AIM – PRODUCTION AND ASSAY OF XYLANASE ENZYME THROUGH SUBMERGED AND SOLID STATE FERMENTATION BY FUNGI

Requirement – Pure culture of *Aspergillus niger*, MSM or Czapek dox agar medium, Oat powder, Sterile Petri plates, Conical flasks, Laminar air flow, Congo red, NaCl, Cork Borer, Centrifuge, Ammonium Sulphate, Spetrophotometer, distilled water

Dr. Pragya Kulkarni
Govt. VYT PG Autonomous College, Durg (C.G.)

Principle

Xylanase producing fungi –

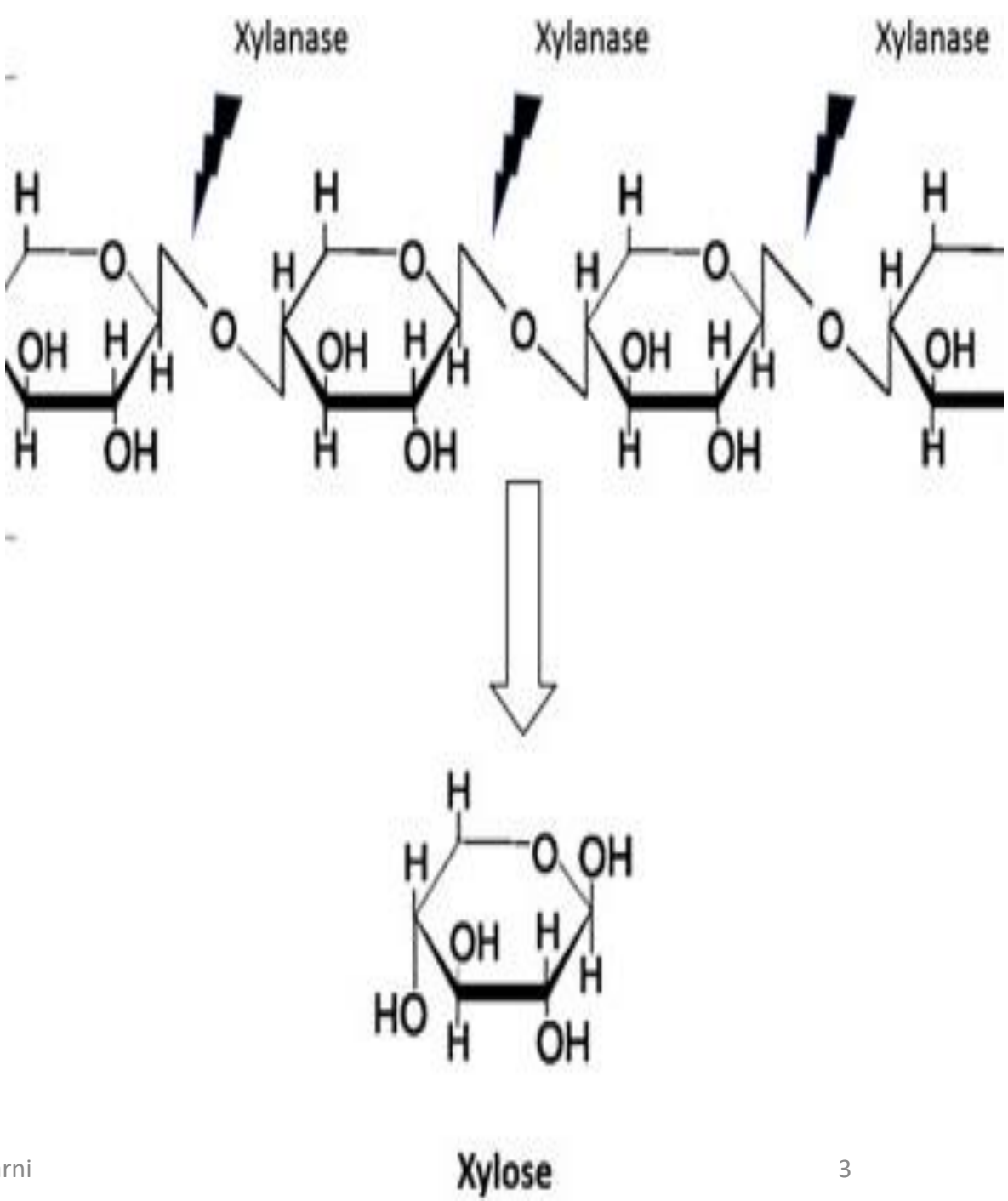
- Filamentous fungi are particularly interesting producers of xylanase enzyme due to the fact that they produce extracellular xylanases into the medium
- Furthermore, xylanase levels from fungal cultures are generally much higher than those from yeasts or bacteria
- In addition to xylanase, fungi typically produce several accessory xylanolytic enzymes, which are necessary for debranching substituted xylans
- An important factor for efficient xylanase production is the choice of an appropriate inducing substrate, either insoluble or soluble, as well as optimization of the medium composition

