COMPARATIVE STUDY FOR DEVLOPMENT OF IDEAL PROTOCOL FOR THE PRODUCTION OF XYLANASE FROM ISOLATED FUNGI USING DIFFERENT SUBSTRATES

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ABSTRACT
The substrate xylan of the enzyme xylanase, is approximately one-third of the renewable organic carbon on Earth and the second most-abundant polysaccharide in nature. The objective was to isolate and identify xylanase producing fungi, optimize substrates and culture conditions for the enzyme production. Xylanase degrade ß -1, 4-xylan in a random fashion, yielding a series of linear and branched oligosaccharide fragments. The fungal strains *Aspergillus fumigatus*, *Aspergillus niger* and *Penicillium spp* were isolated in pure form from agro-industrial waste like cotton, paper, beverage and pharmaceutical industries. The same was confirmed by screening with Congo red test, based on the clear zone of enzyme activity formation in Birchwood xylan Czapek's agar plates. These strains were selected and optimized for xylanase production in mineral's medium using different substrates like wheat bran, rice bran, oat bran, corn cob and sugarcane baggase. Maximum enzyme activity was observed in rice bran and corn cob as substrate at optimum pH-5.5 and temperature (30°C) for xylanase. Thus our present study suggests that fungal strains *Aspergillus fumigatus*, *Aspergillus niger* and *Penicillium spp*. were potential and useful for xylanase production and could be used for various applications of paper, bio-ethanol, bread, food and pharmaceutical industries.

KEYWORDS: Xylanase, Solid State fermentation, Submerged fermentation, substrates

INTRODUCTION
Agro-industrial wastes represent a good resource for generation of energy and production of fibres, chemicals, feed and other products. They are mostly cellulose, hemi-cellulose and lignin, which presents in all lingo-cellulosic materials. Hemi-cellulase is essential enzyme mixtures that hydrolyse the carbohydrates of lingo-cellulosic substrates (Silva *et al*., 2005). Xylan is the major hemi-cellulose constituent of hard wood and soft wood and is the next most abundant renewable polysaccharide after cellulose (Nair *et al., 2008*), So it is not unrealistic to see that coal and crude oil are likely to be substituted by agro-industrial biomass in another 50 years (Kulkarni *et al.,* 1999.

Xylanases are extracellular enzymes produced by microorganisms such as mycorhizic fungi, bacteria, and some yeasts. It's also found in protozoa, insects, crustaceans, snails, seaweed and also seeds of plants during the germination phase in soil (Wong *et al*., 1988).

In the present study protocol has been formulated starting with the isolation, screening and identification of xylanase producing fungi. After identification the strains has been characterised for growth at different pH, temperature and non-metals and surfactants. The optimum cultural parameters of the best strain have been looked into and data on optimum parameters has been evaluated. Results will be expected to isolate a fungal strain capable of producing xylanase of good grade.

MATERIAL AND METHOD
Collection of Sample and Isolation of Fungi
Samples were collected as soil from different agro-industrial waste (Cotton, Paper, Beverage and Pharmaceutical industry) and suspended in double distilled water and after serial dilution (Dilutions 10^{-2}, 10^{-3} and 10^{-4}) 0.100ml spread over SDA (Sabouraud Dextrose Agar media = Peptone – 10g/l, Dextrose – 40g/l, Agar – 15%, pH = 5.6 ± 0.5) (Nair *et al*., 2008) and incubated at 30 ± 2^0C and sub-cultured for purify and preserve under refrigeration conditions on SDA for further study.

Screening and Identification of Xylanase Producing Fungi
The isolates were screened for their ability to produce xylanase by growing them on modified Czapek's agar media (birchwood xylan– 2.0g/l, peptone – 5.0g/l, yeast extract – 5.0g/l, K_2HPO_4 – 1.0g/l, $MgSO_4.7H_2O$ – 0.2g/l, Agar – 15%, pH = 5.2 ±0.5) medium. After inoculation, plates were incubated for 7 days at 30˚C and their ability was confirmed by using Congo red 0.1% (w/v). After 30

minutes of incubation, plates were washed with 1.0 M NaCl (Nakamura *et al*., 1993). After confirmation of xylanase producing fungi, these were identified by morphology, mycelial growth under microscope by Lactophenol Blue staining and available literature and manuals (Ellis *et al*., 2007, Thom and Raper, 1945).

