Different Types of Enzyme Production Ability by Halophilic Bacteria Isolated from Bay of Bengal Water

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Abstract

Halophiles are excellent sources of enzymes that are not only salt stable but also can withstand and carry out reactions efficiently under extreme conditions. The aim of the study was to isolate and study the diversity among halophilic bacteria producing enzymes of industrial value. Screening of halophiles from various saline habitats of India led to isolation of 108 halophilic bacteria producing industrially important hydrolases (amylases, lipases and proteases). They belonged to moderately halophilic group of bacteria exhibiting salt requirement in the range of 3–20%. There is significant diversity among halophiles from saline habitats of India. Preliminary characterization of crude hydrolases established them to be active and stable under more than one extreme condition of high salt, pH, temperature and presence of organic solvents. It is concluded that these halophilic isolates are not only diverse in phylogeny but also in their enzyme characteristics. Halophilic enzymes are potentially useful in many industries, particularly in food fermentation, pharmaceutical, textile, and leather for the treatment of saline and hypersaline wastewaters. In this study, halophilic bacteria were isolated from saline water. Two isolates were selected on the basis of their colony morphology and gram staining and screened for their ability to produce commercially important hydrolytic enzymes as amylase, protease and lipase. The result show that *Enterobacter soli* strain MP2 and *Enterobacter asburiae* strain are the highest amylase, protease and lipase producing strain. Bacteria were isolated from three hypersaline sites, in solid medium supplemented with various salt concentrations ranging from 0 to 330 g/L. Physical and chemical characteristics of samples from the isolation site were determined to suggest eventual correlations with the occurrence of the halophilic bacteria. Assays on enzymatic activities were performed in submerged cultures in the presence of various salt concentrations and appropriate substrates.

Keywords: *Halophilic Bacteria, Amylase, Protease, Lipase.*

Introduction

The three-fourth part of the earth is consumed by water in which both fresh water and saline water are present and the rest one-fourth is covered by soil in which the soil of coastal areas are saline. The bacterial flora habitat to the saline environment is called Halophilic bacteria. Halophilic bacterial flora is classified into three categories according to their different

environmental salt concentrations as slightly, moderately and extremely halophilic bacteria. The marine water is always considered as an extremely halophilic environment. In such environment the bacterial community is different in their potential and activities from the other bacterial communities because salt can rupture bacterial cell by creating ionic activities and osmotic stress. As in spite of having such environment halophilic bacteria can grow easily, their potentiality can be used in industrial purposes. This research activity is based on the enzyme production by halophilic bacteria. For this experiments, had been done here, sea water sample was collected from the Bay of Bengal of Digha and the saline soil was collected from a grassland of Digha coastal area. Halophilic microorganisms possess stable enzymes that function in very high salinity, an extreme condition that leads to denaturation, aggregation, and precipitation of most other proteins. Halophilic bacteria are efficient source of hydrolytic enzymes which are very essential in biochemical industrial production because they have the potential to fight against any harsh environmental conditions with 100% activity. In biochemical industries some time some such conditions are happened or on behalf of production quantity of some factor are needed to increase which can cause unfavourable environment where production may be effected by degradation of enzymes. In this case the enzymes, derived from halophiles can work smoothly. For this research activity sea water sample was taken from where eight hydrolytic enzyme producing halophilic bacteria can be isolated among them three were amylase producer, four were lipase producer. For the experiments of enzymes production saline water sample was collected from that the crude culture was collected by streak plate method, spread plate method and centrifugation and incubation. For both enrichment and carrying out of the entire experiment the utilized favourable medias are Selective Halophilic Agar Media. In comparison to other groups of extremophiles, and especially the thermophilic and the alkaliphilic prokaryotes that are extensively used for the production of valuable enzymes, the extremely and moderately halophilic microorganisms are to some extent a neglected group when considering the number of their biotechnological applications. This is even more true when considering the great diversity of halophiles: they are found in all three domains of life, Archaea, Bacteria and Eukaryotes and they contain representatives of many different physiological types, adapted to a wide range of salt concentrations up to salt saturation. Halophiles can be use in different industries. The objective is - To study the enzyme production of halophiles. Most of us are unaware of how much diversity there is in the corners of the globe since the beginning of life on earth. Although we know the existence of some organisms, we still do not know their significance. Knowing the unknown, discovering the unknown is science. Scientists are constantly researching the unknown in a perfect way. There is no limit to diversity in the world. Just as they can survive in very hot water, many organisms are born in ice and that is their favourable place. A simple definition of extreme environments is that they are hostile, or even lethal, for most living creatures. The organism which are grow in this environment are known as extremophiles. Extremophiles are just not only survive in extreme conditions but they prefer this environment for their living. Enzymes, membranes, and nucleic acids can be adapted to perform their functions under very different conditions, the creature becomes an extremist. Most extremists are confined to extreme environments because the adaptation process usually reduces their ability to grow normally. Earth environment changes continuously till now, extremophiles has been forced to be that to survive and adapt in this extreme conditions in every condition in every corner of the earth. We do not know how many different organisms are born in salt water and how many of them have significance. Screening of new source and it‘s identification is the key of research. Saline ecosystem offers a habitat for organisms that can adapt to survive in a adverse environment, in