Xylanase Enzyme

- **Xylanase [E.C.3.2.1.8] are a class of hydrolytic enzymes which can hydrolyze the straight polysaccharide -1, 4-xylan in hemicelluloses which is a noteworthy component of secondary cell wall of plants**
- **Xylan, the most abundant in lignocellulosic feedstock in the form of hemicelluloses containing hetero polysaccharides, comprises of a backbone of -1-4-linked xylopyranose residues and forms an interface between lignin and other polysaccharides**

Xylanases have gained a unique importance in the biotechnology and industries due to their potential applications:

- **In textile industry for biopolishing of fabrics and producing stonewashed look of denims**
- **In household laundry detergents for improving fabric softness and brightness**
- **In food, leather, paper/pulp industries, in the fermentation of biomass for the biofuel production, in ruminant nutrition for improving digestibility, in fruit juices processing and in de-inking of paper**



Principle

Fermentation –

- **The growth of microorganism (mainly fungi) on moist solid materials in the absence of free flowing water is called Solid state fermentation**
- **In this process xylanase is an inducible enzyme and the xylan present in substrate (oat, wheat bran) acted as good inducers for enzyme production**
- **Industrial production of enzymes and other substrates are also done by growing the microorganisms in a submerged culture (broth) is called Submerged state fermentation**
- **Submerged cultures allows control over the degree of aeration, pH and temperature of the medium as well as control over other environment factors required for optimum growth of organisms**

Procedure

1. For selective isolation of xylanase producing fungi:

- Prepare Mineral Salts Medium (MSM) with NH_4NO_3: 1.5g, KH_2PO_4: 2.5g, NaCl: 1.0, $MgSO_4$: 1.5g, $CaCl_2$: 0.05g, $MnSO_4$: 0.01g, $FeSO_4$: 0.005g in 1000 ml distilled water and were amended with xylose: 10.0 g/L or birchwood xylan 0.1%.pH 5.0

2. Primary Screening of isolates for xylanase production:

- After 5 days of incubation, flood the plates with 0.01% of 10-15 ml Congored solution (Teather and wood 1982) and allowed to stand for 10 to 15 min and destained with 1% NaCl
- Observe the zone of hydrolysis and measure in cm/mm

3. Secondary screening:

- Select the cultures with zone of hydrolysis more than 2 cm for further screening under solid state fermentation (SSF) and/ or submerged state fermentation (SmF) for the production of xylanase

Solid State Fermentation Technique –

1. Add 40 gm of substrate (whole oat powder) to 50 ml MSM medium (Czapek dox medium can also be used) in a flask
2. Autoclave it at 121°C for 15 minutes and inoculate with the test organism
3. Incubate the flask at 28° C for 7 days
4. Harvest the enzyme by filtration through cheese cloth followed by centrifugation at 10000 rpm for 20 minutes at 4°C
5. Prepare agarose gel plates, make wells in the center of plates with the help of cork borer and load the wells with the harvested enzyme
6. Incubate the plates for 24 hour at 37 C
7. Add 1% Congo red after 24 hours incubation and wait for 10 minutes and de-stain with 1% NaCl solution if required

Submerged State Fermentation Technique -

1. Prepare 30 ml of liquid MSM medium with 0.1% of birch wood xylan as carbon source and inoculate with 5 agar plugs of 0.5 mm size fungal culture

2. After of 7 days of incubation at 30 C and 150 rpm in an orbital shaker, withdraw the filtrate through Whatman No.1 filter paper

3. Centrifuge the filtrate at 8000g at 4°C for 10 min and collect the supernatant and used as enzyme source for the assay of xylanase activity

4. Prepare agarose gel plates, make wells in the center of plates with the help of cork borer and load the wells with the harvested enzyme

