SCREENING OF POTENTIAL ISOLATE FOR THE PRODUCTION OF AMYLASE WITH DIFFERENT AGRO WASTES AS SUBSTRATE

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Abstract: Amylolytic bacteria were isolated from decayed pomegranate. The bacteria were screened for amylase activity using starch agar plate assay. Five different substrates were used namely banana peel, *Gossypium* oil cake, groundnut oil cake, cassava, and wheat bran; for microbial production of amylase by Submerged fermentation (SmF) and it was found that maximum amylase activity (2.005 U/ml) specific activity (0.520U/mg) was produced by bacterial isolate 8 using cassava as a substrate.

***Index terms*- Amylase, submerged fermentation, agro-waste substrates**

I. INTRODUCTION

Amylolytic enzymes are of great significance in present-day biotechnology, which are obtained from various plant sources, animals and microorganisms. The short growth period of microbial sources made them to meet the industrial demands. The microbial amylases have almost completely replaced the chemical hydrolysis in starch producing industries and these enzymes have a wide application in fermentation, food and paper industry (Pandey *et al*., 2000). The first industrially produced enzyme in 1994 was an amylase from fungal source that was used for gastric disorder treatment (Cruege*et al.,* 1989). α-Amylases (endo-1,4- α-D-glucan glucohydrolase EC 3.2.1.1) are endo-extracellular enzymes that randomly cleave the 1,4- α-D-glucosidic linkage in a linear amylose chain of adjacent glucose units. Amylase has a wide distribution in the living system and has particular substrates (Guzmán-Maldonado *et al.,* 1995; Gupta *et al.,* 2003). Amylases are categorized into endoamylases and exoamylases. Starch molecules are hydrolyzed by endoamylases in an unknown manner thereby causing oligosaccharide formation in branched or linear manner. Shorter end products are resulted consecutively when substrates are hydrolyzed by exoamylase from non-reducing ends (Gupta *et al.,* 2003). The enzyme production has been done by submerged fermentation (SmF) because the Solid State Fermentation (SSF) system appear promising to the offered advantages and the natural potential (Pandey *et al.,* 1999). Presently, the isolates of *Rhizopus, Bacillus*, and *Aspergillus* are the most significant industrial amylase producing sources (Gupta *et al.,* 2003; Pandey *et al.,* 2000).Fermentation methods like solid-state fermentation and submerged fermentation are used in amylase production which uses various agro-wastes as substrate for the fermentation process. SSF technique is believed to be not confined to bacterial cultures because of its higher water activity requirements (Lonsane *et al.,* 1985).In the planet, the residues of Agricultural and Agro-Industrial practices generated round a year represent the most energy rich resources and are accounts for the best-fixed carbon reservoirs in nature (Nigam *et al.,* 2001). Inexpensive Industrial substrates are provided by these energy-rich resources that offer in removing the biomass accumulation of a large scale. The potential raw material for the enzyme production includes crop residues like bran, husk and oil cakes. They are used because of their essential nutrient supply and further, provide an excellent growth substratum (Pandey *et al.,* 2000; Pandey *et al.,* 1998).Since conventional media used for α-Amylase production is expensive, agricultural wastes can be used as an equally efficient substrate that is cheap and easily available (Miranda *et al.,* 1999; Norouzan *et al.,* 2006; Bhatnagar et *al.,* 2010). Amylase has to be purified completely for its use in clinical and pharmaceutical sectors (de Souza et *al.,* 2010).The fermentation process can be developed by their physical and chemical parameters of optimizing the fermentation condition that owes economical impact and process practicability. The cell growth production is permitted by the interaction of fermentation media components with that of the cells metabolism in addition to a greater amount of media constituents (Wenster-Botz*et al.,* 2000). The use of agro waste residues as substrates cleans up the pollution problems (Pandey *et al.,* 2000a, b, c).The emergence of biotechnological novelties caused opening of new ground for utilization of their raw materials for producing high-quality products in the zone of enzyme and fermentation technology (Pandey *et al.,* 2001). The substrate value can be improved to animal feed by the microbial degradation of the safe strain residues.

