ISSN No: 2348-4845
International Journal & Magazine of Engineering, Technology, Management and Research
A Peer Reviewed Open Access International Journal

Effect of mutagens on gene expression of amylases producing Bacilli SPS

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Abstract: ***Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents. Although amylases can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand. Microbial amylases have successfully replaced chemical hydrolysis of starch in starch processing industries. Besides their use in starch saccaharification, they also find potential application in a number of industrial processes such as in food, baking, brewing, detergent, textile and paper industries. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields, such as clinical, medical and analytical chemistry. The objective of this paper is selection of a suitable strain for the production of amylase by comparative study of the strains such as bacilli and cocci Screening of different agricultural byproducts as substrates for maximum enzyme production, application of different combinations of these substrates for enzyme production, and optimization of cultural conditions for the production of glucoamylase.***

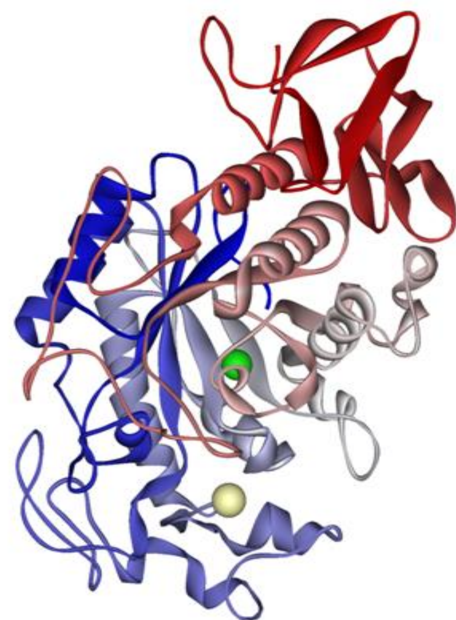
Keywords: ***Amylase, glycoside hydrolase enzymes , Starch, strain, bacilli, cocci, glucoamylase.***

Introduction:

Amylase is the name given to glycoside hydrolase enzymes that break down starch into maltose molecules. It is mainly found in the saliva, which is the reason why we can feel the sweetness when eating rice (contains glucose). However, the glucose is absorbed into the bloodstream from the small intestines, not in the mouth. Although the amylases are designated by different Greek letters, they all act on α-1, 4-glycosidic bonds.

There are three sub classifications of amylases they are

1. α-Amylase: - The α-amylases are calcium metalloenzymes, completely unable to function in the absence of calcium. By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. Because it can act anywhere on the substrate, α-amylase tends to be faster-acting than β-amylase. In animals, it is a major digestive enzyme and its optimum pH is 6.7-7.0. In human physiology, both the salivary and pancreatic amylases are α-Amylases. Also found in plants (barley) , fungi (ascomycetes and basidiomycetes) and bacteria (Bacillus).



Human salivary amylase

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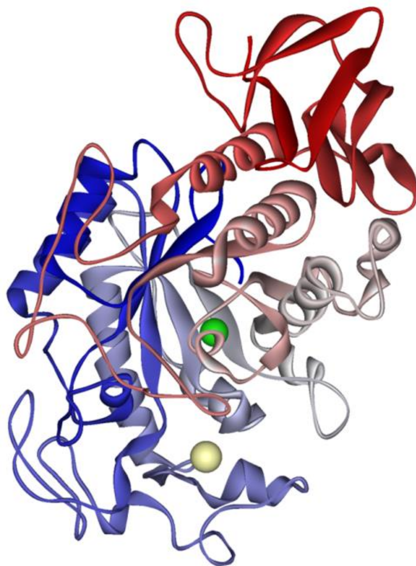

Human pancreatic amylase

Calcium ion visible in pale khaki, chloride ion in green.

2. β-Amylase: - Another form of amylase, β-amylase is also synthesized by bacteria, fungi, and plants. Working from the non-reducing end, β-amylase catalyzes the hydrolysis of the second α-1, 4 glycosidic bond, cleaving off two glucose units (maltose) at a time. During the ripening of fruit, β-amylase breaks starch into sugar, resulting in the sweet flavor of ripe fruit. Both are present in seeds; β-amylase is present prior to germination, whereas α-amylase and proteases appear once germination has begun. Cereal grain amylase is key to the production of malt. Many microbes also produce amylase to degrade extracellular starches. Animal tissues do not contain β-amylase, although it may be present in microrganisms contained within the digestive tract.

3. γ-Amylase: - In addition to cleaving the last α(1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose, γ-amylase will cleave α(1-6) glycosidic linkages. Unlike the other forms of amylase,γ-amylase is most efficient in acidic environments and has an optimum pH of 3.

During the last decade, an increased attention was paid to the use of various agro industrial wastes for value addition using solid-state fermentation (SSF) by filamentous fungi. SSF is the most appropriate process due to the advantages it offers. The hyphal mode of growth and good tolerance to low water activity and high osmotic pressure conditions, make fungi most efficient for bioconversion of solid substrates.

Starch is also present in waste produced from food processing plants and industries like paper. Starch waste causes pollution problems. Biotechnological treatment of waste water by immobilization of amylase degrade the starch in the waste which can produce valuable products such as microbial biomass protein and also purifies the effluent

Microbial amylases are exploited for the following purposes:

1. High Fructose Corn syrup preparation
2. Additives to detergents for removing stains
3. Saccharification of starch for alcohol production
4. Starch and cellulose in waste and its treatment.
4. Brewing

The enzyme amylase is widely distributed in the intestinal track of fresh water fishes and plays an important role in the digestion of starch. Enzyme found in intestinal lumen of fish could potentially have come from either pancreas or the secretary cells in the gut walls of the fish. In addition, enzymes from intestinal micro flora potentially could have a significant role in digestion, especially for substrates such as starch and cellulose. The intestinal track of fish is generally colonized by great number of heterotrophic bacteria, including aerobes and anaerobes.

