International Journal of Advanced Research in Engineering and Applied Sciences

ISSN: 2278-6252
Impact Factor: 7.388

EFFECT OF SUB MERGED FERMENTATION ON PRODUCTION OF AMYLASE THROUGH FRUIT ISOLATES.

VINIT SINGH BAGHEL[1], [1]Government Nagarjuna Post Graduate College of Science Raipur, MP, India

CHITRANSHU PANDEY[2], R&D, MRD LifeSciences Pvt. Ltd. Lucknow, UP, India.

PALLAVI SHARMA[3], R&D, MRD LifeSciences Pvt. Ltd. Lucknow, UP, India.

ABSTRACT:

The isolation of amylase producing bacteria from the soil and identifying the gene responsible was done. The gene was then mutated for the enhanced production of amylase; the study was carried out using starch (2%) as the substrate of enzyme. The production of enzyme was carried out under submerged fermentation. The best condition for the production of enzyme were, incubation temperature 37^{o}C, pH 6, incubation period of 72 hours, the starch as carbon source and the ammonium sulfate as nitrogen source. The amylase was purified using ammonium sulfate (40%) and dialysis. The refined amylase had a maximum activityat pH 6. The enzyme was active between pH range of 6-8 and temperature range of 30^{o}C to 40^{o}C.

1. INTRODUCTION:

Amylase is an enzyme that breaks (cleaves) starch. Total of 19 enzymes have been ranked that relate to the microbial amylase: hydro lases such as glucoamylase,α-amylase,β-amylase,α-glucosidase,debranchingenzymesand transferases (EC 2) such as CGT ase, 4-α-glucanotransferase. First, the enhancing of break down properties of each of the nineteen enzymes were explained to express the discrimination among them and their microbial sources. Industrial usages of amylase were seen with an attention on the function of α-amylase and gluco-amylase in the starch saccharification industry. There have been recent developments in trehalose production and other cyclic-glucans. Many enzymes work on starch or on the oligosaccharides derived from them, and manipulate them into maltose, a disaccharide and few monosaccharide like glucose. These disaccharides and monosaccharides moves into cytoplasm of the bacterial cell through semi permeable cell

membrane and are then employed by endo-enzymes. Starch is a composite carbohydrate made up of two constituents – amylose, a straight chain polymer of 200 – 300 glucose sub-units, and amylopectin, a larger branched polymer with phosphate groups.

Amylase exist in the saliva of humans and few other mammals, where it begin the chemical process of digestion. The foods like rice and potatoes that include the starch in higher quantity in contrast to other sugars may tastes sweet when chewed because the amylase present in the saliva will break the starch into simple sugars. The amylase in the body of humans is secreted by pancreases and salivary glands and is ranked as alpha amylase. Alpha amylase help in the breakdown of the dietary starch and convert the in to disaccharides and trisaccharides, which are further hydrolyzed into simpler glucose such as glucose by other enzymes. Amylase production is known in some bacteria while well known in fungi. Amylase commercially produced from various *aspergillus*are used in the initial steps in the several food fermentation process to convert starch into fermentable sugars. They are also used to partially predigest foods for young children, to clarify fruit juices and in the manufacture of corn and chocolate syrups.

2. MATERIALS AND METHODOLOGY:

2.1. Collection of sample:

The soil sample was collected from the areas near to the fruit stalls, which are enriched in starch presence.

2.2. Isolation of amylase producing bacteria:

The sample was serially diluted in 0.85% sterilized saline and then spread over the sterilized nutrient agar plates. Then the cultures were incubated at 37°C for 24 hours. Once the growth observed then these cultures were selected on the basis of different morphological parameters and streaked over sterilized nutrient agar plates. Further the screening of amylase producing bacteria was performed by the hydrolysis of starch and iodine test.

2.3. Strain identification:

The identification of positive culture was done by using biochemical test based on Bergey's manual.

2.4. Strain improvement by mutation:

The physical and chemical mutation was given for improving the production of amylase. The sources for physical and chemical mutations are UV rays and Ethidium bromide.

2.5. Selection and optimization of media:

The best media was selected on the basis of highest growth of culture. Further each component of the media was optimized by applying one factor at a time method.

Table1: Optimized media for amylase production by OFAT methods.

S no.	Factors	Media composition	Quantity
1	Production media 1	Peptone	6 g/l
		$MgSO_4.7H_2O$	0.5 g/l
		KCl	0.5 g/l
		Starch	10 g/l
2.	Production media 2	Starch	10 g/l
		Nutrient broth	8 g/l
		$(NH_4)_2SO_4$	2.25 g/l
		NaCl	0.85 g/l
		$MgSO_4.7H_2O$	0.25 g/l
3	Production media 3	Starch	10 g/l
		K_2HPO_4	9 g/l
		KH_2PO_4	2 g/l
		$(NH_4)_2SO_4$	5 g/l
		Sodium citrate	1 g/l
		$MgSO_4.7H_2O$	0.2 g/l
		$FeSO_4.7H_2O$	0.1 g/l
		$ZnSO_4.7H_2O$	0.1 g/l
4	Production media 4	Yeast extract	5 g/l
		Starch	10 g/l
		$MgSO_4.7H_2O$	0.5 g/l
		K_2HPO_4	1 g/l
5		Starch	0.5%, 1%, 2.5%, 2%
6		Nutrient broth	1%, 1.3%, 2%, 2.3%
7		$(NH_4)_2SO_4$	0.1%, 0.2%, 1%, 1.2%
8		NaCl	0.1%, 0.8%, 1%, 1.8%
9		$MgSO_4.7H_2O$	0.02%, 0.1%, 1%, 1.25
10		pH	6,7,8,9

2.6. Bacterial growth curve:

The bacterial culture was inoculated in sterilized optimized media and incubated at 37ºC for one week. Then OD was taken in spectrophotometer, at 620 nm, after 1 hour of time interval.

