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Screening, Isolation and Characterization of Amylase Producing Bacteria and optimization for Production of Amylase

Alka Ekka[1], Neha Namdeo[2]
[1, 2](Department of Biotechnology, Guru GhasidasVishwavidyalaya, Bilaspur, Chhattisgarh, India)
Corresponding Author: Alka Ekka

Abstract: *Amylase is (E.C.3.2.1.1-1,4-alpha D-glucanohydrolase) an extracellular enzyme, which is involved in the starch processing industries where it breaks starch into simple sugar constituents.Amylase has extensive application in starch processing, brewing and sugar production, in textile industries and in detergent manufacturing processes. Interestingly, the first enzyme produced industrially was an amylase.In the present study, amylase producing bacteria were isolated from rice field, sugarcane field and sugarcane dump area and characterized for their morphological and biochemical properties. Then amylase activity of isolated bacterial cultures were determined and it was concluded that 3 (NN1, NN2, NN5)out of 6 bacterial colonies(NN1, NN2, NN3, NN4, NN5, NN6) were potent and their enzyme activity was more than other colonies. The potent colonies were also optimized for enzyme activity under certain conditions like different carbon sources, nitrogen sources, pH, incubation time and chlorides.Agro-industrial wastes were used as substrate for amylase production by Solid-State FermentationSSF) and we have found that wheat bran was the suitable substrate for amylase production.*

Key words: *Amylase, solid state fermentation, agro- industrial wastes*

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I. Introduction

Amylase is (E.C.3.2.1.1-1,4-alpha D-glucanohydrolase) an extracellular enzyme, which is involved in the starch processing industries where it breaks starch into simple sugar constituents. Alpha amylase have extensive application in starch processing, brewing and sugar production, in textile industries and in detergent manufacturing processes. Interestingly, the first enzyme produced industrially was an amylase from fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (Pandey *et al*., 2000). Amylases are among the most important enzyme and account for about 30% of the world's enzyme production (*Kandra*, 2003). These enzymes are found in animal, plant, bacteria, and fungi. The different types of microorganisms like bacteria, fungi and yeast have been reported as the source of amylase and their properties are described (Gupta*etal*., 2008). Although there are many microbial sources available for amylase production,*Bacillus* strain have capability to produce large quantities of amylase. They produce about 60% of enzymes that arecommercially available. The most widely used thermostable amylase in the starch industries is produced by *Bacillus subtilis* and *Bacillus mesentericus.* Enzymatic conversion of starch to dextrin and sugar take place by the enzyme obtained from plant, animal, bacteria, and fungi.

Amylase plays an important role in the biogeochemical cycle of carbon and also has a wider application in the biotechnological based food, detergent and pharmaceutical industries. Amylase has ability to hydrolyze glycosidic linkage in polysaccharide. Amylase present in human saliva, where it begins the chemical process of digestion, food that contain much starch but little sugar, such as rice and potato. Taste slightly sweet as they are chewed because amylase turns some of their starch into sugar in the mouth. The Pancreas also makes amylase (alpha amylase) to hydrolyze dietary starch into disaccharides and tri-saccharides which are converted by other enzyme to glucose to supply the body with energy. Alpha amylase can also be derived from various sources such as plant, animal and microorganisms. In recent year, a number of new enzymes associated with degradation of starch and related polysaccharides structures have been detected and studied.

II. Material and Methods

2.1 Soil sample collection: Soil samples were collected from rice field, sugarcane field and sugarcane dump area of Bilaspur, Chhattisgarh.

2.2 Isolation of amylase producing bacteria: Amylase producing bacteria were isolated from serial dilution method. 1g ofeach soil sample was mixed in 10 ml of distilled water and transfer in test tubes for dilution i.e.$10^{-1}$to 10^{-10} and further o.1ml of sample was inoculated into sterilized nutrient agar media plates and spreads on the

plates. All the plates were incubated at 37ºC for 24hours.