The other organism enzyme producing. Amylase production was checked and activity was measured. Production of amylase was compared with the reference of crude enzyme kinetics. Amylase was separated from the crude enzyme by dialysis and ion – exchange chromatography. The presence of amylase was conformed by performing SDS-Poly Acrylamide Gel Electrophoresis. The results were interpreted by visualizing the protein bands.

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MATERIALS AND METHODS

ISOLATION OF MICROORGANISMS FROM DIFFERENT SOURCES

Organisms ranges from bacteria to nematodes and includes diverse species of algae and protozoa. soil microorganisms are responsible for the breakdown of organic matter, including hydrocarbons, conversion of inorganic components from one form to another and the production of humus. Although this group is large and diverse, soil microorganisms are thought of as being of three distinct types: fungi actinomycetes, and bacteria. Cow dung microorganisms form a robust community capable of surviving and functioning under extremes of temperature, water availability, pH, energy resources, nutrient availability and salt concentration.

Isolating individual microorganisms and allowing them to grow and produce colonies is another method of enumerating microorganisms in soil. **Nitrogen-fixing bacteria** form symbiotic associations with the roots of legumes **Nitrifying bacteria** change ammonium (NH4+) to nitrite (NO2-) then to nitrate (NO3-) – a preferred form of nitrogen for grasses and most row crops. **Denitrifying bacteria** convert nitrate to nitrogen (N2) or nitrous oxide (N2O) gas. Denitrifies are anaerobic. **Actinomycetes** are a large group of bacteria that grow as hyphae like fungi (Figure 3). They are responsible for the characteristically “earthy” smell of freshly turned, healthy soil. Actinomycetes decompose a wide array of substrates, but are especially important in degrading recalcitrant (hard-to-decompose) compounds, such as chitin and cellulose, and are active at high pH levels. Fungi are more important in degrading these compounds at low pH. A number of antibiotics are produced by actinomycetes such as Streptomycin.

Municipal and rural water supplies can transmit human diseases such as cholera (*Vibrio cholerae*), typhoid fever *(Salmonella typhi*), shigellosis (*Shigella*), salmonellosis (*Salmonella*), and gastroenteritis (*Campylobacter jejuni, Escherichia coli, Giardia lamblia*). The threat of such disease transmission becomes more serious as the population density increases and more sewage pollutes public water supplies, carrying with it human intestinal pathogens. Municipal and rural water supplies can transmit human diseases such as cholera (*Vibrio cholerae*), typhoid fever *(Salmonella typhi*), shigellosis (*Shigella*), salmonellosis (*Salmonella*), and gastroenteritis (*Campylobacter jejuni, Escherichia coli, Giardia lamblia*). The threat of such disease transmission becomes more serious as the population density increases and more sewage pollutes public water supplies, carrying with it human intestinal pathogens.

Isolation and enumeration of microorganisms can be done from different sources such as cow dung, soil, water, and air. Serial dilution agar plating method is one of most routinely used procedure for the isolation and enumeration of microorganisms.

SERIAL DILUTION

AIM: To isolate and enumerate microorganisms from natural cow dung source.

PRINCIPLE: This method is based on the principle that when material containing bacteria is cultured, every viable bacterium develops into a visible colony on a nutrient agar medium .In this procedure a small measured volume (or weight) is mixed with a large volume of saline called the diluents or dilution blank.
The number of organisms developed on the plates after an incubation period of 24-48h per ml is calculated as follows:
Number of cells/ml= number of colonies/volume plated*dilution.

PROCEDURE:

1. Label the dilution blanks as 10^{-1}, $10,^{-2}10^{-3}$, 10^{-4}, 10^{-5}, 10^{-6} and $10^{-7.}$

2. Prepare the initial dilution by adding 1ml or 1g of the sample into a 9ml dilution blank labeled 10^{-1} thus diluting the original sample 10 times.

3. Vortex the tubes to obtain uniform distribution of organisms.

4. From the first dilution, transfer 1ml of the suspension to the dilution blank 10^{-2} with a sterile 1ml pipette.

5. From the 10^{-2} suspension, transfer 1ml of the suspension to the 10^{-3} dilution blank with a sterile pipette, thus diluting the original sample to 1000 times(1:1000 or 10^{-3})

6. Repeated this procedure till the original sample has been diluted 10,000,000(10^{-7}) times using every time a sterile pipette.

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International Journal & Magazine of Engineering, Technology, Management and Research
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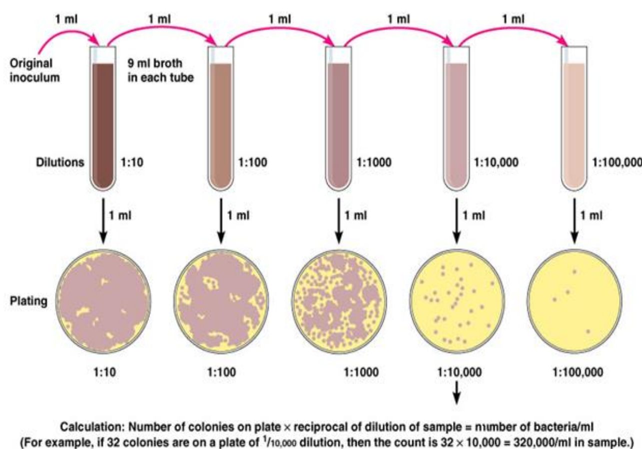
7. From the appropriate dilutions ($10^{-2,}$ $^{10-4}$, 10^{-6}, and 10^{-7}) transfer 1ml or 0.1ml of suspension with the respective pipettes to the sterile petriplates.

