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RESEARCH ARTICLE

Cloning and expression of α-amylase producing gene from *S. aureus* into *E. coli*

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Abstract

Among the top commercial enzymes utilized for industrial applications, amylases are the most widely used and accepted. The aim of present work was to isolate amylase producing bacteria from the soil samples collected from area around the plant roots. Bacterial species were isolated by serial dilution and cultured on the nutrient agar medium plates. The isolated bacteria were evaluated for the amylase activity using starch hydrolysis test. The bacterial isolate with maximum hydrolytic activity was considered for further study. Based on the morphology and biochemical tests, the species was confirmed to be *Staphylococcus aureus*. Amylase of *S. aureus* was amplified using PCR with the help of specific primers; and a PCR product of approximately 1.9 kb was obtained. This was cloned into pUC 18 vector which was transformed into competent *E. coli* DH5α cells. The ability of transformed bacteria to grow on agar plates with ampicillin confirmed successful transformation. Furthermore, these transformed bacteria were able to produced active amylase that is revealed by starch hydrolysis test. Thus *S. aureus* amylase gene was successfully cloned into *E. coli*..

Introduction:-

For industrial use, the enzymes stable during extreme conditions such as pH and temperature are usually accepted since majority of enzymes are degraded at temperature more than 55-60°C. α-Amylase is one of the best thermostable enzyme whose protein structure allows to preserve its activity at high temperatures. Therefore, they are widely used in starch industry. Among various extracellular enzymes, α-amylase ranks first in terms of commercial use (Babu and Satyanarayana, 1993) and commands 12% of world market (Baysal *et al*., 2003). α-Amylase (E.C.3.2.1.1) is a hydrolase that catalyzes the hydrolysis of internal α-1, 4-glycosidic linkages of starch to yield glucose and maltose. It is a calcium metalloenzyme that is dependent on a metal co-factor and calcium for its activity. There are two types of hydrolases: endo-hydrolases that act on the interior whereas exo-hydrolases that act on terminal non-reducing ends of the substrate (Gupta *et al*., 2003).

Amylases from fungal and bacterial sources have many commercial applications, particularly in the food and detergent industries. The amylases from halophilic bacteria have optimal activities in high salt containing medium; and therefore, preferred for industrial use. For large scale industrial use, amylases are extracted from bacterial species *Bacillus*. They are of special interest for biotechnological processes owing to their thermostable property and ability to be expressed at high levels (Ali *et al*., 1998).

Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis, and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-amylase, and have widely been used for commercial production of the enzyme for various applications (Prakash *et al.*, 2009)**.**

S. aureus can colonize and survive in a wide variety of environmental niches. It is capable of causing a range of mild to life-threatening conditions including septicemia, meningitis, toxic shock syndrome, food poisoning, and skin abscesses. It colonizes tissues using a repertoire of virulence factors including cell surface-associated protein and extracellular proteins (Greene *et al*., 1995)**.** *S. aureus,* one of the prominent Gram-positive human pathogens, secretes many surface and secretary proteins including various enzymes and pathogenic factors that enable them to colonize and infect host tissue. α-Amylase of *S. aureus* catalyzes the breakdown complex sugars into monosaccharides, which are required for the bacterial colonization and survival (Lakshmi *et al*., 2013).

An α-amylase gene from *S. bovis 148* was cloned into *Escherichia coli* MC1061 (Satoh *et al.,* 1990). The α-amylase produced by the recombinant *E. coli* strain showed similar raw starch-hydrolyzing capability that of the α-amylase of *S. bovis* 148. Recently, an additional α-amylase gene of *S. bovis* was cloned into *E. coli* MC1061 which seemed to code an intracellular α-amylase of this strain (Satoh *et al*., 1993).

The screening for a single amylase is difficult because of production of multiple amylases with different specificities or very low amount of amylase by a single strain. Thus the cloning of a gene that expresses the desired amylase in a well-characterized host like *E. coli* or *B. subtilis* greatly helps in the characterization of new amylases and also in significantly increasing the yield (Ohdan *et al*., 1999; Haddaoui *et al*., 1999).

This study was aimed to clone an α-amylase of *S. aureus* isolated from soil into *E. coli.*

Materials and Methods:-

Chemicals and Media - All the bacterial culture media used were from HiMedia, India. The chemicals were from Merck. Restriction endonucleases were purchased from Sigma-Aldrich, St. Louis, USA and used as recommended by the manufacturers.

Isolation of *S. aureus* and its culture maintenance:-

The soil samples were collected from Indira Nagar region of Lucknow, India. Serial dilutions up to 10^{-8}-10^{-7} fold of the soil samples were made and cultured on nutrient agar plates and incubated at 37 ℃ for 24 h. The bacterial colonies were tested for amylase producing capability using starch hydrolysis test. The bacteria positive for starch hydrolysis test were characterized by their morphological and biochemical characteristics. The bacterium was identified according to the standard key of Bergey's manual of Determinative Bacteriology (Holt *et al.,* 1994).