highly or moderately salt concentration. These organisms are called halophilic or salt tolerant. Halophile is a Greek derived word in which halo means salt and phyllo's means loving . Halophilic organisms are salt loving organisms that thrive well in various ranges of salt concentrations They live around comfortably in this harsh condition. These halophilic bacteria are categories in three group according to their optimum growth. Slight halophiles, are those bacteria that can survive in two to five percent salt or NaCl concentration. Moderate halophiles are live in 5- 20 %, of NaCl concentration and extreme halophiles are comfortable to live in 20-30% of NaCl concentration. Any organism living in a hypersaline environment must have a number of environmental stressors related to the high ionic concentration and the presence of high amount of electrolyte in the medium creates various effects. First, there is a decrease in water activity. Salinity in this case similar to desiccation and explains it, for example, the similarity between plants from desert and saline environments. Salt, on the other hand, is the normal level of cytoplasmic solution containing efficient osmolytes and cells forming an osmotic imbalance which allows the lipid membrane to expand rapidly most other biological molecules of water molecules inside and outside the cell while presenting an effective barrier. This situation implies the loss of water and an irreversible retraction of the cytoplasm. In addition, an organism adapting to life in hypersaline habitats, in which salt concentrations change over wide ranges, must be able to function over those ranges. In other words, the capacity for osmoregulation is an important characteristic of inhabitants of hypersaline environments. As with other environmental variables the ability to grow at different salt concentrations is varied in microorganisms. Most freshwater and marine habitat microorganisms are rather stenohaline, the sharpest growth around them is the best in natural salt content. However, some creatures do not have a normal habitat. Hypersaline can tolerate relatively high salt concentrations, although the growth rate decreases. Some halotolerant microorganisms can grow in high salt concentrations, and are often found in hypersaline habitats. They tolerate hypersaline concentration but they are still negatively affected by salt. Their orientation is incorporated. Hydrolase forms a class of enzymes widely distributed in nature Eukaryotes superior to bacteria. Halotolerance of many enzymes derived from halophilic Bacteria can be used wherever enzymatic transformation is needed to work Under physical and chemical conditions, such as in the presence of organic solvents and Extremes of temperature and salt content. In recent years, various screening programs The novel was performed in a saline habitat to isolate and characterize enzymatic Activation with conventional enzymes with different properties. Contains halophilic enzymes known as Extremozymes produced by halophilic microorganisms They have identical enzymatic properties similar to non-halophilic replicas, but they exhibit different properties. Mainly in structure. The following is a review of various outreach to communication Hydrolytic enzymes have been created from halophilic microorganisms and their economic values with the basic properties of protein chemistry in media with high concentrations of sodium chloride and other salts. The goal of the study was to isolate and study diversity Among the halophilic bacteria that produce enzymes of industrial value. There are significant variations in the halophile from the saline habitat of India. Its initial characterization Crude hydrolase have established themselves as active and stable in multiple extremes Salt, pH, temperature and presence of organic solvent. It is concluded that these are not halophilic isolates Varied not only in phylogeny but also in the properties of their enzymes. Solvent stability amid Halophilic enzymes seem to have a generic fancy feature that makes them potentially useful in non-aquatic fields Enzymology.