8. Add approximately 15ml of the nutrient medium, melted and cooled to 45^{0}c, to each Petri plate containing the diluted sample. Mix the contents of the plate by rotating clock wise and antilock wise.

9. Allow the plates to solidify.

10. Incubate the plates in an inverted positon for 24 to 48h at 37^{0}c.



OBSERVATION

1. Observe all the plates for the appearance of bacterial colonies.
2. Count the number of colonies in the plates, that have colonies in the 30-300 range,by placing each plate on the colony counter.

RESULTS

Calculate the number of bacteria per ml of the sample as follows:
Organisms per gram of the sample= number of colonies/volume plated *dilution
60 colonies were counted on a $1:10^{5}$ dilution, then
Number of cells=60 colonies/1ml*10^{-5}=6000000
= $6*10^{6}$ bacteria /ml or gm of sample.

SPREAD PLATE METHOD:

The number of bacteria in solution can be readily quantified by using the spread plate technique. In this technique, the sample is appropriately diluted and a small aliquot transferred to an agar plate. The bacteria are then distributed evenly over the surface by a special streaking technique. After colonies are grown, they are counted and the number of bacteria in the original sample is calculated.

PRINCIPLE:

The principle behind this technique is that as the Petri dish spins at some stage, single cells will be deposited with the bent glass rod on to the agar surface. Some of these cells will be separated from each other by a distance sufficient to allow the colonies that develop to be free from each other.

PROCEDURE:

1. Prepare nutrient agar. Autoclave at 121^{0}c, 15lbs, for 15min.
2. Pour the agar in the petriplates (15ml).
3. Allow it to solidify
4. 0.1 ml of bacterial suspension is placed in the center of the plate using a sterile pipet.
5. The glass rod is sterilized by first dipping it into a 70% alcohol solution and then passing it quickly through the Bunsen burner flame. The burning alcohol sterilizes the rod at a cooler temperature than holding the rod in the burner flame thus reducing the chance of you burning your fingers.
6. When all the alcohol has burned off and the rod has air-cooled, streak the rod back and forth across the plate working up and down several times.
7. Turn the plate 90 degrees and repeat the side to side, up and down streaking. Turn the plate 45 degrees and streak a third time.
8. Do not sterilize the glass rod between plate turnings. Cover the plate and wait several minutes before turning it upside down for incubation. This will allow the broth to soak into the plate so the bacteria won't drip onto the plate lid.

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RESULT: - . The bacteria are then distributed evenly over the surface. Colonies grown are counted and the number of bacteria in the original sample are calculated.
Organisms per gram of the sample= number of colonies/volume plated *dilution
60 colonies were counted on a 1:10^5 dilution, then
Number of cells=60 colonies/1ml*10^{-5}=6000000
= 6*10^6 bacteria /ml or gm of sample.

THE STREAK PLATE TECHNIQUE

Streak plates allow for the growth of isolated colonies on the surface of the agar. An isolated colony is a colony that is not touching any other colonies and is assumed to be a pure culture. These colonies are easily accessible for performing staining and Identification procedures. They also show colonial morphology that may be useful in identifying the organism.

PRINCIPLE:

An original inoculum containing a mixture of bacteria is spread into 4 quadrants on solid media.

The goal is to reduce the number of bacteria in each subsequent quadrant. Colonies are masses of offspring from an individual cell therefore streaking attempts to separate individual cells.

Discrete colonies form as the individual cells are separated and then multiply to form isolated colonies in the later quadrants.

PROCEDURE:

1. Pick up a loopful of your inoculum from either a broth or an agar culture. Using a sterile agar medium plate (lift the lid just enough to insert the loop), streak a vertical line straight down.

2. When streaking the agar, keep the loop horizontal and only streak the surface of the agar: DO NOT DIG INTO THE AGAR.

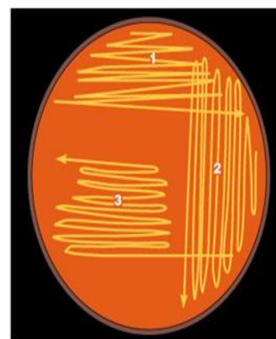
3. Move the loop in a zig-zag pattern across the agar until 1/3 of the plate is covered, finishing the first section.

4. Sterilize the loop in the flame and let it cool before continuing to spread the bacteria. You can do this by 1) sticking the hot loop in the agar at the edge of the agar away from the bacteria, or 2) just holding the loop for a few seconds while it cools.

5. Rotate the plate about 90 degrees and spread the bacteria from the first streak into a second area using the same zig-zag spread technique.

6. Sterilize the loop again. Rotate the plate about 90 degrees and spread the bacteria from the second streak into the 3rd area in the same pattern.

7. Sterilize the loop again. Replace the lid and invert the plate. Incubate the plates at 37^0c



(a) The direction of streaking is indicated by arrows. Streak series 1 is made from the original bacterial culture. The inoculating loop is sterilized following each streak series. In series 2 and 3, the loop picks up bacteria from the previous series, diluting the number of cells each time. There are numerous variants of such patterns.

