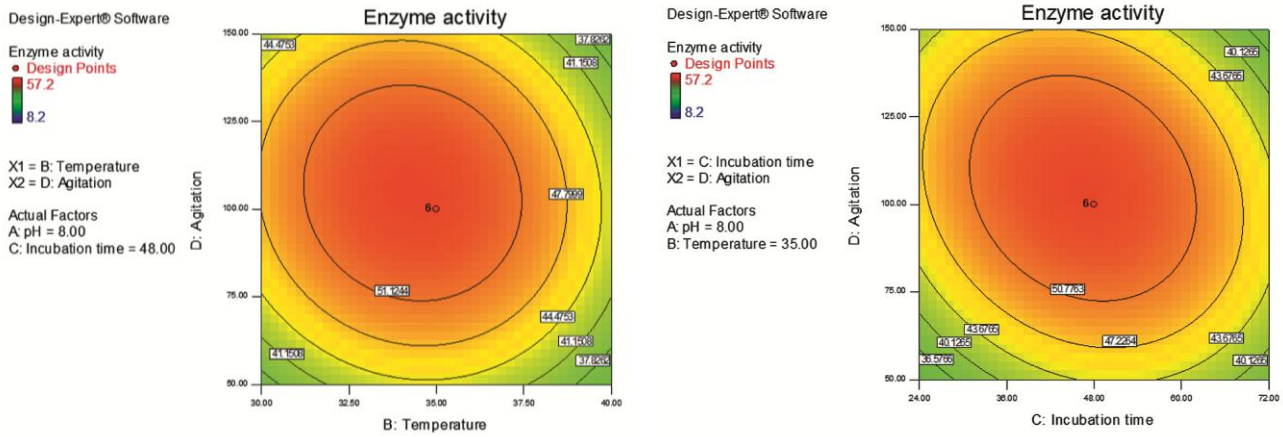

Fig. 9 — Contour plot for the effect of (incubation time and agitation; temperature and agitation) on the activity of amylase enzyme

Table 3 — Actual and predicted enzyme activity for various runs

A: pH	B: Temperature	C: Incubation time	D: Agitation	Enzyme activity	
				Actual	Predicted
				IU/ml	
8	35	48	100	54.2	54.23333
4	35	48	100	22.1	20.685
10	30	72	50	21.5	21.96583
6	30	24	50	8.9	9.699167
8	35	48	100	55.6	54.23333
8	35	48	100	54.3	54.23333
8	35	0	100	20.8	21.145
10	40	72	50	16.4	17.3325
10	40	24	150	16.8	18.04917
10	30	72	150	14.8	15.91583
6	30	24	150	25.7	26.84917
10	30	24	150	32.9	32.5325
8	35	96	100	18.9	17.85167

(*Contd.*)

Table 3 — Actual and predicted enzyme activity for various runs (*Contd*.)

A: pH	B: Temperature	C: Incubation time	D: Agitation	Enzyme activity	
				Actual	Predicted
				IU/ml	
10	30	24	50	28.36	26.2225
8	45	48	100	16.4	15.485
6	30	72	150	24.8	23.2225
10	40	24	50	16.8	16.99917
8	35	48	100	52.9	54.23333
8	35	48	200	22.5	21.96833
6	40	72	150	19.4	23.61917
8	25	48	100	24.1	24.31167
6	40	72	50	25.1	24.08917
6	40	24	50	9.8	10.76583
8	35	48	100	57.2	54.23333
8	35	48	100	51.2	54.23333
6	30	72	50	17.6	18.4325
10	40	72	150	8.2	6.0225
12	35	48	100	18.9	19.61167
8	35	48	0	16.3	16.12833
6	40	24	150	24.5	22.65583

Table 4 — ANOVA for response surface quadratic model

Source	Sum of squares	df	Mean square	F - value	*p*-value Prob > F	
Model	6522.432	14	465.888	102.3942	< 0.0001	significant
A - pH	1.728067	1	1.728067	0.3798	0.5470	
B - Temperature	116.8651	1	116.8651	25.68494	0.0001	
C - Incubation time	16.26907	1	16.26907	3.575663	0.0781	
D - Agitation	51.1584	1	51.1584	11.24374	0.0044	
AB	105.8841	1	105.8841	23.27151	0.0002	
AC	168.7401	1	168.7401	37.08619	< 0.0001	
AD	117.5056	1	117.5056	25.82572	0.0001	
BC	21.0681	1	21.0681	4.630408	0.0481	
BD	27.6676	1	27.6676	6.080865	0.0262	
CD	152.7696	1	152.7696	33.57615	< 0.0001	
A^2	1991.635	1	1991.635	437.7274	< 0.0001	
B^2	2020.958	1	2020.958	444.172	< 0.0001	
C^2	2068.32	1	2068.32	454.5815	< 0.0001	
D^2	2122.259	1	2122.259	466.4362	< 0.0001	
Residual	68.24917	15	4.549944			
Lack of fit	46.59583	10	4.659583	1.075951	0.4987	Not significant
Pure error	21.65333	5	4.330667			
Cor total	6590.682	29				
Std. dev.	2.13306		R^2		0.989645	
Mean	26.56533		Adj R^2		0.97998	
C.V. %	8.029487		Pred R^2		0.954546	
PRESS	299.5728		Adeq Precision		31.96367	

conditions of the experimental value of the enzyme activity was ranging from 8.20. to 57.2 IU/g (Tables 3). The multiple correlation coefficient R^2 (98 %), adjusted R^2 (98 %) and the predicted R^2 (0.95) were in justifiable agreement (Table 4).

Conclusion

Halophilic bacteria (or) halotolerant bacteria are a special group of extremophilic bacteria and are present widespread. They adapt well to the harsh, hypersaline environments and possess enzyme

capacity to cope with salt denaturing effects. Halophiles have the potential to offer major biotechnology opportunities. The findings of this study suggested that the halophilic *Aquabacterium* sp. strain AS02 isolated from the local marine environment could be used for the production of amylase enzyme, and its characterization can enable us to use it for many biotechnological purposes.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

AS - Conceived and designed the analysis, collected the data, contributed data analysis tools, performed the analysis, and manuscript preparation; ML, AVS, PJJC, SS & GN - Conceived and designed the analysis, collected the data, contributed data or analysis tools.

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