2.3 Screening of amylase producing bacteria: Starch hydrolysis test was done for screening of amylase producing bacteria. Bacterial isolates were streaked on the starch agar medium plates and incubated at 37^0C for 24-48 hours.After incubation Iodine solution was flooded in the plates with the help of dropper.The plates were then kept undisturbed for 5-10 minutes and then the iodine solution was discarded from the plates.

2.4Morphological and biochemical Characterization of bacteria: Screened amylase producing bacteria were morphologically characterized by Gram's staining method and characterized biochemically by catalase test, oxidase test (Filter paper method), indole Test, methyl red test ,Voges-Proskauer (VP) test,citrate utilization test,gelatin hydrolysis test, urease test.

2.5 Production of amylase and enzyme assay: The isolates showingclear zone around them was propagated in broth supplemented with 1% starch medium in shaking incubator at 150 rpm at 37°C for 24 hrs.After incubation, the resultant broth was centrifuged at 10,000 rpm for 10 minutes at 4°C.The supernatant was recovered and was used as source of crude enzyme.1ml of crude enzyme and 1ml of 1% soluble starch in Sodium phosphate buffer(pH 7) was added in a test tube.The test tubes were covered and incubated at 35°C for 10 minutes.Then 2ml DNS reagent was added in each tube to stop the reaction and kept in boiling water bath for 10 minutes.After cooling at room temperature, final volume was made to 10ml using distilled water.The absorbance was read at 540 nm by spectrophotometer against maltose as standard. (Pokhrel B. et al, 2013).

2.6 Optimization for amylase production: Amylase producing bacteria were optimized for amylase production. For optimization different carbon source (lactose, fructose, sucrose and dextrose), nitrogen source(Alariyaet al,2013), nitrogen sources **(**potassium nitrate, ammonium sulfate, sodium nitrate, ammonium nitrate, ammonium chloride, casein, peptone, urea, gelatin and yeast extract),at different pH (5, 6, 7 and 8), at different incubation time **(**0, 24, 48,and 72 hrs), examined at different chlorides (potassium chloride, sodium chloride, magnesium chloride, barium chloride, ammonium chloride, ferric chloride and calcium chloride) to test its ability to induce amylase productionin the production medium. The enzyme activity is measured after 24h of incubation. (Alariya et al,2013).

2.7 Uses of agro industrial wastes as substrate: Different types of agro-industrial wastes were used as substrate like Wheat bran, Gram husk, Rice bran, Potato peel, Sugarcane baggase, etc. Experiments were conducted in 100ml Erlenmeyer flasks containing 5g of the substrate impregnated with 10ml of sterile liquid nutrient medium containing(%):[KH_2PO_4-0.1, NaCl-0.25, $MgSO_4.7H_2O$-0.01, $CaCl_2$-0.01] with agro-industrial wastes by solid state fermentation process and inoculated with 1ml of the prepared inoculums, thoroughly mixed and followed by incubation at 37°C for 5 days. Then the enzyme assay was carried out. (Saxena and Singh, 2011).

III. Result and Discussion

3.1 Isolation and Screening of amylase producing bacteria: After performing serial dilution method, numerous colonies were obtained from different soil samples. From those colonies, 12 colonies were picked and streaked in NAM plates and pure culture of those colonies were prepared. 12 colonies were grown in Starch agar plate and out of 12 colonies, 6 colonies showed clear zone around them and thus it shows the presence of amylase producing bacteria. They were named as Colony NN1, NN2, NN3, NN4, NN5 and NN6 respectively. Colony NN1, NN3 and NN4 are isolated from soil sample of Sugarcane field, Colony NN2 from soil sample of Rice field and Colony NN5 and NN6 from soil sample of Sugarcane dump area. Oseni and Ekperigin, 2013 isolated the bacteria from forest soil, Pokhrel*et al*, 2013 isolated amylase producing bacteria from sewage enriched soil, Verma et al, 2011 isolated amylase producing bacteria from waste potato dumpsite.