Materials And Methods

Collection Of Samples

For the isolation of halophilic bacteria the water sample was taken from Bay of Bengal at Digha costal area.

Isolation Of Enzyme Producing Halophilic Bacteria

The collected sea water sample was inoculated on mineral salt agar media by streak plate method where pH was kept 7.4 to 7.6 and the plates were kept for incubation at room temperature during 10 to 15 days. After the incubation period many bacterial colonies were detected from where they were reinoculated into halophilic broth. This is the pure culture of halophilic bacteria collected from sea water and it was preserved at -18°C temperature.

Screening Of Hydrolase Activity

For the screening of hydrolase activity of the isolated pure culture plate assay was done where the plate were prepared with starch for the identification of amylase and tributyrin agar for the identification of lipase production.

Starch nutrient agar media was prepared for the screening of amylase activity because starch, a polysaccharide is composed of amylose and amylopectine which were degraded by amylase enzyme. Then the pure culture was inoculated by applying streak plate method and kept for incubation at 30°C for 48h. After the incubation period clear zones were detected by adding iodine solution(0.2% iodine, 0.4% KI, 100 ml H2O).

For the screening of proteolytic activity the pure culture was inoculated on SM agar media because SM agar contains casein, a type of proteins that is break down by protease enzyme. The pure culture was inoculated by streak plate method and incubated at 30°C for 7 days. After the incubation the clear zones were detected.

The bacterial cultures were inoculated onto tributyrin agar medium and the plates were incubated at 40°C, for 24hrs. Isolates that showed clear zones of hydrolysis were considered as lipase producing bacteria.

Enzyme Assay

The halophilic bacteria with hydrolytic enzyme activity were isolated as zone of clearence. For getting further conformation this quantitative enzyme assay was done in broth medium. For this assay mineral salt broth media were prepared where the isolates were seeded. They were kept for incubation at 37°C for 48h. After this incubation period the cultures were centrifuged at 7000 rpm for 20min at 4°C by which the microbial cells remained as pellet and their produced enzymes were seperated that remained in the cell free supernatant which were collected.

Amylase Assay

In case of amylase activity DNSA (Dinitrosalicylic acid) is used where starch is used as substrate. In this method 30microgram of supernatant was mixed with 0.3ml of 1% starch solution. Then the reaction mixture was left for incubation at 37°C during 30min. After that 3ml of DNSA reagent was added that indicates the stop of the enzymatic reaction and the reaction mixture was allowed for water bath for 10min and cooled at normal room temperature. Then its absorbance was taken at 540nm. The quantity of amylase was determined as 1unit of amylase is equivalent to the 1microgram of maltose that was liberated by the action of amylase per minute.

Protease Assay

In case of protease activity assay the reaction mixture was prepared by adding 30 microgram of supernatant and 2ml of 1% insoluble casein solution in a buffer containing 60mM Tris Cl, and

200mM NaCl with 8.3 pH value where casein is the substrate. Then the isolate was added and the mixture was left for incubation for 30min at 37°C. Then 3.5ml of 5% trichloroacetic acid (TCA) was added to stop the reaction. After that this mixture was centrifuged at 8000 rpm for 5min. The supernatant was collected and quantified by Lowery method where absorbance was measured at 650nm by keeping tyrosine as standard. The quantity of protease was determined as 1unit of protease is equivalent to the 1microgram of tyrosine per mL of the reaction mixture, liberated in per minute of the reaction.

Lipase Assay

Among 1-6% (v/v) tributyrin, Halomonas salina grows maximally at 4% and secretes maximum lipase at 3% substrate concentration. Tributyrin is an inducer for lipase production.