In this study, five agro wastes were used as substrate namely Groundnut oil cake, Gossypium oil cake, Cassava, Wheat bran and Banana peel and were used as substrate for the production of amylase using bacteria isolated from decayed pomegranate by submerged fermentation.

II. MATERIALS AND METHODS

1. Sample collection:

Decayed Pomegranate was collected from Kollam, Kerala, India.

2. Isolation of the bacteria:

Isolation of bacteria was done by serial dilution method and dilutions were made up to 10^{-6}. Different dilutions were placed on Nutrient Agar medium and kept for incubation at 37°C for 24hours. Bacterial cultures were observed after incubation and seven different cultures were selected which differed in their shape, structure, elevation etc.

3. Screening and of amylase producing bacteria

All the seven bacterial isolates were observed for amylase production by streaking the bacterial isolate on Starch agar media, which have soluble starch as the carbon source. After incubation, the plates were flooded with 1% iodine solution and kept for 1 minute until the media got colored in violet. The isolates 2, 4 and 8 showed the wider degrading zone around the bacterial colony, so these were chosen for further study because of its better starch degrading capability indicating amylase produced by bacteria.

4. Substrates

Five different types of agro wastes were used as substrates which include Banana Peel, Groundnut oil cake, Gossypium oil cake, Cassava and Wheat Bran. These were obtained from Kollam market. Submerged Fermentation (SmF) was performed with all the five substrates and their enzyme production was examined by DNS assay.

5. Identification of bacteria

Bacterial characterization was done by gram staining, Sugar (Sucrose, lactose and glucose) fermentation tests, IMVIC test, catalase activity test and nitrate reduction test.

6. Preservation of the bacterial colony:

The selected bacterial isolate was sub-cultured in Nutrient agar (0.4g-peptone, 0.24g- Beef extract, 0.4g- NaCl, 1.2g- Agar, 80ml distilled water) slants and preserved at 4°C.

7. Fermentation media for Amylase production:

500ml of amylase production media was prepared ([g/L]: Bacteriological peptone-6g, $MgSO_4$-0.5g, KCl-0.5g and substrate-4g). 100ml of the media was transferred to each of the five conical flasks, each for different substrate- banana peel, groundnut oil cake, Gossypium oil cake, cassava, wheat bran etc. 2g of each substrate were suspended to the corresponding conical flasks. After that, the medium was inoculated with the bacterial culture and it was kept for 48 hours incubation on a bench top shaker at 37°C.

8. Crude enzyme extraction:

After the incubation, it was centrifuged for 10 minutes and the supernatant was collected which is the crude enzyme. The crude enzyme was checked for enzyme activity by amylase assay.

9. Amylase assay:

Amylase assay was done by DNS method. 1ml of the crude enzyme from different substrate of isolate 2 was taken, and 1ml of starch solution was pipetted to respective test tubes. It was incubated at 27°C for 15 minutes. The reaction was stopped by adding 2ml of Dinitrosalicyclic reagent. The solutions were heated in a boiling water bath for 5 minutes, and 1ml of potassium sodium tartrate was added to the warm test tubes. The solutions were cooled in running tap water. The volume was made to 10ml by distilled water and absorbance was read at 560nm using glucose as standard. The same procedure was done for isolates 4 and 8 (Sadasivam *et al*., 2006).

$$\text{Amylase enzyme activity (U/ml)} = \frac{\mu g \text{ of maltose released} \times \text{Total volume of assay (ml)}}{\text{Incubation time (min)} \times \text{Volume of enzyme used (ml)}}$$

$$\text{Specific Activity(U/mg)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

10. Protein assay:

Protein estimation was performed by Lowry's method. 1mL of crude enzyme from different substrate of isolate 2 is pipetted into a series of test tubes, and 5mL of alkaline copper solution was added. It was mixed well and allowed to stand for 10 minutes. 0.5mL of Folin-Coicalteau reagent is added, mixed well and incubated at room temperature in the dark for 30minutes. The absorbance was measured at 660nm using bovine albumin serum as standard. The same procedure was done for isolates 4 and 8 (Sadasivam et al., 2006).