Comparative Study of Solid State and Submerged Fermentation for Xylanase Production

Enzyme production compared at different substrate (wheat bran, rice bran, corn cob, oat bran and sugarcane baggase) with isolated culture by solid state fermentation (SSF) and submerged fermentation (SMF). In SSF and SMF, all isolated cultures were inoculated in selected substrate with pH-5.2 ±0.2 media and remain composite of media is KCl – 0.5, $MgSO_4.H_2O$ – 0.5, $(NH_4)_2HPO_4$ – 2.5, NaH_2PO_4 – 0.5, $CaCl_2.2H_2O$ -0.01, $FeSO_4.7H_2O$- 0.01, $ZnSO_4.7H_2O$ – 0.002, along Birchwood xylan – 1.0 g/l as control or reference. In 25 ml of culture, add 4 discs of 5.0 mm obtained from isolates and incubated at 30±2˚C for 7 days. After incubation, fermented medium is filtered with filter paper and subjected to centrifugation at 10,000 x g for 20 min at 4˚C. The obtained supernatant used for enzyme assay.

Activity of Xylanase

Using known concentration of xylose as standard curve, Xylanase activity was determined by mixing 0.9 ml of 0.1% (w/v) birch wood xylan (prepared in 50 mM Na-Acetate buffer, pH - 5.2±0.2) with 0.1 ml of enzyme and mixture was incubated at 50°C for 5 min and stopped reaction by addition of 1.5 ml of 3, 5-dinitrosalicylic acid (DNS) and contents were boiled for 5 min (Miller, 1959, Bailey et al., 1992). After cooling, develop colour was read at 540 nm. The amount of reducing sugar liberated was quantified using xylose as standard. One unit of xylanase is defined as the amount of enzyme that liberates 1.0 µmol of xylose equivalents per minute under the assay conditions.

Mycelia Dry Weight

Mycelia dry biomass was collected on a pre-weighed Whatmann filter paper 5, dried to a constant weight at 60°C and reweighed. The difference in weight denoted the mycelial growth of fungus.

Effect of Temperature

The effect of temperature on xylanase produced was determined by growing fungal isolates on different temperatures *viz*.20°C, 30°C, 40°C, 50°C and 60°C on SSF.

Effect of pH

The effect of pH was determined by growing the isolates on different pH ranges, maintained by buffer solutions of different pH like Glycine buffer (3.0), Acetate buffer (4.6), Sodium acetate buffer (5.5), Tris-HCl buffer (7.4) and Glycine-NaOH buffer (8.6) on SSF.

Effect of Non-metals, Surfactants

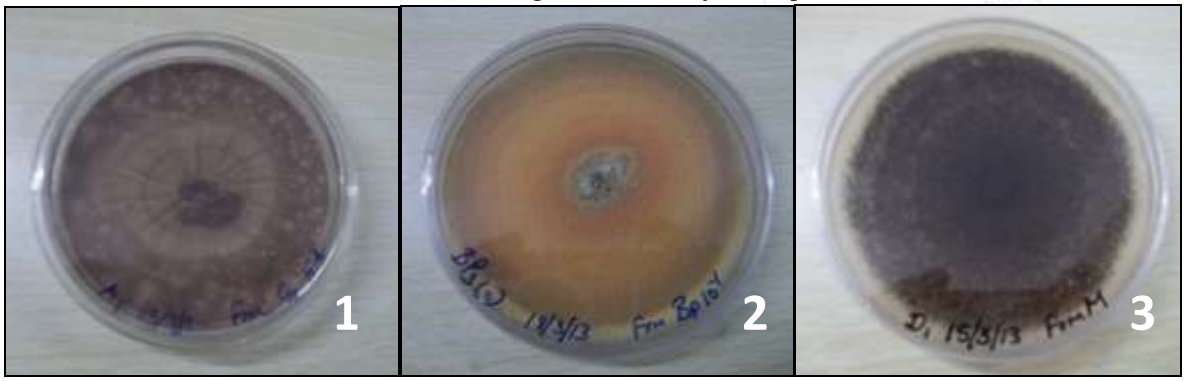
The effect of non-metals on xylanase activity was determined by using different non-metals and surfactants in the production medium like NaCl, $CaCl_2$, KCl, $FeSO_4$, $BaCl_2$, $MnSO_4$, $ZnSO_4$, $MgSO_4$, SDS, and Tween 20 on SSF.