3.2 Morphological and Biochemical characterization of amylase producing bacterial isolates

Table 1: Gram's staining of Amylase producing bacterial isolates

Isolate	Result
NN1	Gram Positive
NN2	Gram Positive
NN3	Gram Negative
NN4	Gram Negative

NN5	Gram Positive
NN6	Gram Positive

Table 2: Biochemical tests of isolates

Test	NN1	NN2	NN3	NN4	NN5	NN6
Catalase Test	+	+	+	+	+	+
Oxidase Test	+	+	+	+	+	+
Indole Test	+	+	+	+	+	_
MR Test	_	+	_	+	_	+
VP Test	_	_	_	_	_	_
Citrate Utilization Test	+	+	+	+	+	_
Gelatin Hydrolysis Test	+	+	_	+	_	_
Urease Test	_	_	_	_	_	_

3.3 Production of amylase and enzyme assay

Table 3-Amylase Activity of amylase producing bacterial isolates

Isolates	Amylase Activity(U/ml)
NN1	0.251
NN2	0.127
NN3	0.094
NN4	0.115
NN5	0.144
NN6	0.098

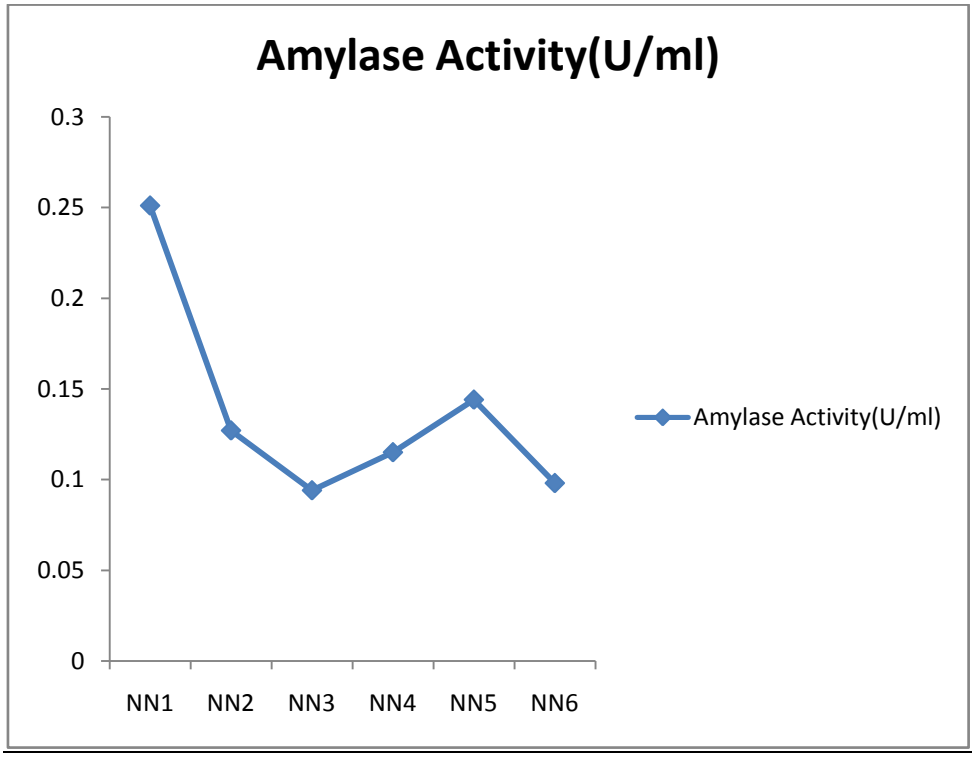

Fig.1. Amylase activity of bacterial isolates

On the basis of amylase activity of 6 amylase producing colonies, it is known that isolate NN1, NN2 and NN5 having amylase activity 0.251U/ml, 0.127U/ml and 0.144U/ml respectively are the 3 potent amylase producing isolates.