III. RESULTS AND DISCUSSION.

In the present study, bacteria were isolated from decayed pomegranate. In a study by Babu *et al*., 1995 of amylase production using *Bacillus coagulans* the bacterial strain was isolated from compost and Vijayan *et al*., 2015 isolated amylase producing bacteria from waste cassava.

Bacteria was serially diluted and isolated on Nutrient Agar in the present study. In a study by Vijayan *et al*., 2015 bacteria were isolated on nutrient agar plates where Cassava waste was used as substrate. Bacteria were isolated on starch agar medium in another study by Dey *et al*., 2001.

Amylolytic bacteria were screened using Starch agar test. In a similar study by Vijayan *et al*., 2015, the amylase producing bacteria was also screened using starch agar assay.
The argo-wastes used as substrates were banana peel, groundnut oil cake, gossypium oil cake, cassava and wheat bran. Banana peel was used as a substrate of alpha amylase production (Krishna *et al*., 1996), different substrates like wheat bran, maize bran, corn bran, millet bran, cassava peel powder, cotton seed oil cake, coconut oil cake, seasame oil cake, groundnut oil cake etc were used as substrate for α-Amylase production (Kalairasi *et al*., 2013).

Among the substrates used, it was found that the maximum amylase activity (2.005 U/ml) specific activity (0.520U/mg) was found with cassava as a substrate using submerged fermentation technique. In a study when cassava was used as substrate, the

amylolytic activity was found to be 2.9U/ml at 30°C (Senthilkumar *et al*., 2012). The bacteria was identified according to Bergey's manual of systemic bacteriology and found out to belong to *Enterobacteriaceae* sp.. Amylase was produced by submerged fermentation using *Bacillus* sp. (Vidyalakshmi Rajagopal *et al*., 2009), *Bacillus amyloliquefaciens* (Basma*et al*., 2015). Cassava was found to show an amylolytic activity of 6.81U/ml at a pH of 7, and a temperature of 35°C using *Bacillus subtilis* (Ebiamadon Andi Brisibe *et al*., 2014).

Table 1 Amylase enzyme activity and specific activity of bacterial isolates on different substrates.

	Banana Peel		Gossypium Oil cake		Groundnut oil cake		Cassava		Wheat Bran	
	EA (U/ml)	SP (U/mg)	EA (U/ml)	SP (U/mg)	EA (U/ml)	SP (U/mg)	EA (U/ml)	SP (U/mg)	EA (U/ml)	SP (U/mg)
Isolate 2	0.674	0.141	1.309	0.278	0.754	0.154	1.728	0.391	1.33	0.26
Isolate 4	0.336	0.035	0.274	0.033	0.233	0.033	0.325	0.055	0.19	0.03
Isolate 8	0.981	0.202	1.462	0.310	0.715	0.175	2.005	**0.520**	1.45	0.37