(b) In series 3 of this example, notice that well-isolated colonies of bacteria of two different types, red and white, have been obtained.

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RESULT: - Isolated colonies are observed on the surface of the agar. An isolated colony is a colony that is not touching any other colonies and is assumed to be a pure culture. These colonies were accessible for performing staining and Identification procedures. They show colonial morphology that may be useful in identifying the organism.

PURE CULTURE

A pure culture consists of a population of only one species of microorganisms derived from a single microorganism. A strain is made up of succession of one kind of microorganism from a single colony. Isolation of one kind of microorganism from a mixture of many different kinds is called pure culture technique.

AIM: To isolate individual colonies including surface and subsurface colonies.

PRINCIPLE: - separation of a particular microorganism from the mixed populations by inoculating the single and pure colony of microorganism in the agar slant

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PROCEDURE:

1) Prepare the agar slants by adding adequate amount of agar in the test tubes and made them solidify by placing in slant position.
2) Take a loop full of pure culture and inoculate in the slant by streaking on the slant.
3) Close the tube with the cotton plug and place in the incubator for overnight at 37c.

RESULT: - Pure cultures of microorganisms were grown in the test-tube which are called pure cultures of specific microorganism which were further studied by colony morphology and confirmed by gram staining.

GRAM STAINING

The gram stain a differential stain was developed by Dr.Hans Christian Gram; Danish physician in 1884.it is a very useful technique for identifying and classifying bacteria into two major groups: the gram positive and gram negative.

PRINCIPLE:

Differential staining requires the use of four reagents that are applied on the heat fixed smear. The first reagent is primary stain (crystal violet).its function is to impart color to all the cells; the second is the mordant (iodine solution) which serves to intensify the color of the stain. The CV-I complex binds to magnesium ribose nucleic acid component of cell wall, which is more difficult to remove .the third is decolorizing agent (95% ethyl alcohol) serves as a lipid solvent and protein dehydrating agent. The last one is the counter stain(saffranin) has a contrasting color to that of primary stain .Following decolourization if the primary stain is not washed out the counter stain cannot be absorbed or if the primary stain is removed the decolorized cellular components will accept and assume the counter stain.

PROCEDURE:

- Make thin smears on a grease free glass slide
- Let the smears air dry
- Heat the slide for few seconds until it becomes hot to the touch so that bacteria are firmly mounted to the slide.
- Add the primary stain crystal violet and incubate 1 minute. This step colors all cells violet
- Add grams iodine for 30 seconds. It is not a stain it is a mordant it doesn't give color directly to the bacteria but it fixes the crystal violet to the bacterial cell wall. All cells remain violet
- Wash with ethanol and acetones.
- Wash with ethanol and acetone, They decolorize. If the bacteia are gram positive it will retain the primary stain. If it is gram negative it will lose the primary stain and appear colorless.
- Add the secondary stain , safranin and incubate 1 min, then wash with water for maximum of 5 seconds. If the bacteria are gram positive the cell will retian the primary stain, will not take the secondary stain., and will appear red-pink.
- Blot dry with absorbent paper.
- Let the slides air dry

OBSERVATION:

- Examine the slides microscopically using oil immersion objective.
- Identify the gram reaction and classify them
- Describe the morphology and arrangement of the cells.

RESULTS:

The bacteria that appear purple are referred as gram positive. And the bacteria were found to be bacillus species. I confirmed the organism according to the BERGEYS MANUAL.

PRECAUTIONS:

Always use fresh and young cultures(less than 24h old).
Excessive heat should be avoided during heat fixing.
Smear should be thin and uniform.

SCREENING OF MICROOGANISM

AIM: - To screen the microorganism by providing the selective medium.

Principle: - organism's shows difference in physiological and biochemical reactions, thus by using this principle organisms were screened by providing the selective medium.

PROCEDURE:-

1) Prepare nutrient agar starch medium. Autoclave at 121^0c, 15lbs, for 15min.
2) Pour the agar in the petriplates (15ml).
3) Allow it to solidify.
4) Take a loop full of pure culture and streak as M shape in the Petri dish
5) Place the plates in the incubator for over night at 37c

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Observation: -

Culture grown was checked with iodine.
Clear zone of white color was formed and the remaining plate was blue in color.

PRILIMINARY TEST

Starch broth:

Beef extract - 3g
Peptone - 5g
Nacl - 5g
Starch - 10g
Distilled Water - 1000ml

From the above sample we took 100ml sample for the production of enzymes.

- Pour the broth into test tubes and it was inoculated with culture and it was allowed for incubation for 24-48hrs at 37^0c.
- We took 1ml of broth and it was centrifuged. Then we collected the supernatant and add iodine reagent to check amylase activity.
-

Observations and Results:

If Blue color is observed by adding the iodine reagent it is negative, if iodine color is observed it is positive.

Type		Amylase production
Vs1	-	positive
Vs2	-	negative
Vs3	-	negative
Vs4	-	positive

ISOLATION AND EXCTRATION

Centrifugation:

- To collect the crude enzymes from the starch broth we use this technique.
- Take 1ml of starch broth into append off tubes and it was allowed for centrifugation at 6000rpm for 10mins.
- Later we collected the supernatant which was used as crude enzyme.

ESTIMATIONS OF STANDARD REDUCING SUGAR BY DNS METHOD:-

Reagents preparation:

1. Standard preparation.

   Maltose standard – 100mg in 100ml water.