3.4 Optimization for amylase production: further optimization study we have chosen three isolates NN1, NN2 **and NN5.**

3.4.1 Optimization of carbon source for amylase production

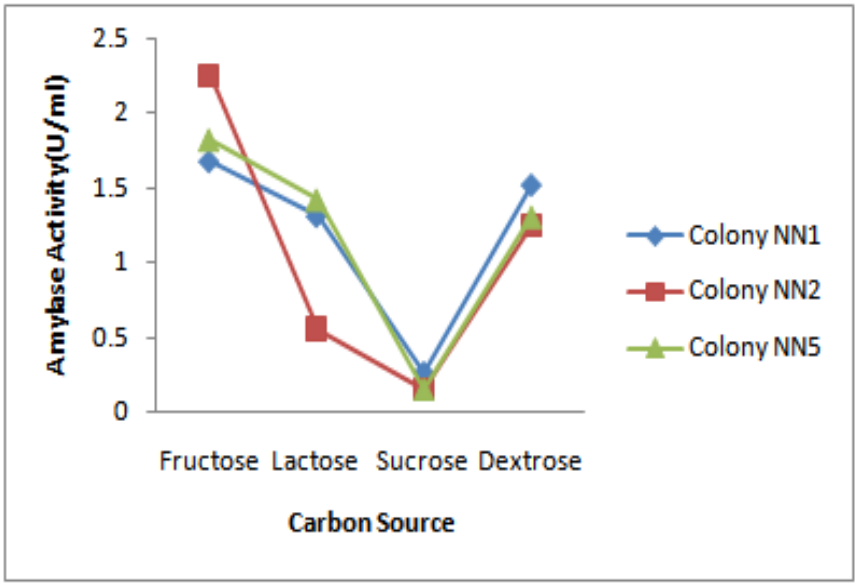

Fig. 2: Various sources of carbon such as Fructose, Lactose, Sucrose and Dextrose were used to replace Starch which was the original carbon source in growth media. Results obtained showed that, Fructose brought the highest amylase production compared to other carbon sources at 24 hrs incubation in all the 3 colonies.