6. Incubate the plates for 24 hour at 37 C

7. Add 1% Congo red after 24 hours incubation and wait for 10 minutes

8. De-stain with 1% NaCl solution if required

Purification of Enzyme –

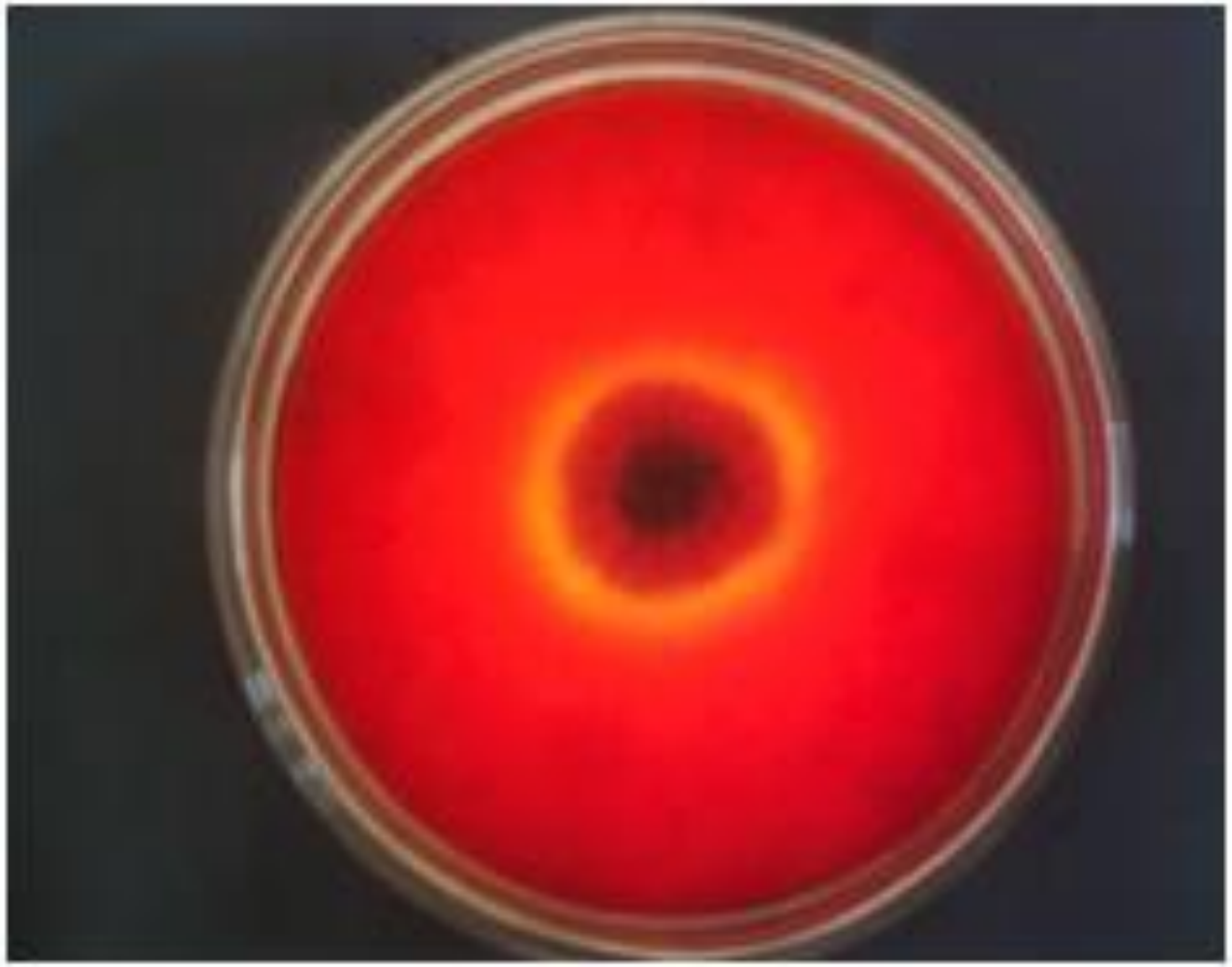
- The crude enzyme received after centrifugations needs purification to remove contaminants
- There may be contamination of unspent media ingredients and other soluble compounds which needs to be removed
- Grow the positive and high potential cultures in starch broth (You need a large amount of culture for purification process)
- Centrifuge the culture and collect the supernatant after 2-4 days incubation
- Add saturated solution of **Ammonium sulphate** to the supernatant for precipitation of proteins, called Salting
- Salting out is a purification method that utilizes the reduced solubility of certain molecules in a solution of very high ionic strength
- Collect the pallet by centrifugation at 10000 rpm for 15 min. (Use cooling centrifuge if available)