RESULTS AND DISCUSSION

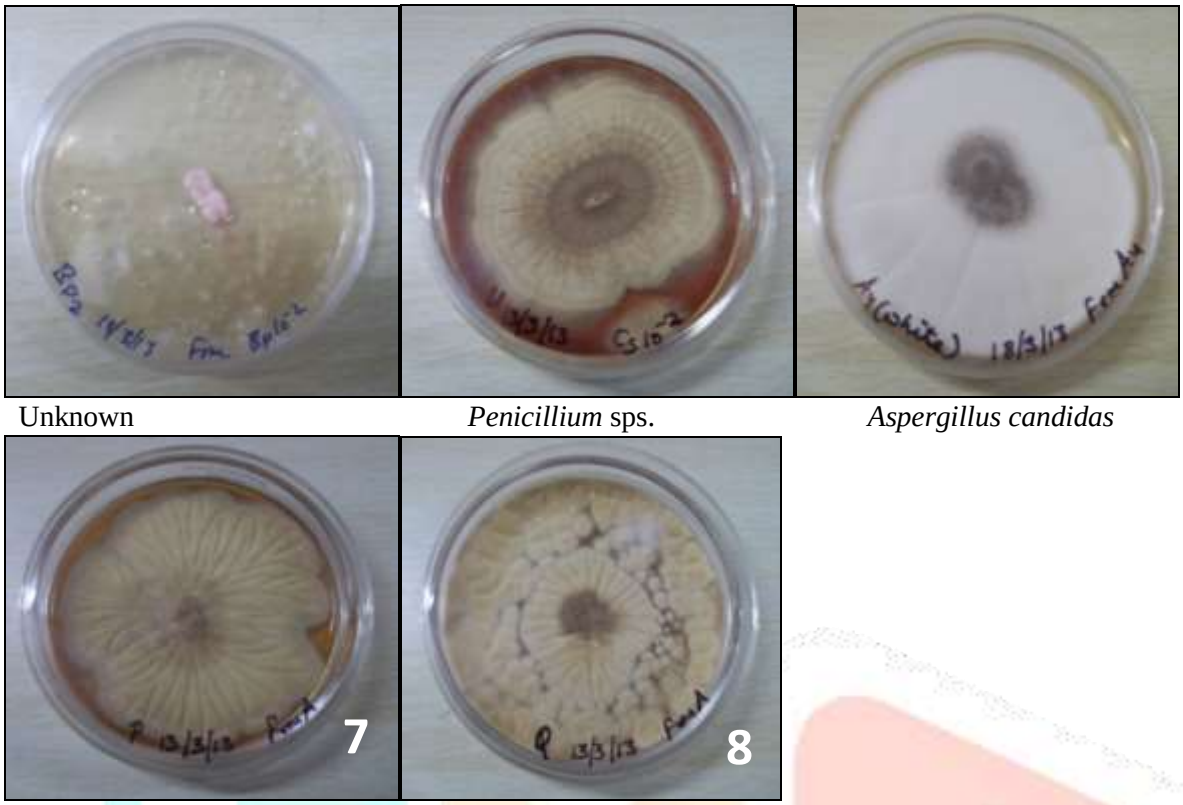
Xylanase is a good source for breakdown of xylan, lignin and cellulose in food, paper industries. Therefore, solid state and submerged fermentation carried out and enzyme activity was measured to distinguish potential xylanase producing fungi and fermentation. A higher efficiency on enzyme production by SSF is described by several authors for various enzymes and microorganisms. On comparative analysis of xylanase production over different substrates, SSF is better than SMF.

Identification and Screening of Fungus

Isolated fungi were identified with lacto-phenol blue staining, morphological structure, available literature and manuals. After the identification, all the fungi were screened for extracellular enzyme activity on czapak dox agar medium containing xylan as carbon source for production of xylanase. When isolates were inoculated in czapak dox agar media and subsequently stain with congo red solution then zone of clearance around microbial growth shown xylanase production.



Aspergillus fumigatus *Aspergillus versicolor* *Aspergillus niger*



Unknown *Penicillium* sps. *Aspergillus candidas*

Penicilium sp. Aspergilus sp.

Fig: 1. - Isolated xylanase producing fungi on SDA.