3.4.2 Optimization of organic and inorganic nitrogen source for amylase production

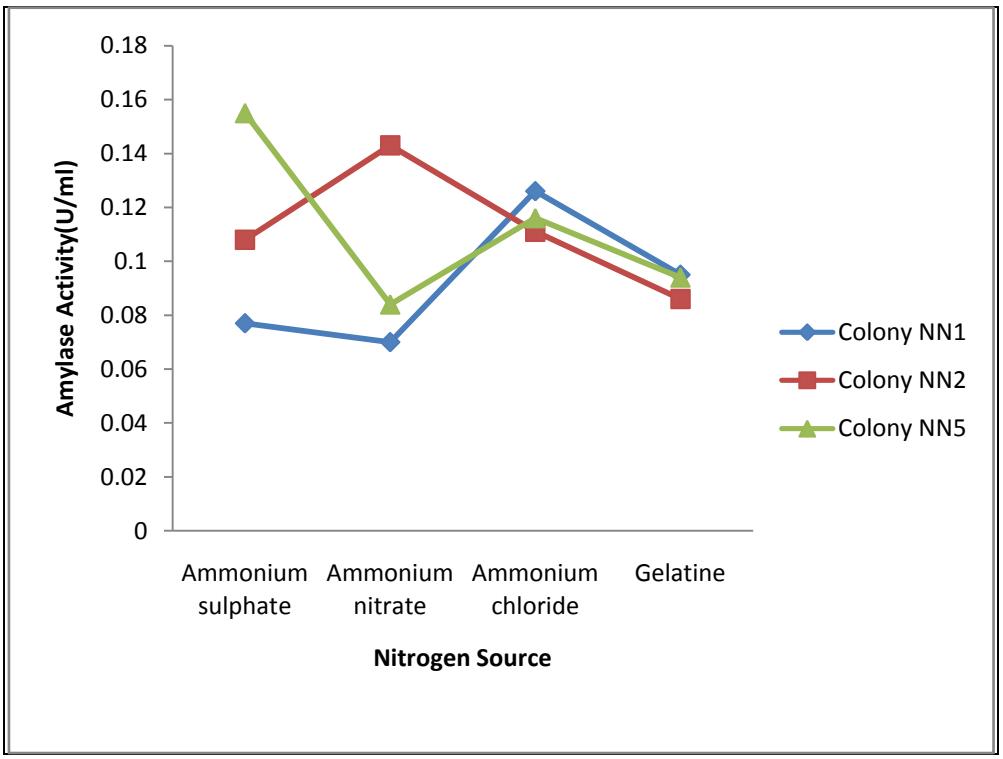

Fig. 3: Various sources of Nitrogen such as Ammonium sulphate, Ammonium Nitrate, Ammonium Chloride and Gelatin were used as nitrogen source in growth media. Results obtained showed that, Ammonium Chloride brought the highest amylase production in Colony NN1, Ammonium Nitrate in Colony NN2 and Ammonium sulphate in Colony NN5 compared to other nitrogen sources at 24 hr incubation.

3.4.3 Optimization of pH for amylase production

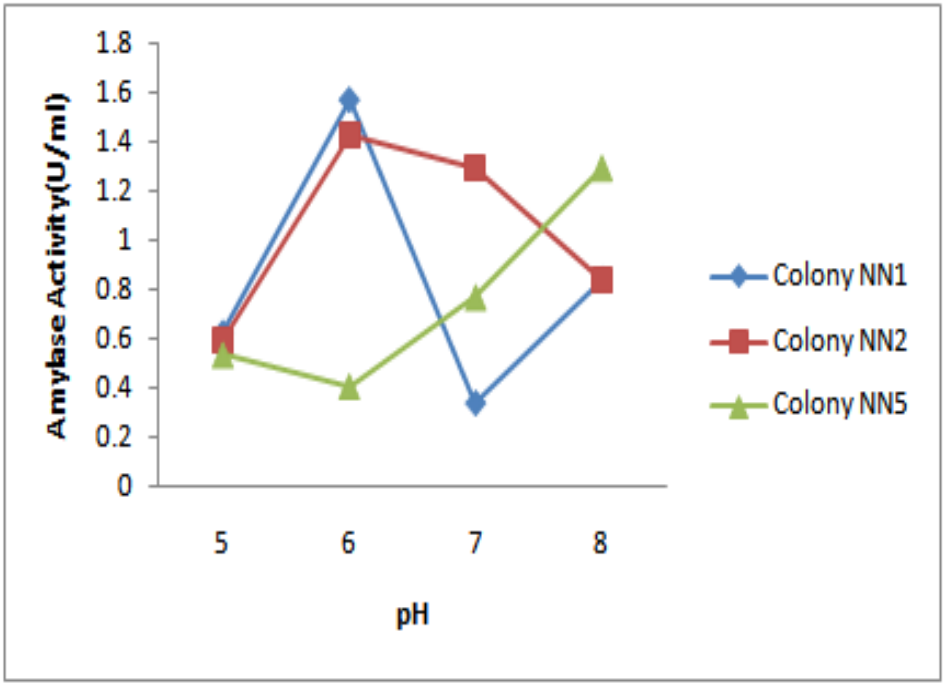

Fig. 4: All the three colonies were allowed to grow in media of different pH ranging from 5.0 to 8.0. Maximum enzyme activity was observed in medium of pH 6.0 in case of Colony NN1 and NN2 whereas Colony NN5 showed maximum enzyme activity in medium of pH 8.0.