   Starch standard - 100mg in 100ml water.

2. Buffer preparation.

- we added 2.78gms in 100ml sodium phosphate solution and it was
- taken as X-solution

- Then we add 0.2M dibasic sodium phosphate 5.365mg in 100ml water and it was taken as Y-solution.
- From solution X we took 87.7ml and from solution Y we took 12.3ml in a conical flask and it was make upto 200ml.

3. DNS(Dinitro salcylate reagent)

   Composition

   NaoH - 1gm
   Sodium sulphate - 50mg
   Crystalline phenol - 400mg
   DNS - 1gm
   Distilled Water - 100ml

By using this composition we prepared 100ml DNS reagent.

4. Sodium-Potassium tartarate

   40% sodium potassium tartarrate

40gms sodium potassium tartarrate in 100ml distilled water

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Preparation of Standard Graph:-

	S. No	Volume of standard sugar	Volume of distilled water	Conc of standard	Incubate at 37°c	DNS	keep in ath at 100°c for 10 min	Vol of Na k tartarate	OD at 550nm
	Blank	nil	1ml			4ml		0.5ml	
	1	0.2	0.8ml	200ug/ml		4ml		0.5ml	0.20
	2	0.4	0.6ml	400ug/ml		4ml		0.5ml	0.40
	3	0.6	0.4ml	600ug/ml		4ml		0.5ml	0.60
	4	0.8	0.2ml	800ug/ml		4ml		0.5ml	0.80
	5	1.0		1000ug/ml		4ml		0.5ml	1.00

Procedure:

- Take a series of test tubes and label them as 1 to 5.
- Then add different concentrations of standard between 0.2 to 1.00 to the test tubes.
- Then make up the test tubes to 1ml by using buffer.
- Incubate the test tubes for 10 minutes at room temperature.
- Then add 4ml of DNS reagent to all the test tubes.
- Then keep the test tubes in hot water bath at 100 c for 10 minutes.
- Maintain the tube, blank as same as, instead of standard add distilled water to adjust the calorimeter to zero.
- Then cool the test tubes and add 0.5ml sodium potassium tartarate to all the test tubes.
- Then take O.D values by using calorimeter at 550 nanometers.
- Then plot the graph between concentration Vs O.D values.

Estimation of reducing sugars:-

Reagents preparation:

- Standard preparation.
- Maltose standard – 100mg in 100ml water.
- Starch standard - 100mg in 100ml water.

Buffer preparation.

- we added 2.78gms in 100ml sodium phosphate solution and it was taken as
- X-solution
- Then we add 0.2M dibasic sodium phosphate 5.365mg in 100ml water and it was taken as Y-solution.
- From solution X we took 87.7ml and from solution Y we took 12.3ml in a conical flask and it was make upto 200ml.

DNS (Dinitro salcylate reagent)

- Composition
- ---------------
- NaoH - 1gm
- Sodium sulphate - 50mg
- Crystalline phenol - 400mg
- DNS - 1gm
- Distilled Water - 100ml
- By using this composition we prepared 100ml DNS reagent.

- Sodium-Potassium tartarate
  40% sodium potassium tartarrate

  40gms sodium potassium tartarrate in 100ml distilled water.

Estimations:-

s.no	Volume of substrate	con of enzyme	Volume of enzyme	Volume of buffer	Incubation at 37°c	Volume of DNS	keep in ath at 100°c for 10 min	Vol of Na k tartarate	OD at 550nm
Blank	0.5ml	500ug/ml	nil	0.5ml		4ml		0.5ml	0.00
sample	0.5ml	500ug/ml	0.1ml	0.5ml		4ml		0.5ml	0.50

Procedure:

Take two tubes and then label them as B and S.

- Then add 0.5ml of standard solutions to the test tubes.
- Then add 0.1ml of enzyme to the S test tube.
- Incubate the test tubes for10 minutes at 37 c .
- Then make up the test tubes to 1 ml by using buffer solution.
- Then add 4 ml of DNS reagent to all the test tubes.
- Maintain the tube blank as same as, instead of standard add distilled water to adjust the colorimeter to zero.
- Keep the test tubes in hot water bath at 100 c for 10 minutes.
- Then cool the tubes and add 0.5 ml of NaK tartarate to all the tubes.
- Then take the O.D values by using colorimeter at 550nm.

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- Then plot the graph between concentration Vs O.D values.

RESULT:-

500ug/ml of reducing sugars were released from starch activity of the enzyme

Effect of U.V mutations:-
Preparation of media:-
Starch agar median preparation:-

- Peptone -----→0.5 gr
- Beef extract ---→0.3 gr
- Nacl ----→ 0.5 gr
- Starch ----→ 1.0 gr
- Agar - - --→ 2.0 gr
- Distill water --→100ml

Prepare starch agar medium plates:-

- Take 5 ml autoclaved water inoculated with loop full of culture.
- Take 0.1 ml from this and it was spreaded on starch agar medium.
- These plates are exposed to UV light in different fume intervals like 5, 10 and 15 minutes.
- Later these plates were incubated at 37 c for 24 hrs.
- Then observe the growth of microbes for mutational changes.
- To identify the type of bacteria we done the process of gram staining and colony morphology.
- Then after comparing this with wild type of bacteria by the preparation of starch broth.