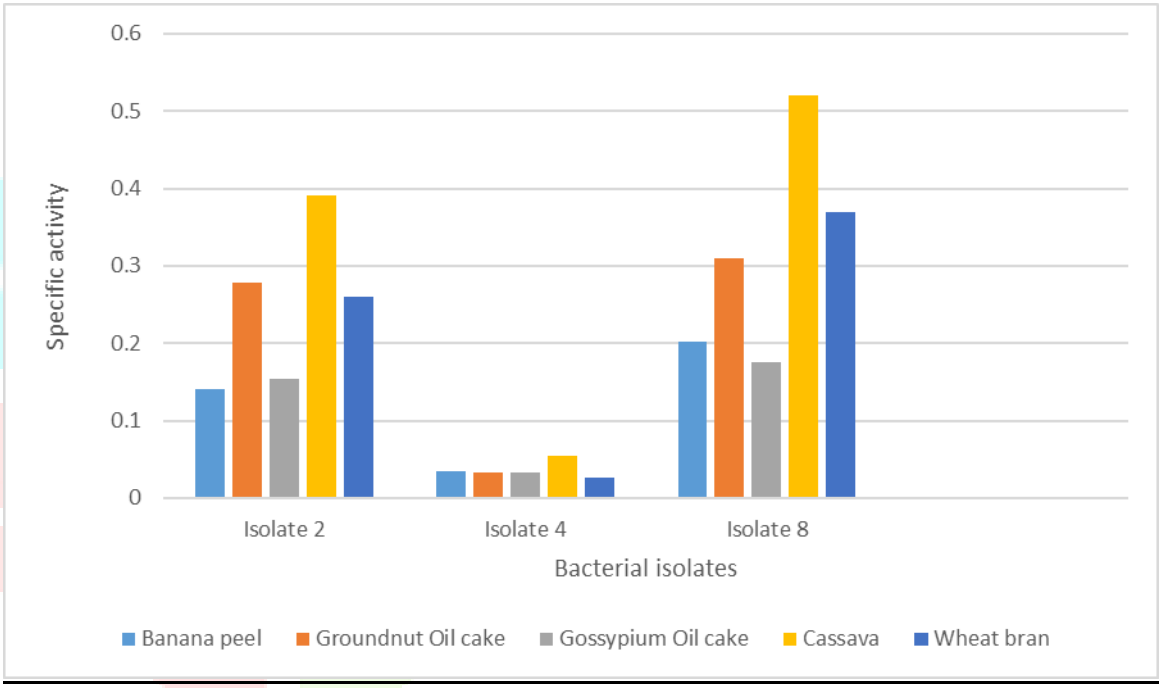
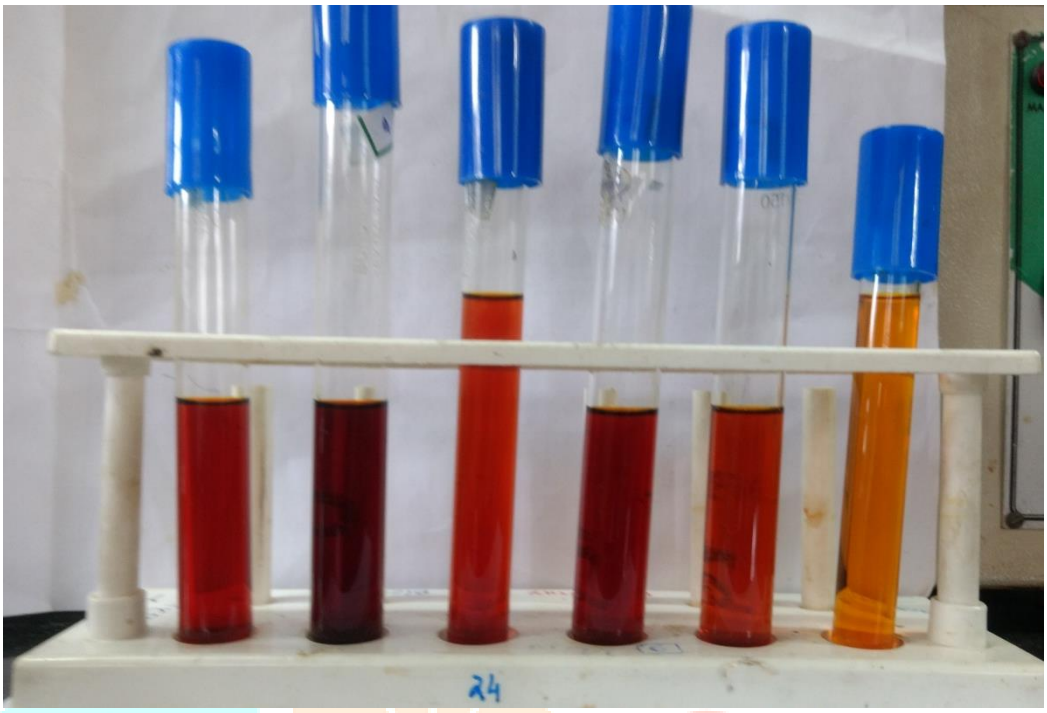