Confirmation of Enzyme Activity –

- Resuspend the pallet in Distilled water or any suitable buffer
- Confirm the enzyme activity by repeating the experiment of Secondary Screening
- The pure enzyme may further used for quantitative estimation and electrophoretic separation for characterization of Protein

4. Enzyme Assay:

- Prepare reaction mixture by mixing 0.9 ml of 1% (w/v) birch wood xylan (standard substrate) in 50 mM Na-citrate buffer (pH 5.3) with 0.1 ml of purified enzyme
- Incubated the reaction mixture at 50°C for 5 min, add 1.5 ml of 3,5-dinitrosalicylic acid (DNS) and boil for 5 mins
- Read the colour developed at 540 nm with the help of Spectrophotometer
- Quantify the amount of reducing sugar liberated using xylose as standard
- One unit of xylanase is defined as the amount of enzyme that liberates 1 µmol of xylose equivalents per minute under the assay conditions
- Plot the graph with the readings of OD obtained



Primary Screening

Secondary Screening

Observation

Primary Screening:

S.No.	Name/ Code of Culture	Clear Zone *

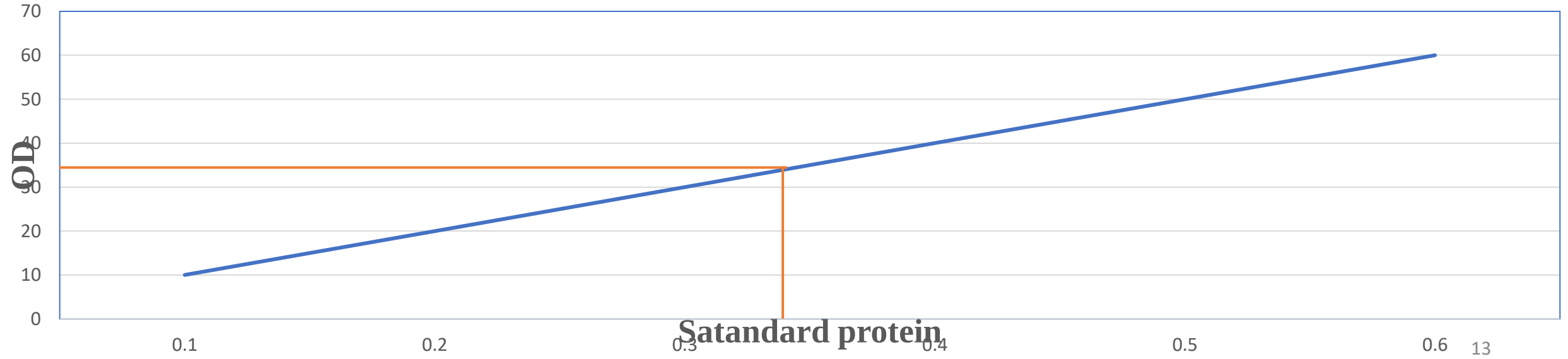
* + Present / – Absent; Observations may be recorded as +/++/+++ or – for low positive, moderate positive or high positive results

Secondary Screening: SSF/ SmF

S.No.	Name/ Code of Culture	Size of Clear Zone (cm/ mm)	
		SSF	SmF

Enzyme Assay:

S.No.	Concentration of Standard (M)	OD at 540 nm
1	0.1	10
2	0.2	20
3	0.3	30
4	0.4	40
5	0.5	50
6	0.6	60
7	Unknown (Enzyme solution)	34



Result – State the result as:

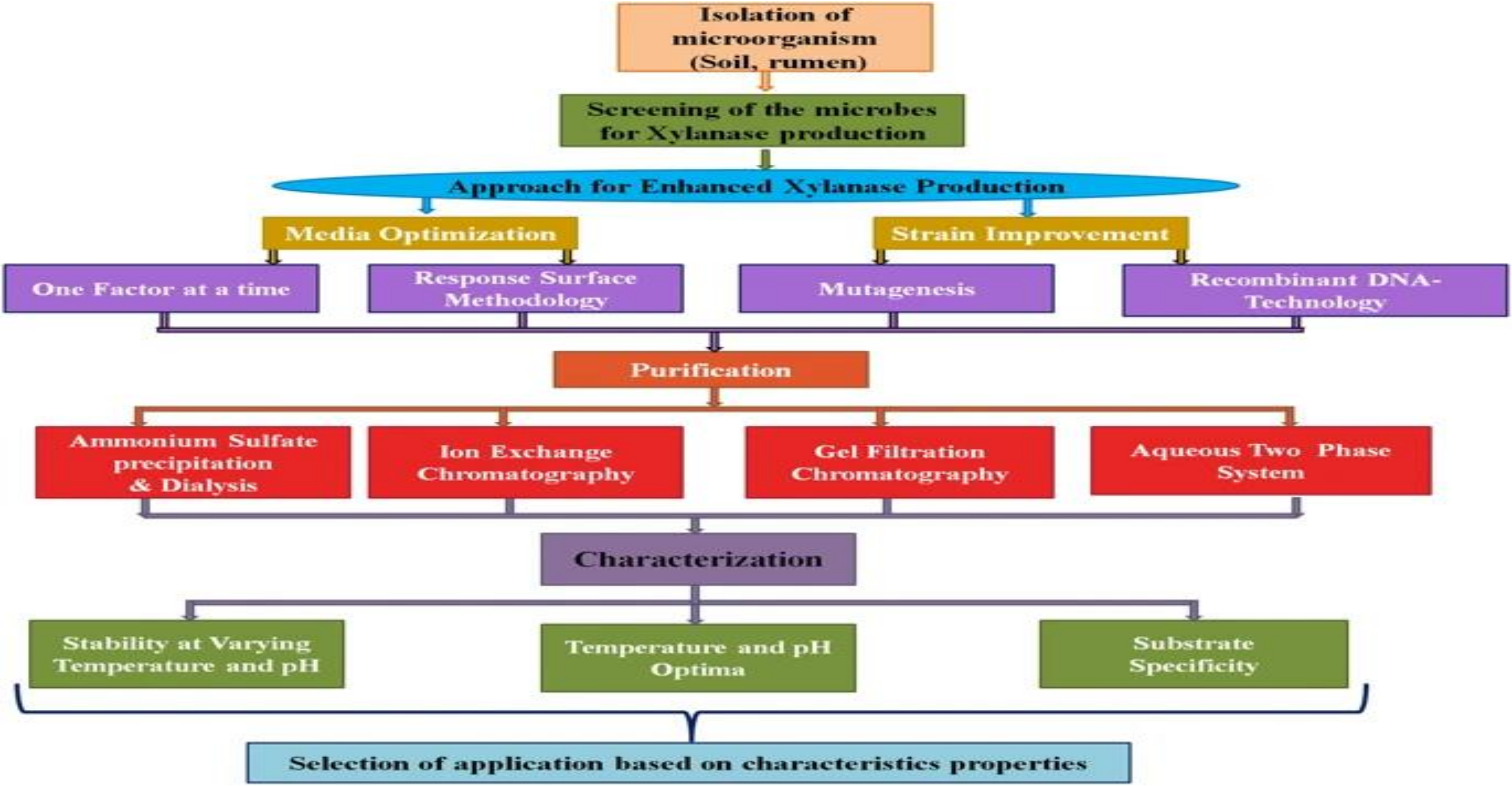
1. Number of positive cultures in primary screening
2. Name of highly positive, moderate positive and least positive cultures in secondary screening
3. Amount of enzyme units /ml of sample

Interpretation –

- Interpret your result as source of microorganism and probability of amylase production

Precautions –

1. All inoculations should be done carefully
2. Care should be taken while handling the centrifuge
3. All observations should be recorded carefully


Isolation of microorganism (Soil, rumen)
Screening of the microbes for Xylanase production
Approach for Enhanced Xylanase Production
Media Optimization
Strain Improvement
One Factor at a time
Response Surface Methodology
Mutagenesis
Recombinant DNA-Technology
Purification
Ammonium Sulfate precipitation & Dialysis
Ion Exchange Chromatography
Gel Filtration Chromatography
Aqueous Two Phase System
Characterization
Stability at Varying Temperature and pH
Temperature and pH Optima
Substrate Specificity
Selection of application based on characteristics properties

Thank You....