Starch broth:-

- Peptone0.5gr
- Beef extract 0.3gr
- Nacl 0.5gr
- Starch 1gr
- Distill water---→100ml
- Prepare starch broth tubes and it was inoculated with mutated organism. For check out the amylase production.
- Incubate in orbital shaker for 24 hrs at 37 c
- Check the amylase production by iodine test
- Find out the amylase production by using iodine test
  Collect the crude enzyme from the broth centrifugation method.

U.V Mutations:-

S No.	Volume of sub	Volume of enzyme	Volume of buffer	Incubated at 37 c for 10 mins.	Volume of DNS	Keep in water bath at 100c	Volume of Na k tartarate	O.D at 550 nm
Blank	0.5ml	Nil	0.5ml		4ml		0.5ml	0.00
5min	0.5ml	0.1ml	0.5ml		4ml		0.5ml	1.03
10min	0.5ml	0.1ml	0.5ml		4ml		0.5ml	0.23
15min	0.5ml	0.1ml	0.5ml		4ml		0.5ml	0.48

Procedure:

- Take series of test tubes and label them as B, Vs5, Vs10 & Vs15.
- Then add 0.5ml of starch solution [sub] to the four test tubes.
- Then add 0.1ml of enzyme to all the test tubes.
- Then add 0.5ml of buffer and make up the tubes to 1ml.
- Incubate the tubes for 1mins at 37 c.
- Then add 4ml of DNS to all the test tubes.
- Maintain the tubes as same as, instead of enzyme add distill water to adjust the colorimeter to zero.
- Keep the tubes in hot water bath with 100 c for 10 mins.
- Then cool the tubes and add 0.5ml of Nak tartarate to all the test tubes
- Then take the O.D values by using colorimeter at 550nm
- Then plot the graph between concentration Vs O.D values.

RESULT:-

After mutating the wild organism with uv forVs5',Vs10'and Vs15' 1030ug/ml, 230ug/ml and 480ug/ml of reducing sugars were released from starch respectively by the activity of the enzyme.

Effect of EtBr mutations
Preparation of starch agar medium:

- Peptone
- Beef extract
- Starch
- Nacl
- Distill water
- ✓ In starch agar medium add 0.01% EtBr then pour this into plate and allow solidifying the plates.
- ✓ Then add 5ml of autoclaved water and get inoculated with loop full of culture.
- ✓ Take 0.1ml from this solution and this was spreaded on starch agar medium.
- ✓ These plates are incubated at37 c for 24hrs.

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- ✓ Then observe the growth of microbes for mutations.
- ✓ To identify the type of bacteria we did the gram staining process.
- ✓ Then after compare this wild type of bacteria by the preparation of starch broth.

Starch broth: -

- ➢ Peptone
- ➢ Beef extract
- ➢ Nacl
- ➢ Starch
- ➢ Distilled water

- Prepare starch broth tubes and these were inoculated with EtBr mutated microbes for the amylase production.
- Find out amylase production by iodine test
- Collect the crude enzyme from the broth by centrifugation.

Etbr mutation:

s.no	Vol of sub	Vol of enzyme	Vol of buffer	Incubated at 37 c for 10 mins.	Volume of DNS	Keep in water bath at 100c	Volume of Na k tartarate	O.D at 550 nm
Blank	0.5ml	Nil	0.5ml		4ml		0.5ml	0.00
Etbr 1	0.5ml	0.1ml	0.5ml		4ml		0.5ml	0.06
Etbr 2	0.5ml	0.1ml	0.5ml		4ml		0.5ml	0.15

Procedure:

- ➢ Take test tubes and label them as Eb1, Eb2.
- ➢ Then add 0.5ml of starch solution [sub] to the test tubes.
- ➢ Later add 0.1ml of enzyme to it.
- ➢ Then add 0.5ml of buffer to the tubes.
- ➢ This was incubated for 10 mins. at 37 c
- ➢ Later to this add 4ml of DNS reagent and it was kept in water bath for10mins 100 c.
- ➢ Later to this add 0.5ml Nak tartarate was added
- ➢ Take O.D values in a colorimeter at 550nm
- ➢ Then plot the graph by taking this O.D values

RESULT:-

After mutating the wild strain with EtBr1, EtBr2, 60ug/ml, 150ug/ml of reducing sugar were released from starch by the activity of the enzyme.

PURIFICATION

AMMONIUM SULPHATE PRECIPITATION

Ammonium sulphate is a method for protein purification by altering the solubility of protein $(NH_4)_2SO_4$ precipitation is a simple and effective means of fractionating protein. it is based on the fact that at high salt concentration the natural tendency of protein not to aggregate is overcome, since the surface charges are neutralized Charge neutralized means that protein will tend to find together, from large complexes and hence are easy to precipitate out by mild centrifugation. Since, each protein will start to aggregate at a characteristic salt concentration, than approach provides a simple way of enriching for particular protein in a mixture and is used

SALTING OUT Increase in the salt concentration implies that there is less and less water available to solubilize protein finally ,protein starts to precipitate when there are not sufficient water molecules to interacts with protein molecule, then phenomenon of protein precipitation in the presence of excess salt is know as salting out.

SALTING IN At Low concentration, the presence of salt stabilizer the various charged groups on a protein molecule, then attracting protein into the solution and enhancing the solubility of protein, then phenomenon commonly know as salting in

FRACTIONATION:

The precipitated proteins is collected and categorized to the concentration of salt solution at which it is formed. This partial collection of the separated protein pellet called Fractionation

For example the attraction of the precipitated protein collected between 20 and 21% of salt saturation is commonly referred to as the 20-21% fraction

Salting out is thought to work by dehydrating the environment

PROCEDURE:

- Crude enzyme extracted was taken and amount of enzyme taken was noted
- 44grams of ammonium sulphate is taken then calculated for 4ml of enzyme.
- The crude enzyme was taken in a beaker with a magnetic bead and the beaker was placed on the magnetic stirrer.