S. Code	Scientific Name	Czapak Dox Agar (CDA) media				
		CM (mm)	MDW	T (^{0}C)	pH	Enzyme Activity (nkatal/m)
A1	*Aspergillus fumigatus*	15.8	1.35	30	5.2	59.132
BP3	*Aspergillus versicolor*	15.6	0.95	30	5.2	55.328
M	*Aspergillus niger*	15.0	1.52	30	5.2	51.326
BP2	Unknown	14.8	1.40	30	5.2	48.22
U	*Penicillium* sps.	15.0	1.50	30	5.2	53.57
A4	*Aspergillus candidus*	15.5	1.45	30	5.2	54.232
P	*Penicillium* sps.	15.2	1.56	30	5.2	52.61
Q	*Aspergillus* sps.	14.3	1.30	30	5.2	49.05

Table-1- Screening of fungi for xylanase production, hydrolysed zone on Czepak media (CM), Mycelial Dry Weight (MDW), Temperature **(^{0}C)**

Selection of Method and Substrate

We compared that which one is best method for production of xylanase enzyme between SSF and SMF with different substrate (wheat bran, oat bran, corn cob, and rice bran and sugarcane bagasse). The isolates which gave maximum production were *Aspergillus fumigatus, Aspergillus niger and Penicillium spp.* over two substrates (rice bran and corn) giving maximum production in SSF.

S.Code	Scientific	Enzyme activity on selected substrate in nkatal/ml

	Name	Solid state fermentation					Submerged fermentation				
		WSSF	RSSF	CSSF	OSSF	SSSF	WSSF	RSSF	CSSF	OSSF	SSSF
A1	*Aspergillus fumigatus*	38.41	72.23	70.76	44.74	69.52	64.27	66.03	67.84	39.16	66.39
BP3	*Aspergillus versicolor*	34.76	68.04	68.94	24.24	15.66	65.77	64.92	51.07	30.38	36.46
M	*Aspergillus niger*	43.88	67.96	71.65	56.04	72.1	67.7	31.2	57.72	64.33	67.29
BP2	Unknown	45.38	71.74	38.3	50.64	55.04	50.49	60.51	48.6	13.73	47.63
U	*Penicillium* sps.	52.89	69.93	72.83	62.44	59.76	52.89	62.45	68.38	40.34	47.96
A4	*Aspergillus candidus*	55.04	71.12	69.05	41.41	59.54	55.04	67.88	52.46	26.28	65.53
P	*Penicillium* sps.	44.74	72.53	68.00	42.06	35.72	44.74	68.06	62.98	40.94	63.96
Q	*Aspergillus* sps.	29.72	70.58	68.88	58.26	67.81	29.72	60.4	64.64	43.99	66.84

TEBLE – 2 - Compararive Xylanase Activity on Different Substrates

Effect of temperature

Aspergillus fumigatus, Aspergillus niger and Penicillium sps were inoculated at different temperature 20, 30, 40, 50, and 60°C in rice bran and corn cob respectively for 7days incubation. The increase in temperature above 30°C not only inhibited the fungal growth but also the production of xylanase. Its indicating that 30°C is the optimum temperature for xylanase production with maximum enzyme activity.

Effect of pH

To optimize pH for xylanase production, *Aspergillus fumigatus, Aspergillus niger and Penicillium* sps were inoculated at different pH with buffer solution Glycine-HCl buffer (pH-3.0), Acetate buffer (pH-4.6), Glycine-NaOH buffer (pH- 5.5), Tris-HCl buffer (pH- 7.4) and Sodium Acetate buffer (pH-8.6). Optimum pH found to exhibit maximum enzyme activity was 5.5 having best growth in both substrates. During fermentation, it was monitored that enzyme activity decreases with increase/decrease in pH.

Effect of non-metal and surfactant

The effect of certain non-metal ions and surfactant on xylanase activity was investigated. Ions like KCl, $BaCl_2$, $MnSO_4$ and ZnSO4 supported growth of fungi and a good enzyme activity was observed in their presence. These ions act as

growth promoters for the xylanase activity. Whereas some ions like NaCl, SDS and surfactant like Tween 20 inhibits enzyme activity. They act as inhibitors for the growth of fungi producing xylanase.

The known fungus are produced a variety of enzyme including xylanase. But our data considered only for xylanase. Temperature and pH are an important parameter for production of xylanase and 20-30ºC at pH 5.5. Xylanase production observed best at 30ºC with fungal isolates and with increase or decrease in temperature or pH, activity of enzyme reduced, probably due to conformation change or denaturation. The influence of certain non-metal ions and surfactant on xylanase activity was investigated. Xylanase activity decreased in the presence of NaCl, SDS and Tween 20. We are aware that irrespective of the medium used in combination with substrate and conditions such as degree of aeration, substrate, pH and temperature during fermentation regulate the enzyme production. Nair *et al*., 2008, Biswas *et al*., 2006 and Gilbert *et al*., 1992 found that screening is carried out on pure and defined substrate for isolate xylanase producing fungi. Therefore in our studies, we have found species of Aspergillus as xylanase producing. So by providing these fungal strains the favourable conditions of substrate i.e. corn cob and rice bran, under optimum pH of 5.5 with inducer non-metal ions $ZnSO_4$, KCl, and $BaCl_2$, activity of xylanase will be satisfactory.