Figure 1. Specific activity of different bacterial isolates on different substrates.



Amylase assay for glucose determination by DNS method of different bacterial isolates after incubation period

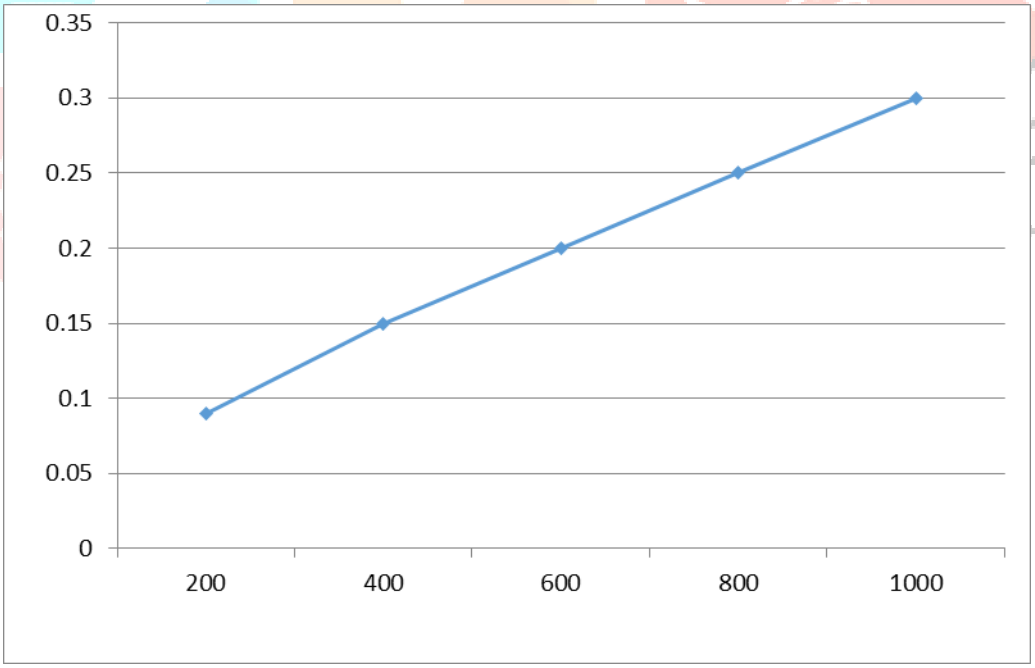
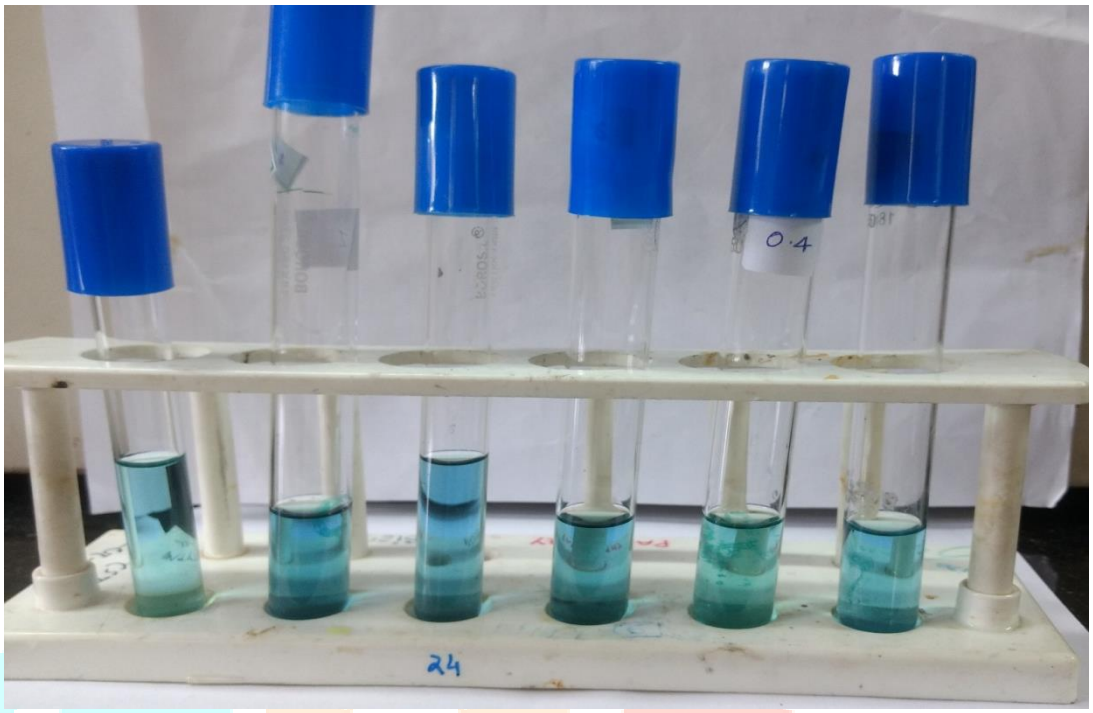