ISSN No: 2348-4845
International Journal & Magazine of Engineering, Technology, Management and Research
A Peer Reviewed Open Access International Journal

- Small amount of ammonium sulphate was added till it dissolved the same process is continued till the total salt was dissolved in the enzyme.
- Then the enzyme was kept in the stirrer for hours
- Then the enzyme was collected and centrifuged at 10000rpm for 6 minutes
- The pellet which contains the protein sample was collected by dissolving it in the buffer of pH-6.
- This protein was further purified by dialysis and ion exchange chromatography.

DIALYSIS

In biochemistry, dialysis is the process of separating molecules in solution by the difference in their rates of diffusion through a semi permeable membrane, such as dialysis tubing.

Dialysis is a common laboratory technique, and operates on the same principle as medical dialysis. Typically a solution of several types of molecules is placed into a semi permeable dialysis bag, such as a cellulose membrane with pores, and the bag is sealed. The sealed dialysis bag is placed in a container of a different solution, or pure water. Molecules small enough to pass through the tubing (often water, salts and other small molecules) tend to move into or out of the dialysis bag, in the direction of decreasing concentration. Larger molecules (often proteins, DNA, or polysaccharides) that have dimensions significantly greater than the pore diameter are retained inside the dialysis bag. One common reason for using this technique would be to remove the salt from a protein solution. The technique will not distinguish between proteins effectively.

Buffer preparation.

- we added 2.78gms in 100ml sodium phosphate solution and it was taken as
  X-solution
- Then we add 0.2M dibasic sodium phosphate 5.365mg in 100ml water and it was taken as Y-solution.
- From solution X we took 87.7ml and from solution Y we took 12.3ml in a conical flask and it was make upto 200ml.
- The pH was adjusted to 6 with HCl and NaOH.

PREPARATION OF DIALYSIS TUBE

1) Boil the tubing on a sitre plate (preferably in the hood) in a 4L volume of 2% (w/v) sodium bicarbonate and 1mM EDTA pH 8.0
2) Rise the tubing in distilled water thoroughly
3) Boil for 10 minutes in 1mM EDTA (pH 8.0)
4) Allow tubing to cool then store it in freezer at 4ºC with tubing submerged.
5) Before use wash out tubing with distilled water
6) Tie one end of the tube with thread and pour the protein the tube
7) Close the other end also tightly with the thread
8) Submerge the tube in the buffer and place the magnetic bead and place it on the magnetic stirrer
9) Change the buffer for every 1 hour and allow the bag in the buffer for 2 ½ hours.
10) Collect the purified protein from the bag after the dialysis is completed.
11) The purified and collected enzyme is further purified and screed by ion exchange chromatography

ION EXCHANGE CHROMATOGRAPHY

Principle: This form of chromatography relies on the attraction between oppositely charged particles. Many biological materials, for eg. Amino acids and proteins have ionizable groups and the fact that they may carry a net positive or negative charge can be utilized in separating mixture of such a compounds. The net charge executed by these compounds is dependant on the pKa and on the pH of the solution in accordance with the Henderson Hasselbalch equation.

Ion exchange separations are carried out mainly in columns packed with an ion exchanger. There are two types of ion exchanger, namely, Cation and Anion Exchangers.

Cation exchangers possess negatively charged groups and these will attract positively charged cations. These exchangers are also called acidic ion exchange materials because their negative charges result from the ionization of acidic groups.

Carboxy methyl cellulose is a cation exchanger, here CM-cellulose is most applicable for the separation of proteins which are positively charged at around pH 4-5.

Anion exchangers have positively charged groups that will attract negatively charged anions. The term basic

ion exchange material is also used to describe this exchanger, as positive charges generally results from the association of the protons with basic groups.

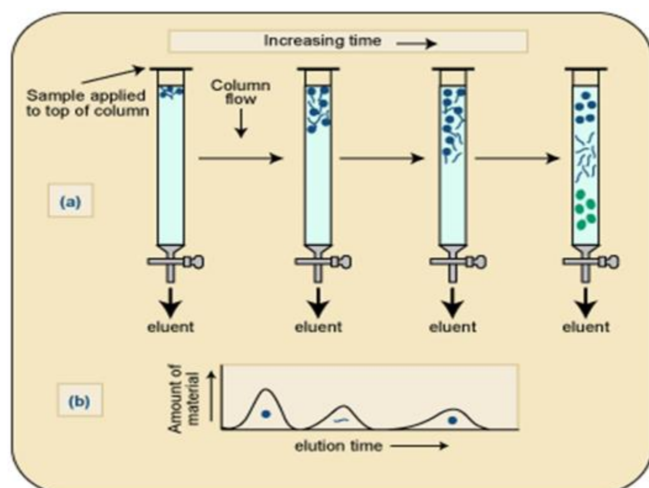
The most frequently used are Diethylaminoethyl (DEAE)-cellulose which is anion exchanger. The DEAE group, -$CH_2H_5NH(C_2H_5)_2$,is highly positive charge at pH 6-8, so DEAE- cellulose is most useful for the chromatography of protein which are negatively charged in this range. Elution of the proteins from the columns may be brought about by changes in either salt concentration of salt (e.g. NaCl) increases, protein is displaced from DEAE by Cl^- ions and from CMC-cellulose by the cation Na^+

The ion exchange mechanism is thought to be composed of five distinct steps:

Diffusion of the ion to the exchanger surface. This occurs very quickly in homogenous solution.

