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MOLECULAR CLONING OF AN α-AMYLASE GENE FROM BACILLUS subtilis AND IT'S EXPRESSION ON E.coli

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Abstract: Amylase are among the most important enzymes and are of great significant for the field of food industry, constituting a class of industrial enzymes having approximately 25% of the world enzyme market. Alpha-amylase have potential application in a wide no. of industrial process such as food, fermentation, textile, paper, detergent and pharmaceutical industries. In this present study we have isolated alpha-amylase producing gene (Alf A gene) from Bacillus subtilis ATCC6051, then this Alf A gene is ligated in T&A plasmid then these plasmid are used in transformation. The transformed bacteria are selected by antibiotic containing plate. These transformed bacteria are checked for the production of α-amylase enzyme by starch hydrolysis test.

***Keywords* – α-amylase, Fermentation, Transformation, cloning.**

INTRODUCTION:

Alpha-amylases are digestive enzymes which hydrolyze glycosidic bond bonds in starch to glucose, maltose, maltotriose and dextrin's. They have a wide range of application in industries such as food, baking, brewing, starch processing, textiles, paper and detergent as representing approximately 25% of the world enzyme production. Alpha-amylase are also used in different fields such as clinical, medicinal and analytical chemistry.

Alpha-amylase belongs to a family of endo-amylases that are found in microorganism, plants and higher organisms. it catalyzes the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of alpha-D-(1-4) glycosidic bond and the end product of configuration is dextrin.

The production of α-amylase by submerged fermentation (SmF) and solid state fermentation (SSF) depends upon different physicochemical factors. SmF is a traditional method used in production of industrial important enzymes for its easy control, where SSF system appear promising due to its natural potential and advantages. As SSF resembles with natural habitat of microorganism, lower capital investment, simpler technique, better product recovery and lack of foam build up, it is preferred for microorganism production.

In recent developments of biotechnology, we can use it in isolation of the alpha-amylase producing gene from Bacillus subtilis ATCC6051, then this Alf A gene is ligated in T &A plasmid then these plasmid are used in transformation. Then these transformed bacteria are selected by screening test and then checked for the production of α-amylase enzyme by starch hydrolysis test.

Numan Ozacan and Adem Altinalann studied that Bacillus subtilisRSKK246 was found to produced alpha-amylase enzyme, the α-amylase producing gene was isolated and insert into pUC18 and pUB110 plasmid for transfer into different Bacillus subtilis. Where the alpha-amylase producing gene was cloned and expressed with its own promoter and this promoter sequence seemed to be function in E.coli and in B. subtilis strains.

A resident plasmid cloning system was developed by Stephen A. Ortlepp, James F. Ollington and David J. McConnel for B. subtilis has been used to isolate recombinant plasmid DNA from B. lichenformis which confer alpha amylase activity. The plasmid contain 3550bp insert at EcoRI site of plasmid pBD64, the DNA was cloned and the evidence of that cloned fragment codes for B. licheniformis sheat-stable alpha amylase with temperature of 93°C and the foreign gene expressed efficiently.

In industrial application alpha amylase enzymes are used in a wide range such as, in starch industry it is use for convert starch into fructose and glucose syrup, in detergent industries used for enhanced the ability, in fuel alcohol industries for the conversion of starch into sugar, in food industry for baking, brewing and production of cake etc. and in textile industries for designing process.

Materials and methods:

1. Preparation of media:

Bacteria stains and plasmid used in cloning of α-amylase gene, B. subtilis strain from our collection of desired gene. Bacteria are growth in L broth which are laterally will be used as a host for the cloning of an alpha amylase enzyme producing gene.

2. Isolation of plasmid DNA from Bacteria (Bacillus subtilis):

Reagents:

1. TE Buffer (PH-8):10mm trisHCL, 1mmEDTA.
2. Solution-1: 1M Tris Base, 20 Mm EDTA, glucose.
3. Solution-2 (Lysis Buffer): 0.2 N NaOH, 1% SDS.
4. Solution-3 (Neutralizing Buffer): 3M Sodium Acetate, 5M Glacial acetic acid.
5. Isopropanol.

Procedure:

Take 1.5 ml of bacterial culture and centrifuge (8000rpm) it. Discard supernatant, add 100µl of solution-1, mix and incubate at room temperature for 5 min. add 100µl of solution-2 and mix by inverting the tube, incubate at room temp. and add 150µl solution-3, mix by inverting and incubate on ice for 10 min. centrifuge at 10,000 rpm for 10 min and transfer the supernatant to a fresh tube. Add 200µl ice cold isopropanol, mix and incubate at room temp. for 30 min and centrifuge (10000rpm) .Discard the supernatant, air dry the palette and add 1X TE Buffer. The extra salt could be removed by 70% ethanol wash before air dry.