In turn, extracellular lipase production by *Rhodotorula glutinis* was maximum in the medium containing olive oil at a concentration of 2%.

Extremophilic microorganisms have been widely explored industrially and biotechnologically for its valuable products. Halophilic microorganisms can secrete salt and thermo-tolerant enzymes protease, lipase, amylase etc.

Optimization of Medium for lipase production

Optimization of salt

Optimization of salt for optimum enzyme production was carried out by varying NaCl concentration in liquid media. NaCl concentration was maintained . After incubation period, growth and enzyme production was monitored.

Optimization of pH

Optimization of pH for optimum enzyme production was carried out by varying pH (adjusted by adding 1N HCl or 20% Na2Co3) concentration in liquid media.

Optimization of Temperature

Temperature was optimized by incubating inoculated media at different temperature.

Optimization of Substrate concentration

Substrate concentration optimization was carried out by inoculating organisms into medium with different substrate (Tributyrin) concentration. All the flasks were incubated in shaking condition followed by measurement of growth and enzyme activity.

Results And Discussion

Halophilic microorganisms are economically important because it produces several bioactive compounds which are useful for many industrial applications. Among the halophilic microbes, the halophilic bacterial forms are typically known for its secondary metabolites such as proteins, amino acids, pigments and enzymes.

Isolation Of Halophilic Bacteria

In this study total two bacteria are isolated from the saline water sample and they are *Enterobacter soli* strain MP2 and *Enterobacter asburiae* strain

Gram Staining

All the 2 isolates were subjected to Gram staining; their results showed that most of the isolates were Gram-positive rod-shaped bacilli.

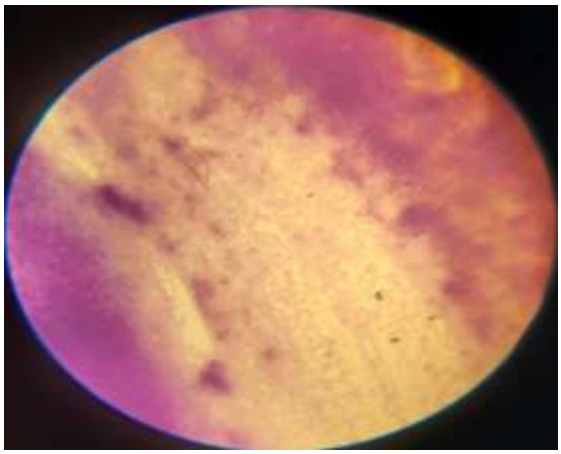

Fig 1: Gram staining of halophilic bacteria

Enzyme Assay

From the sea water sample total two halophilic bacteria could be isolated in which *Enterobacter soli* strain MP2 was detected as amylase producer and other as protease producer.

Amylase Test

The eight selected isolates were screened for their ability towards amylase enzyme production by starch hydrolysis method. The present study showed that the isolates namely *Enterobacter soli* strain MP2 was found to have clear zone in starch agar medium which indicates their amylase production. At the same time, isolates *Enterobacter asburiae* strain did not show any zone in the same medium.

Fig 2: Amylase production activity of halophilic bacteria

Table 1: Gram nature

Name of Isolates	Gram Nature
Enterobacter soli strain MP2	Gram positive
Enterobacter asburiae strain	Gram negative

PROTEASE TEST

After incubation, the isolates such as *Enterobacter asburiae* strain showed protease activity in the medium by a zone formation. Halophilic and halotolerant bacteria produce several commercially important enzymes like amylase, protease and lipases. In the present study, all the selected ten isolates were screened for their ability towards amylase and protease production.

Fig 3: Protease production activity of halophilic bacteria

Lipase Test

After enrichment and pure culture isolation, twenty-four moderate halophiles were obtained from excreta of wild Ass and designated. All the isolates were screened on tributyrin agar media and on the basis of zone-ratio.

Fig 4: Lipase production activity of halophilic bacteria

Media optimization

Optimization of media is important at industrial scale to improve the efficiency of the process without increasing the cost . Media optimization is necessary practices to use available resources maximally.