Figure 2. Standard curve of glucose



Protein assay by lowrys method of different bacterial isolates after incubation period

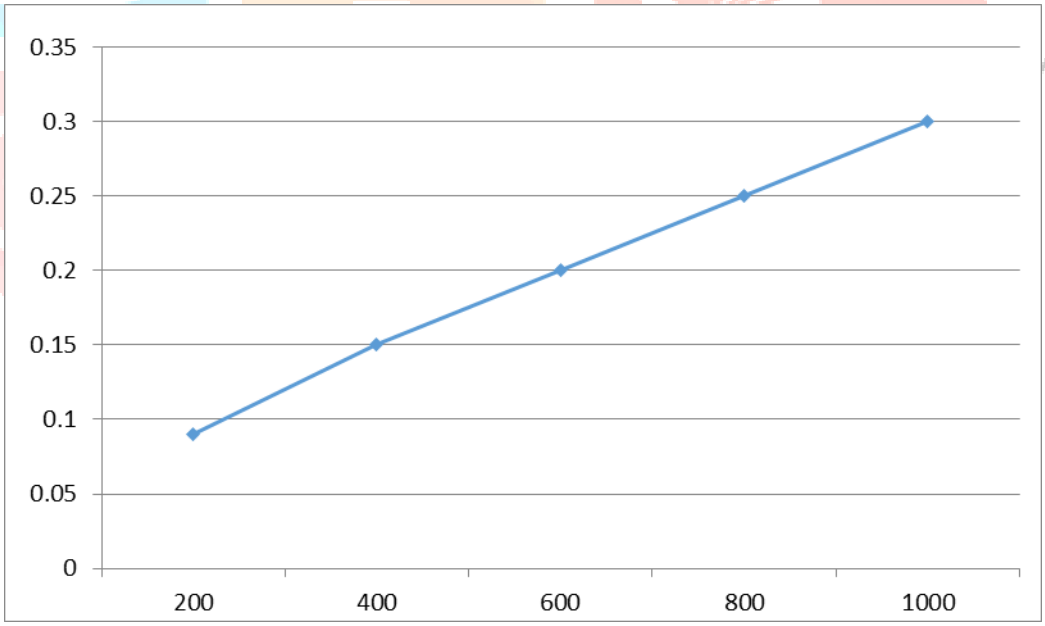


Figure 3. Standard curve of Bovine Albumin Serum (BSA)