3. Gel Electrophoresis:

It is a technique to quickly visualization of the DNA. The method use a gel electrophoresis tank with an external power supply, analytical-grade agarose, an appropriate running buffer and a dye along with appropriate sized DNA strand. Isolated DNA is loaded into well of agarose gel and then exposed to an electric field. DNA fragments are separated according to their size. After run the gel should be placed on the UV-trans illuminator to visualize the DNA and check for orange colored bands in the gel. After visualization of DNA bands, the DNA bands are isolated. Cut the desired DNA band and transfer to a microfuge tube. Add elution buffer, freeze it for 15min then melt the slice by incubating at 65℃. Add equal volume of phenol, centrifuge at 12000rpm for 10min. Transfer the aqueous layer into a new tube with ice cold isopropanol, freeze for 30 min, centrifuge at 5,000rpm and discard the supernatant. Wash with 70% ethanol and preserved with 1X TE buffer.

4. Restriction digestion of bacteria DNA by restriction endonuclease:

10X restriction buffer (20µl), sample DNA (2µl), Distilled water and restriction endonuclease (1µl), take in a Eppendorf tube and incubate at 37℃ for 1-4 hr. then again incubate at 65℃ for 20 min. store it at -4℃ after incubation.

5. Preparation of host cell bacteria:

A bacterial colony was taken in 100ml of LB broth and incubate at room temperature for 3 hour. Transfer 0.5 ml E, coli bacterial cell to another flask contain 50ml LB broth, store it at 0℃ for 30min. Recover cell by centrifuging at 8,000 rpm. Decant the media from the cell pellet and re-suspend the cell in 25ml of 100mM of ice cold CaCl2 solution. Centrifuge at 8,000rpm and discard the supernatant, add 2.5ml of ice cold 10 mM CaCl2 by gently vortexing.

6. Ligation of restricted fragment into plasmid:

Take 10X ligase buffer, DNA, 10mM ATP, T4 DNA ligase, Distilled water in a microfuge tube (vial) total reaction volume 20µl. Kept the vial in micro centrifuge and spin for few second, incubate at 32℃ for 2 hours. The ligate mixture taken for doing transformation.

7. Polymerase Chain Reaction:

The technique for amplification of a specific fragment of DNA of interest by a series of successive cycles. The cycle involves, denaturation of DNA (94-95℃, the double stranded DNA are separated and form single stranded), Annealing (binding of the primer to the template), Extension (72℃ the addition of nucleotides in direction of template take place) . The cycle repeated to produce a number of copy of interested DNA.

8. Transformation of host cell:

In the process of transformation, the component cells are incubated with the DNA in ice. Then it is placed in a water bath at 42 degree Celsius and further plunging them in ice. This process will take up the DNA into the bacterial cell. Then it is plated in an agar plate containing appropriate antibiotics.

Procedure: Transform the CaCl2 treated cells directly transfer 100µl of competent cells to a sterile, chilled polypropylene tube using a chilled micro pipette tips. 10µl of DNA added, mixed well and keep it in ice for 30min. placed the tube in pre-heating water bath at 42℃ for 90 sec. Transfer the tube immediately into ice for 1-2 min. add 500µl LB broth and incubate at 37℃ for 45 min. Add 40µl of X-Gal and 7µl of IUPTG to the LA-amp plate, then appropriate volume of transformed competent cell into the plate and spread them uniformly & allow liquid to be absorbed. Invert the plate and incubate it at 37℃, after 12 hour colonies will appear.

9. Confirmation test of cloning (starch hydrolysis test):

After preparation of growth media for bacterial growth, mix nutrient agar with starch and pour on a petri plate. After solidify of the starch media inoculate bacteria over starch plate by streaking. Incubate the plate for one day in incubator and placed few drops of iodine on plate in next day. Observed the changing in color near the streak area.

RESULT:

Result-1:-

Plasmid DNA are isolated successfully from bacteria and treated with restriction enzymes. After gel electrophoresis DNA strands are visible and could be again isolated from the agarose gel.

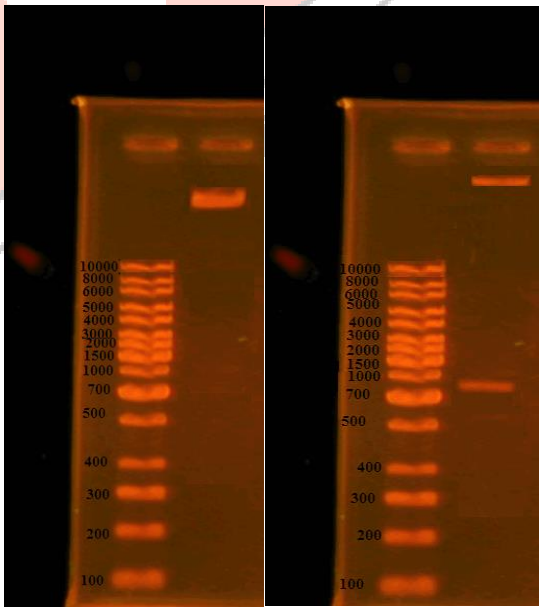


Figure showing bands of DNA of bacterial plasmid under UV –transilluminator with the help of agarose gel electrophoresis.