Culture preparation and growth conditions:-

E. coli and *S aureus* cultures were maintained in Luria-Bertani (LB) medium, pH 7.2 incubated at 37 °C. The bacteria were subcultured and maintained on agar plates for further use as inoculum for DNA isolation.

DNA extraction and quantification:-

DNA was extracted from bacteria positive for amylase production that were grown in the culture broth (Nutrient broth), using phenol:chloroform, Tris HCl, Sodium dodecyl Sulphate, Tis-EDTA. The DNA was separated on 0.8% agarose gel by electrophoresis and its size was estimated using the DNA marker of GeneRuler™1 kb. The isolated DNA was quantified using UV spectrophotometer.

Amylase gene amplification and cloning:-

The α-amylase gene from *S. aureus* chromosomal DNA was amplified by PCR using following primers specific for the α-amylase:

Forward: 5'-CATGAATAAGCAATGG-3' Reverse: 5'- TTAATTTAGTTCGAT-3'

The PCR reactions were performed using a 15 µL reaction mixture containing 10X Taq DNA polymerase buffer with 2 mM $MgCl_2$, 2.5 mM dNTPs, 5 µM primers, 4 µL Taq DNA polymerase, 30 to 40 ng DNA, and sterile distilled water. The temperature cycles used for amplification include an initial denaturation step for 3 min at 94 °C; 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 56 ºC and amplification for 1 min at 72 °C followed by a final extension step at 72 °C for 5 min in a gradient Thermocycler. The PCR amplified products were stored at 4 ºC until further use. The PCR amplified products were separated by electrophoresis on 1.2% agarose gel with 1X TAE buffer containing ethidium bromide (EtBr). Amylase gene was digested with *Eco*RI enzyme and cloned into

the pUC 18 plasmid using T4 DNA ligase and transformed into *E. coli* DH5α obtained from CytoGene Research Laboratory.

Competent cell preparation and transformation:-

The isolated colonies of *E. coli* were inoculated in the Erlenmeyer flask containing LB broth and were incubated overnight at 37 ^{o}C. The overnight grown culture was then centrifuged in Eppendorf tubes and treated with 0.1 M calcium chloride and 40% glycerol. The competent cells prepared were stored at 4 ^{o}C. The ligated vector (pUC 18) containing amylase insert was transformed into the competent cell (*E. coli* DH5α) by heat shock treatment. For selection, the transformed bacteria were grown on ampicillin (100µg/mL) containing LB media.

Results and discussion:-

The bacteria isolated from soil by serial dilution were screened for amylase producing capability on starch media. The isolates with cream colored colonies showed positive catalase activity and starch hydrolysis. They were Gram-positive and coccus shaped. The clear zone was formed due to hydrolysis of starch around the bacterial colonies grown on iodine stained agar plates; this confirmed the amylase activity of the bacterial strain. These strains were considered as amylase producing; and after characterizing, *S. aureus* was used for further study.

Table 1: Morphological and biochemical test results of the isolate

S.No.	Biochemical test	Result
1	Cell wall	Gram +ve
2	Morphology	Coccus
3	MR/VP	+/–
4	Catalase	+
5	Citrate utilization	+
6	Oxidase	–
7	Motility test	+
8	Indole test	–

(* + Positive and – Negative)

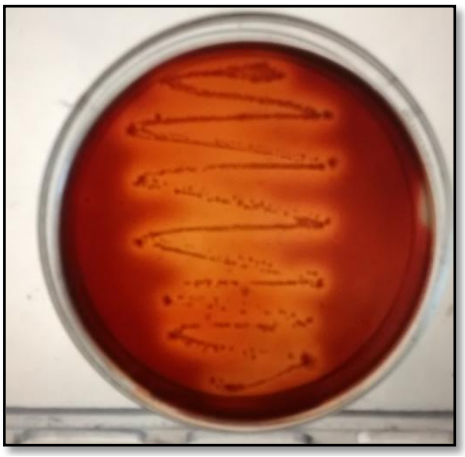


Figure 1: Starch hydrolyzing ability of isolated *S. aureus*

The identified bacterial species was cultured in nutrient agar medium and genomic DNA was extracted and separated on 0.8% agarose gel. The size of genomic DNA corresponds *S. aureus* (Figure 1a). The DNA yield was found to be very good with average concentration of the isolated DNA as 20µg/µL.Using this genomic DNA as a template, with the help of specific primers directed at amylase gene was amplified by PCR. The size of the 1983 bp long PCR product after separation on a 1.2% agarose gel confirmed the amplification of the amylase gene (Figure 1b).