Effect of salt

Halomonas salina shows maximum biomass production at 10% NaCl and maximum lipase at 14% NaCl. Higher or lower salt affects extracellular lipase secretion. This optimum salt concentration for *Halomonas salina* is not compatible with optimum salt requirement by Bacillus for lipase secretion .

Effect of pH

Halomonas salina secretes maximum lipase at pH 5 while maximum growth was observed at pH 7. Alkaline pH found to be unsuitable for growth and **e**nzyme secretion . Halophilic produced maximum lipase after 7 days culture using olive oil .Lipase production was maximum at pH 7 .

Temperature optimization

Among the range of temperature tested, *Halomonas salina* grows maximally at 40˚C temperature and secrete maximum lipase at 30-40˚C affects growth and enzyme. Above optimum temperature is lower as compare to *Bacillus sp.*

Optimization of tributyrin

Among 1-6% (v/v) tributyrin, *Halomonas salina* grows maximally at 4% and secretes maximum lipase at 3% substrate concentration. Tributyrin is an inducer for lipase production.

Conclusion

From this above experiment it can be concluded that halophilic bacteria is a good and beneficial source of hydrolytic enzymes. Both moderated halophilic bacteria and extreme halophilic bacteria are very beneficial source of industrially essential enzymes but moderated halophilic bacteria get more importance that of extreme halophilic bacteria because in case of culturing extreme halophilic bacteria, extreme environment have to be created that is cost effective in many times and in industrial production extreme conditions are created very rare. There is no place in the earth that can be found as microbes free. So the microbial community can be used no doubtly in industrial fields. In industrial production amylase, protease and lipase are three of the most important and useful enzymes.

Amylase is needed to producing some products, derivative of starch. Starch is a polysaccharide, composed with amylose, a linear chain of glucose with α-1,4 linkages and amylopectine, also linear chain of glucose with both α-1,4 and α-1,6 linkages. Protease is used to producing proteinatious compound which break down protein and lipid molecules. And lipase enzyme is essential for degaration of fat. Although people have discovered many things of the world, till now so many things are remain as u discovered. So to for discovering the usefulness of microbial community more research work is needed. As the prevalence of antimicrobial resistance increases, researchers are developing new technologies and strategies to find alternatives that reduce the morbidity and mortality caused by the MDR bacteria. The current and future of natural product discovery is the application of a combination of multi-omics approaches. Depending on the phase of the study, it is foreseen genomics, metagenomics, transcriptomics, proteomics, and metabolomics to reveal the biosynthetic capabilities of a single microorganism or microbial communities in hypersaline environments.

The general features of halophilic microorganisms are the low nutritional requirements and resistance to high concentrations of salt with the capacity to balance the osmotic pressure of the environment.

Different halotolerant and moderate halophilic bacterial cultures were isolated from water.Based on the studied phenotypic characteristics, all of them were found to be diverse. *Bacillus licheniformis* grew in the presence of 15% NaCl concentration and showed polyenzyme production potential under the experimental conditions. Hence, it is the organism of choice, which can be used to produce these enzymes in saline conditions. From application point of view, this study provides useful information about halotolerant bacteria prevailing in the coastal ecosystem having potential industrial significance.

Future Aspects

As the prevalence of antimicrobial resistance increases, researchers are developing new technologies and strategies to find alternatives that reduce the morbidity and mortality caused by the MDR bacteria. The current and future of natural product discovery is the application of a combination of multi-omics approaches. Depending on the phase of the study, it is foreseen genomics, metagenomics, transcriptomics, proteomics, and metabolomics to reveal the biosynthetic capabilities of a single microorganism or microbial communities in hypersaline environments. Nowadays everything that we use in daily life most of this are synthetic or artificial. Altogether we know the impact of using this artificial product we forgot it carelessly. Taking fresh and organic food is absolutely necessary, artificial colour may cause severe disease like cancer too. Halophilic source of pigment can be use industrially for food colouring agents should be a good step toward the health purpose. Be healthy mentally and physically is very important.

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