Result-2:- competent cell preparation

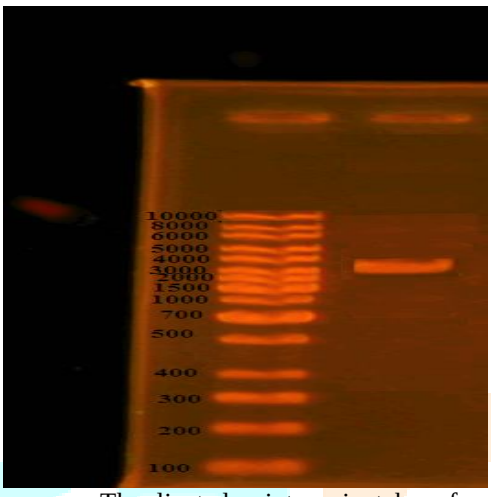
At this point, either use the cells directly for transformation or dispense into aliquots and freeze at -80^{0}c.

Result-3:- PCR

Withdraw a sample (5-10 micro lt.) from the test reaction mixture and the four control reactions, analyze them by electrophoresis through an agarose gel and stain the gel with ethidium bromide to visualize the DNA.

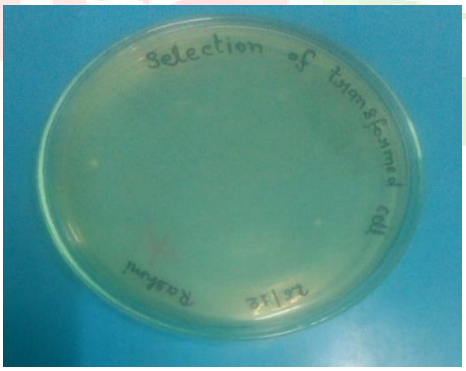
A successful amplification reaction should yield a readily visible DNA fragment of the expected size. The identity of the band can be confirmed by the DNA sequencing, Southern hybridization and restriction mapping. If all is well, lanes of the gel containing sample of two positive controls and the template DNA under test should contain a prominent bands of DNA of the appropriate molecular weight. This bands should be absent from the lanes containing samples of the negative controls.

Result-4:- Ligation of the DNA



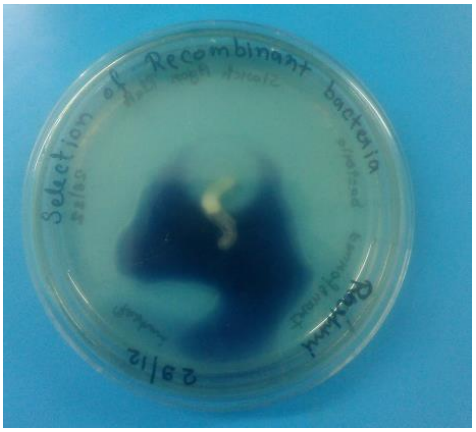
- The ligated mixture is taken for doing transmission.
- T4DNA ligase buffer contains ATP, so repeated freeze thaw cycles can degrade ATP, thereby decreasing the efficiency of ligation.
- It is better to vortex or spin the T4 DNA ligase enzyme before pipetting to ensure that it is mixed well.

Result-5:-Transformation



Transformed colonies are prepared after incubation period and observed by starch hydrolysis test.

Result-6:-Starch Hydrolysis Test



Blue-Black color appears on whole place except streaked area showing the action of the enzyme secreted by bacteria to digest the starch Observe clear zone shows positive result.

DISCUSSION:

According to Numan OZCAN, Adem Altinalan, B. subtilis was found to produce approximately 65 kDa alpha-amylase gene. A gene was isolated in coding alpha –amylase activity was inserted into pUC18 plasmid which contains a whole alpha amylase gene, was excised and inserted into pUB110 and then transferred into the different B. subtilis strains RSKK246, RSKK243, RSKK244, YB886 and ORBAM. Specific activity of cloned enzyme was found to be higher than the native enzyme.

Fahim Afzaljavan, Mohsen Mobini- in their research the gene of alpha amylase enzyme of native isolated B. subtilis was amplified with specific primer by PCR and then sequenced. Purified PCR product and shuttle episomal vector were cut by restriction enzyme and cloned into E. coli and yeast hosts. Based on this similarity and our bioinformatics evaluations this mentioned alpha amylase gene can be expressed in S. cerevistae as extracellular enzyme. The aim of this research project was to isolate alpha amylase gene from Bacillus species are among bacteria that are widely used for the production of commercial enzyme. Result showed that B. subtilis produced alpha amylase well.

The generation time of B. subtilis is 120 min. which are too long time for production of alpha amylase enzyme whereas the doubling time of E. coli is of 20 min. only. For this purpose only we have transferred the gene of alpha amylase from B. subtilis to E. coli to improve the productivity of alpha amylase enzyme.

CONCLUSION:

In this present study alpha amylase producing gene (Amy R gene) is isolated from Bacillus subtilis and transformed to E. coli successfully .selection of transformed cell is made by antibiotic sensitivity test, then the selection of recombinant one is done by starch hydrolysis test clear zone observed on the addition of iodine which is indicate the recombinant of E. coli.

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