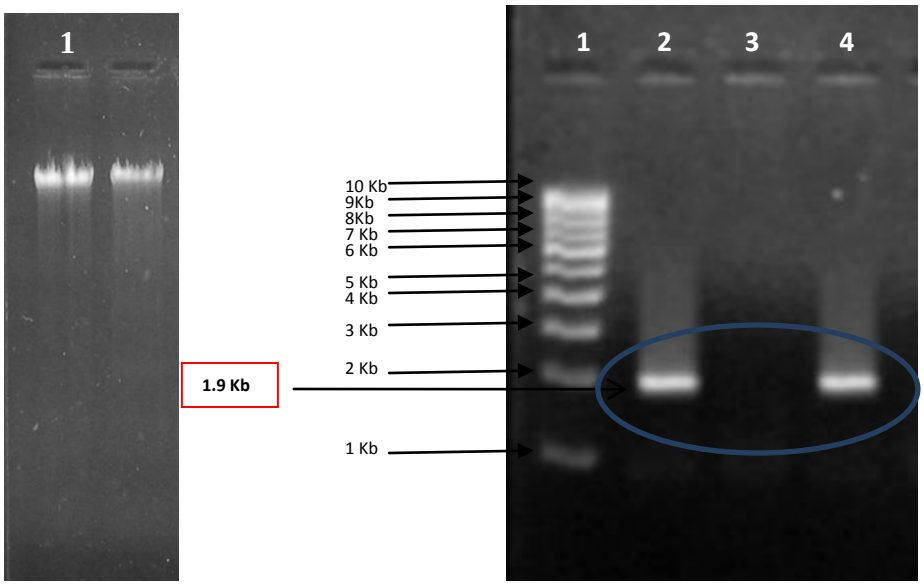
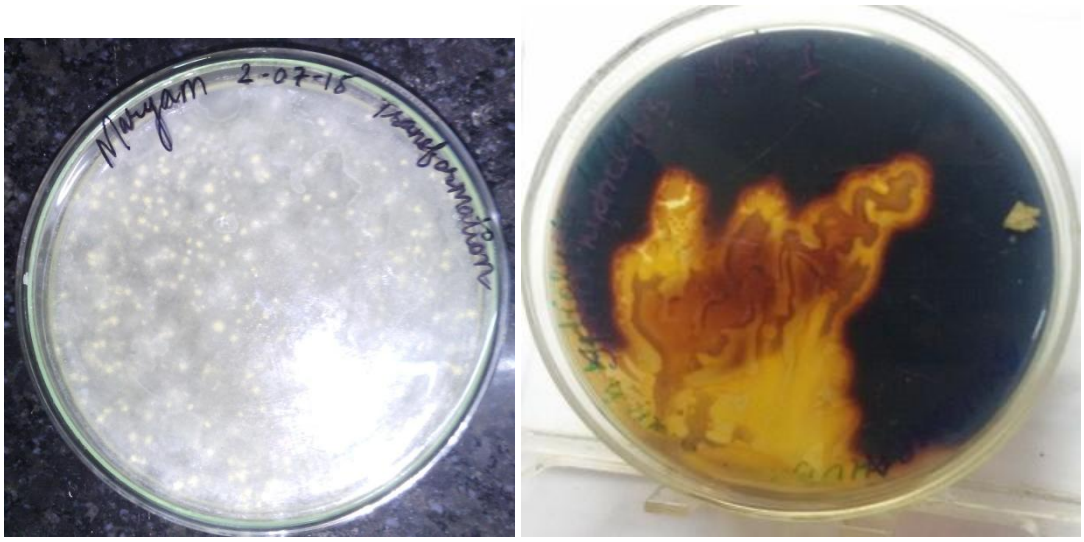


Figure 2: Amylase gene cloning from *Staphylococcus aureus* into *E. coli*; **a.** 0.8% agarose gel, showing genomic DNA isolated from *S. aureus*; Lane 1 and 2 bacterial genomic samples **b.** PCR amplified amylase gene of *S. aureus* separated on 1.2% agarose gel; Lane 1: 1 Kb marker, Lane 2 and 4 – PCR amplified product

The amplified product was then digested using *Eco*RI enzyme and inserted into pUC 18 vector with the help of T4 DNA ligase. The host cells, *E. coli* DH5α, were treated with calcium chloride to make competent. The competent *E. coli* DH5α cells were transformed with pUC vector carrying the *S. aureus* amylase gene using heat-shock method. In order to identify transformant carrying *S. aureus* amylase gene, the transformed *E. coli* cells were plated on agar plates supplemented with ampicillin. The growth of colonies on ampicillin supplemented agar plates after overnight incubation at 37 ºC indicated successful transformation of *E. coli* (Figure 3a).

Furthermore, the successfully transformed *E. coli* cells were analyzed for their starch hydrolytic activity by growing them on fresh media containing starch as a sole source of carbohydrate (Starch agar plates). After 24 h of incubation at 37 ºC, the plates were stained with iodine to reveal starch hydrolysis. The clear zone formed around bacterial colonies indicates presence of active amylase in the bacteria confirming a successful cloning.



(a) *(b)*

Figure 3: Transformed cells incubated overnight at 37 °C **(a)** Transformed *E. coli* grown on agar plates supplemented with ampicillin (100 µg/mL) **(b)** Starch hydrolysis test confirming active amylase producing capability of the transformed *E. coli*

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Conclusion:-

In this study, we have successfully cloned amylase of *S. aureus* into *E. coli.* The amylase produced by cloned bacteria possesses hydrolytic activity and was able to hydrolyze starch. In future, commercial use of these cloned bacteria would be evaluated.

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