3.4.4 Optimization of incubation period for amylase production

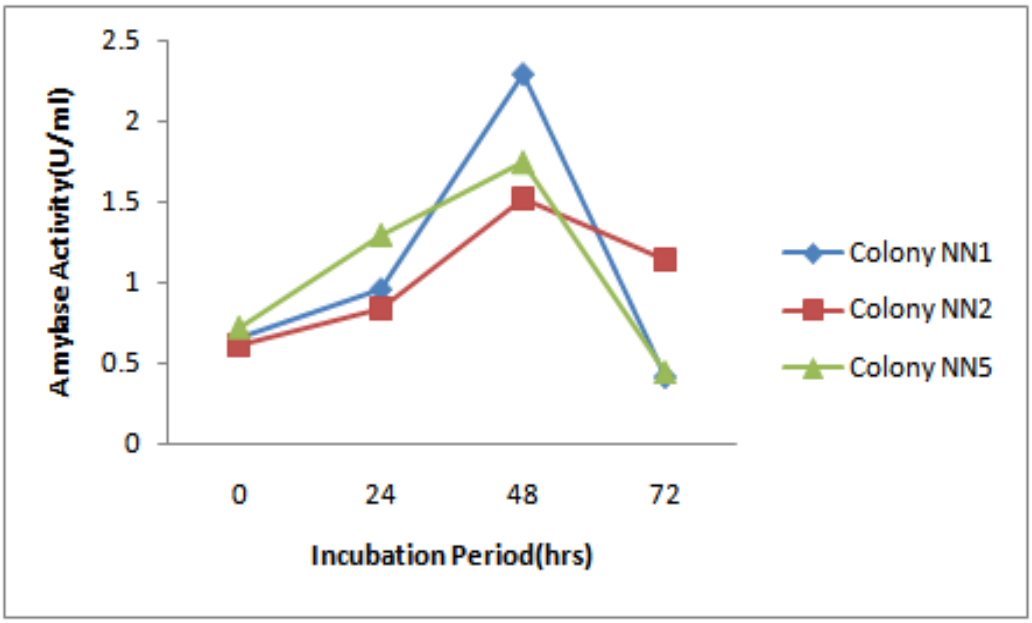

Fig. 5: Effect of incubation period on amylase production showed that 48 hours was the optimum duration for maximum amylase activity for all the three colonies. Above this period the amylase activity started to decrease. This is because, the cells may reach the decline phase and displayed low amylase synthesis.