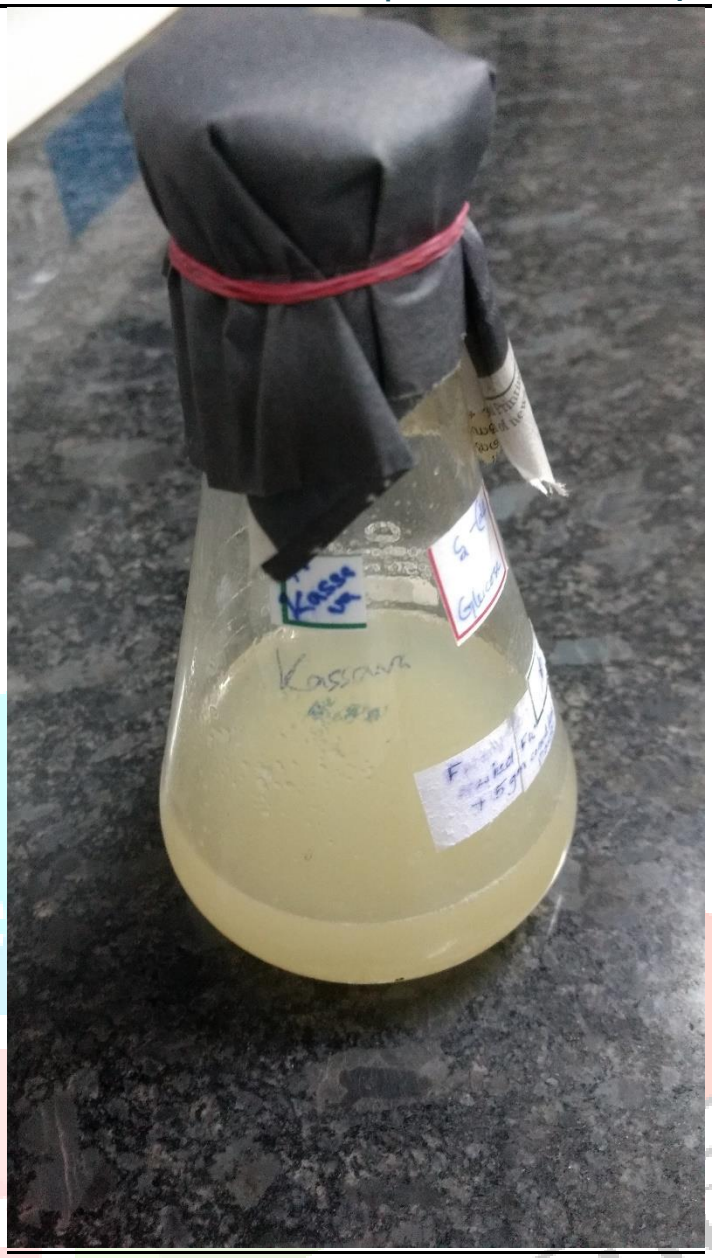


Figure 4. Bacterial culture of isolate 8 on Cassava substrate

IV. CONCLUSION

The bacterial isolates isolated from decayed pomegranate which vary in their shape, texture, color etc was taken and screened for amylase using starch agar. Three bacterial isolates were selected which were sub-cultured and amylase production by submerged fermentation using five agro-wastes as substrates which includes Banana peel, Groundnut Oil cake, Gossypium oil cake, Cassava and Wheat bran. Among the five substrates, it was found that the maximum amylase specific activity (0.520U/ml) was produced by bacterial isolate 8 with cassava as substrate.

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