Diffusion of the ion to the matrix structure of the exchanger to the exchange site. This is dependant upon the degree of cross linkage of the exchanger and the concentration of the solution. This process is thought to be the feature that controls the rate of the whole ion exchange process.

Exchange of ions at the exchange site. This is thought to occur instantaneously and to be an equilibrium process.



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- Then we add 0.2M dibasic sodium phosphate 5.365mg in 100ml water and it was taken as Y-solution.
- From solution X we took 87.7ml and from solution Y we took 12.3ml in a conical flask and it was make upto 200ml.

ESTIMATION OF TOTAL PROTEIN BY LOWRY's METHOD

Preparation of reagents:

1. BSA (Bovine Serum Albumin) - 100mg per 100ml
2. A) 2% Na_2Co_3 in 0.1N NaoH

B) 0.5% $CuSo_4.5H_2O$ in 1% NaK tartarate.

Reagent C:

Take 50ml of reagent A mix with 1ml of reagent B

FC Reagent:

Prepare before use 1:1 dilution.

Estimation of std protein

sno	Vol of protein std	Vol of distilled water	Con of protein	Vol of reagent C	Incubate for 10min	Vol of FC reagent	OD values at 660nm
Blank	nil	1ml	nil	4ml		0.5ml	0.00
1	0.2	0.8ml	400ug/ml	4ml		0.5ml	0.5
2	0.4	0.6ml	600ug/ml	4ml		0.5ml	1.0
3	0.6	0.4ml	800ug/ml	4ml		0.5ml	1.5
4	0.8	0.2ml	1000ug/ml	4ml		0.5ml	2.0
5	1.0	Nil	nil	4ml		0.5ml	2.5

Procedure:

- Take a series of test tubes and label them as 1 to 5.
- Then add different concentrations of standard between 0.2 to 1.00 to the test tubes.
- Then make up the test tubes to 1ml by using buffer.
- Then add 4ml of Reagent C to all the test tubes.
- Incubate the test tubes for 10 minutes at room temperature.
- Add 0.5ml FC Reagent to all the tubes.
- Then keep the test tubes at room temperature for 30min
- Maintain the tube, blank as same as, instead of standard add distilled water to adjust the calorimeter to zero.

ISSN No: 2348-4845
International Journal & Magazine of Engineering, Technology, Management and Research
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- Then take O.D values by using calorimeter at 660 nanometers.
- Then plot the graph between concentration Vs O.D values.

Estimation of Protein (Enzyme)

sno	Test tube con	Buffer in ml	Nacl in ml	Distilled water in ml	Total vol	Incubation for 10min	OD values at 660nm
1	25	0.25	0.125	4.625	5ml		0.15
2	50	0.25	0.25	4.5	5ml		0.06
3	75	0.25	0.375	4.375	5ml		0.04
4	100	0.25	0.5	4.15	5ml		0.05
5	125	0.25	0.625	4.125	5ml		0.03
6	150	0.25	0.75	4.0	5ml		0.04
7	175	0.25	0.875	3.875	5ml		0.03
8	200	0.25	1	3.75	5ml		0.03
9	225	0.25	1.125	3.5	5ml		0.04
10	250	0.25	1.25	3.25	5ml		0.08

Procedure:

- Take a series of test tubes and label them as 1 to 10.
- Then add different concentrations of protein is 0.5ml
- Then make up the test tubes to 1ml by using buffer.
- Then add 4ml of Reagent C to all the test tubes.
- Incubate the test tubes for 10 minutes at room temperature.
- Add 0.5ml FC Reagent to all the tubes.
- Then keep the test tubes at room temperature for 30min
- Maintain the tube, blank as same as, instead of standard add distilled water to adjust the calorimeter to zero.
- Then take O.D values by using calorimeter at 660 nanometers.
- Then plot the graph between concentration Vs O.D values.

RESULT AND DISCUSSION:-

From standard graph:

It is observed that at every 200ug of maltose serially increasing the O.D values from 0.20 to 0.40 .

Estimation of reducing sugars (wild type):

It is observed that 0.5ml VS1 culture can release 500ug/ml of reducing sugars.

Estimation of reducing sugars (UV mutated):

Vs(15min) mutated with u.v has concentration of 480ug/ml reducing sugars liberation.

Vs(10 minutes)mutated with u.v has concentration of 230ug/ml reducing sugars liberation.

Vs(5min) mutated with u.v has concentration of 1030ug//ml reducing sugars liberation.

Estimation of reducing sugars (ETBR mutated):

Vs mutated with ETBR has concentrations of 60ug/ml,150ug/ml reducing sugars liberation.

Estimation of protein:

The amount of protein obtained in 0.15mm concentration is 150µg/ml.

CONCLUSION

The genomic expression of the bacilli is very sensitive to the mutagens therefore the selection of effect of UV is essential for the production of amylase. In this study the effect of UV decreases the hydrolysis of starch by producing the sugars. It means that amylase producing gene is highly affected due to the effect of UV.

Another study of mutagen that is ETBR affected on the production of amylase by decreasing the hydrolysis of starch by producing the sugars. It means that amylase producing gene is highly deactivated by the effect of ETBR.

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