3.4.5 Optimization of chlorides for amylase production

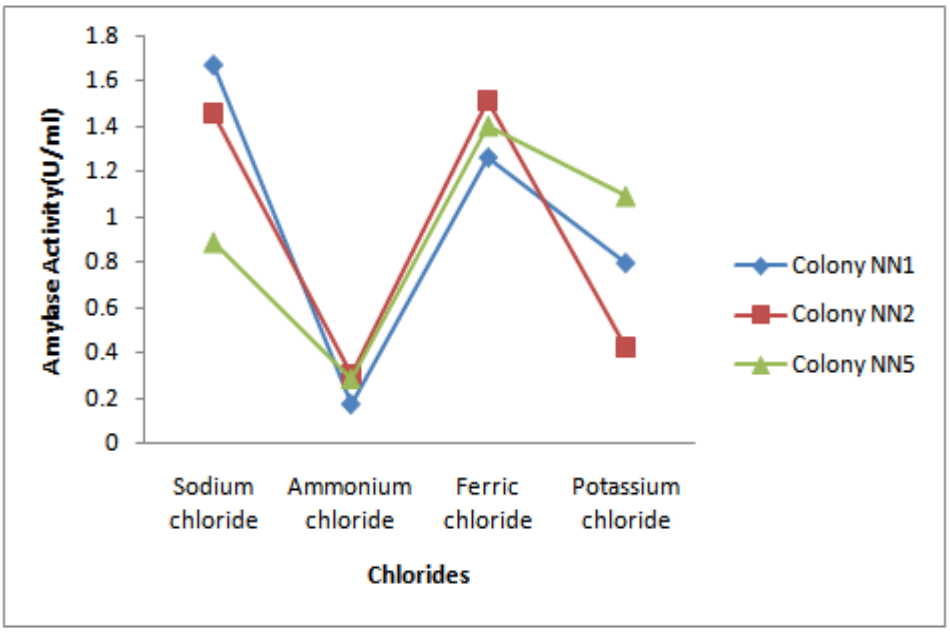

Fig. 6: Ferric chloride was found to be the most suitable chloride source for Colony NN2 and NN5, whereas Sodium Chloride for Colony NN1. Supplementation of salts of certain metal ions provided good growth of microorganisms and thereby better enzyme production.

3.5 Uses of agro industrial wastes as substrate: For the production of amylase agro wastes such as wheat bran and potato bran were employed in solid state fermentation technique. And find out the enzymatic activity.

Table 4: Amylase activity of potent bacterial colonies in SSF technique

Substrate	Amylase activity (U/ml)		
	NN1	NN2	NN5
Wheat bran	0.902	0.880	1.027
Potato peels	0.581	0380	0593

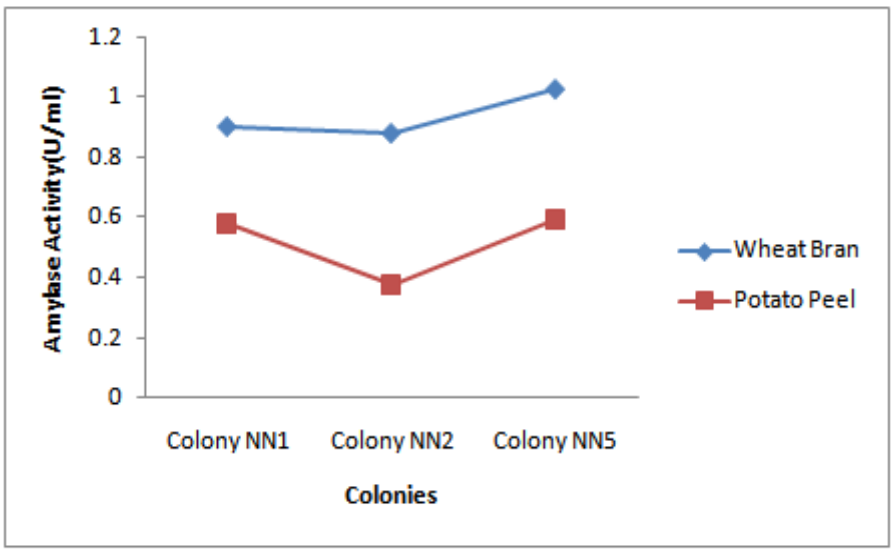

Fig. 7: It shows the enzyme production of the 3 potent colonies with two substrates (Wheat bran, Potato peel). The best productivity was observed with Wheat bran for all the 3 colonies with enzyme activity of **0.902U/ml, 0.880U/ml and 1.027U/ml** respectively for Colony NN1, NN2 and NN5 as compared to potato peel.

IV. Conclusion

In the present dissertation, the amylase producing bacteria were isolated from different soil samples. The isolated bacteria were characterized through morphological characterization by Gram staining and several biochemical tests such as Catalase test, Oxidase test, IMViCtest (Indole test, MRVP test, Simmon's citrate test); etc. Morphologically the isolated bacteria were rod-shaped. Amylase enzyme activity was determined and it was concluded that 3 out of 6 bacterial colonies were potent and their enzyme activity was more than other colonies. The potent colonies were also optimized under certain conditions like different carbon sources, nitrogen sources, pH, incubation time and chlorides. Amylase production by Solid-State Fermentation of Agro-industrial wastes was also done using cheap substrates and Wheat bran was found as the suitable substrate.

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