Conclusion

In the present study we had aimed to isolate the strains of fungi that produce xylanase. Total 8 strains of fungi were isolated from five different agro-industrial waste producing industries of Punjab. Out of 8 isolated fungal strains, 3 fungi were finally selected by using Congo red assay. Seven isolates of fungi were identified on the basis of their morphology of genera *Aspergillus* and *Penicillium*. Selected 3 strains produced xylanase without an apparent decrease in their activity. Out of these *Aspergillus fumigatus* Fresensius showed remarkable activity. Therefore these strains can be suggested for selection for further production of xylanase that is demanded by paper industry, textile industry, food industry, pharmaceutical industry and deinking of waste water.

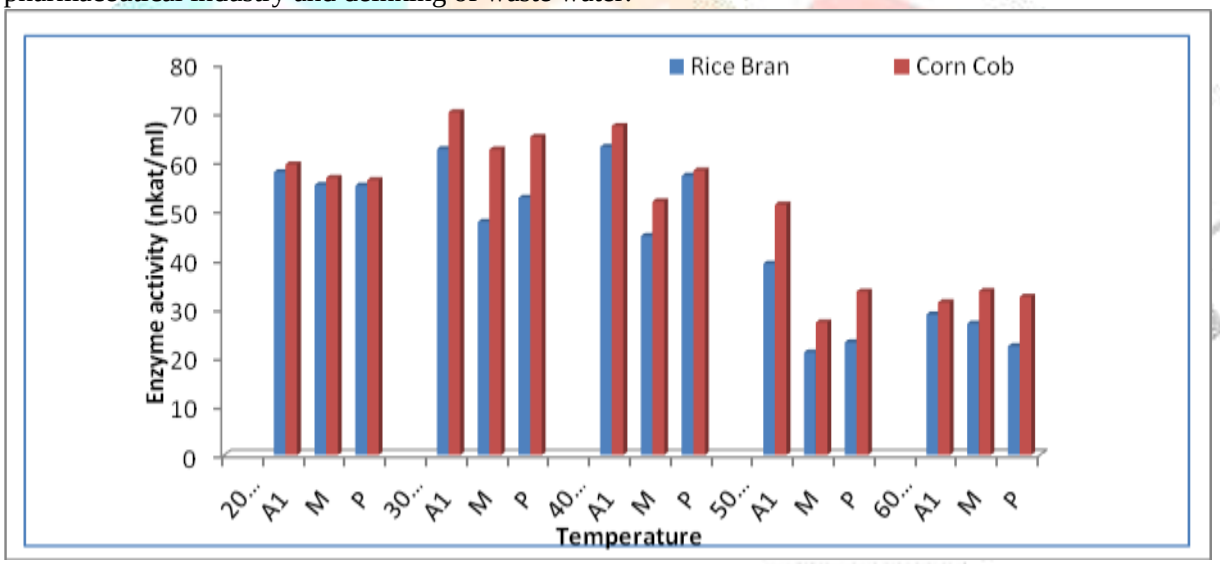


Fig. –2- Effect of temperature on production of xylanase

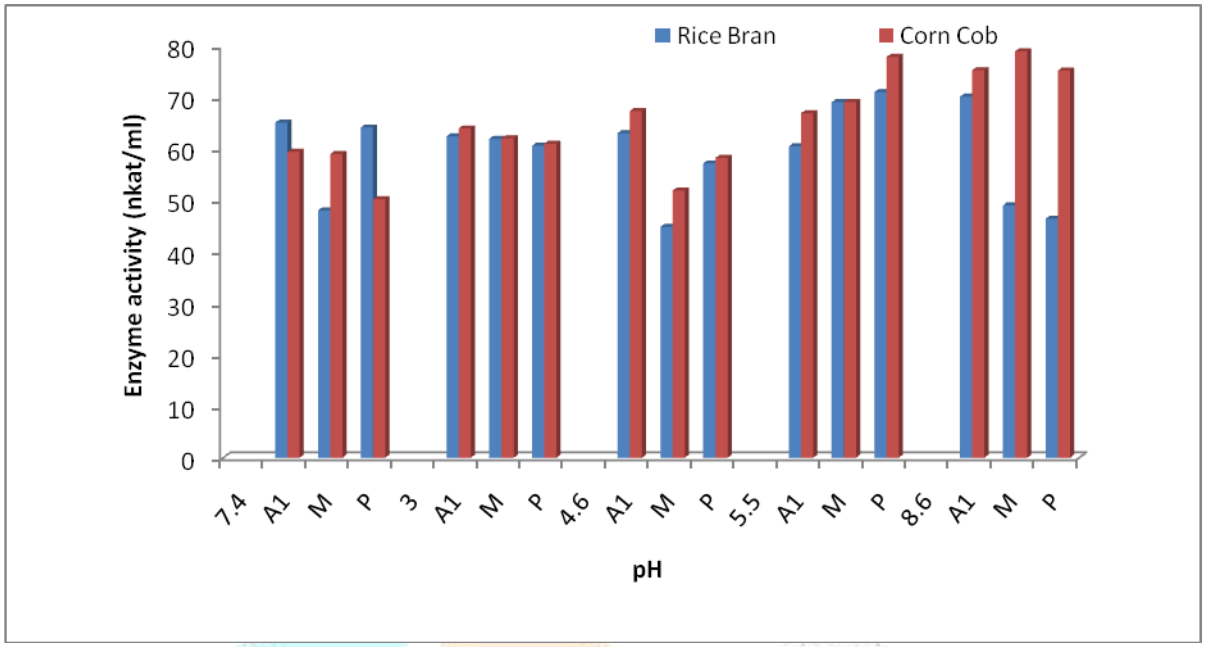


Fig. –3 - Effect of pH on enzyme activity

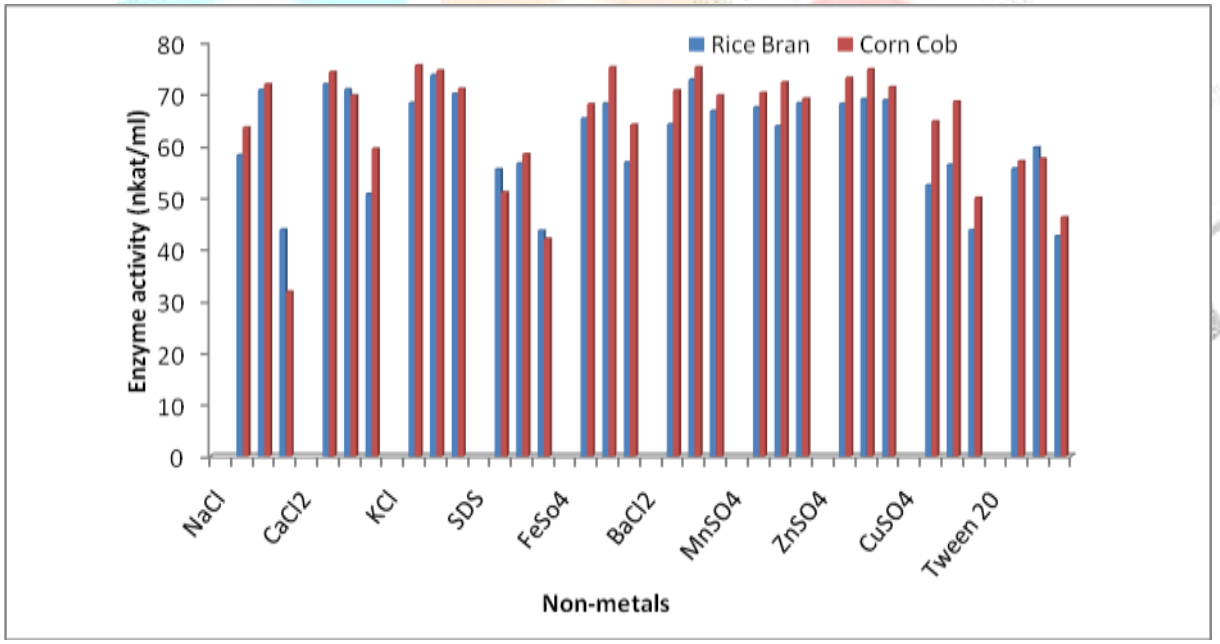


Fig. –4 - Effect of non-metal and surfactant

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