2.7. Fermentation and downstream processing:

The fermentative media was prepared by using optimized components and then culture was inoculated.Submerged fermentation and shake flask method was used for the production of amylase. Purification was carry out by using salt precipitation with 40% ammonium sulfate and then dialysis for obtaining the partial pure enzyme.

2.8. Enzyme assay:

The estimation of enzyme was performed by using DNS method. 1 ml of perfectly diluted (in acetic acid buffer solution; pH=5) enzyme sample are hatched to 15 min at T=37°C with 1 ml of soluble solution of starch 1 % w/v. Following, the created quantity of reducing sugars discharged from starch is determined with the help of DNS standard graph. In the way that a unit of activity (unit, U) of the enzyme amylase, is promptly appointed, the amount of the enzyme needed for the production of 1 µmole of maltose in 1 min, when the enzyme is hatched along with the substrate at pH=5 and T=37 °C.

3. RESULTS:

3.1. Sample Collection:

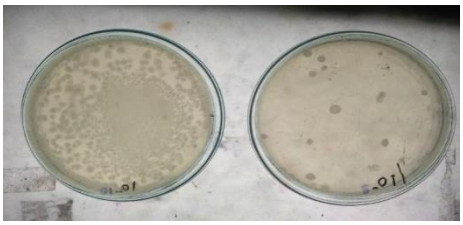
Soil sample containing rotten fruits was collected from fruit stall, Gomti Nagar, Lucknow. Sample was taken from 2-2.5 inches below ground level in sterile polybag and was brought to laboratory. Sample was blackish brownish in color.

3.2. Isolation amylase producing bacteria:

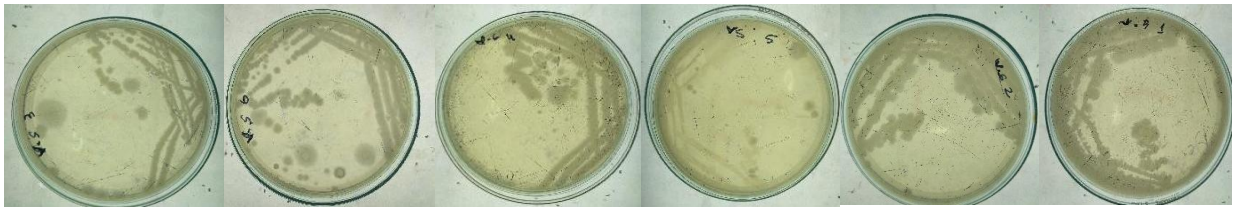
The white colonies were observed representing the bacterial growth, which were then streaked to sterilized nutrient agar media to make the pure culture. Further these cultures was streaked over minimal salt agar media supplemented with 1% starch and then incubated at 37ºC for 48 hours. Iodine flood was carrying out to visualize the zone of

ISSN: 2278-6252
Impact Factor: 7.388

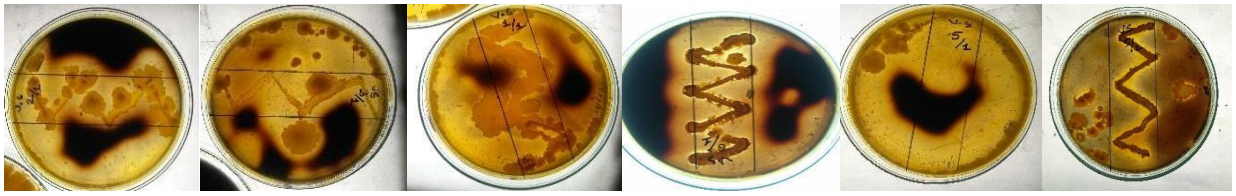
hydrolysis. On the basis of the bacterial growth and enzyme production, primary and secondary screening was performed.



a. Bacterial culture after serial dilution



b. Pure culture by streaking method



c. Screening for amylase producing bacteria.

Figure 1: isolation of bacteria from fruit sample by serial dilution, its purification and screening for amylase enzyme production.

Table 2: Primary and Secondary screening for amylase producing bacteria

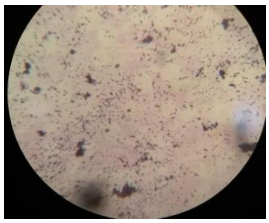
S no.	Isolated cultures	Primary screening	Secondary screening
1	CPVS19001	+	+
2	CPVS1900 2	+	++
3	CPVS1900 3	+	+
4	CPVS1900 4	+	+
5	CPVS19005	-	-
6	CPVS1900 6	++	+++

ISSN: 2278-6252
Impact Factor: 7.388

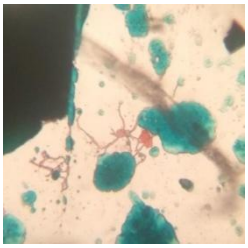
3.3. Strain identification:

Table 3: Biochemical test for strain (CPVS19006) identification.

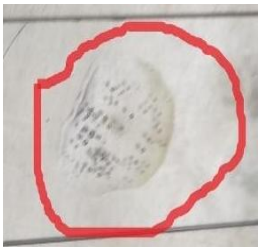
S no.	Isolated cultures	Results
1	Gram's staining	Positive, *Cocci*
2	Endospore's staining	Negative
3	Catalase test	Positive
4	Glucose fermentation test	Positive
5	Mannitol test	Positive
6	MR-VP test	Negative



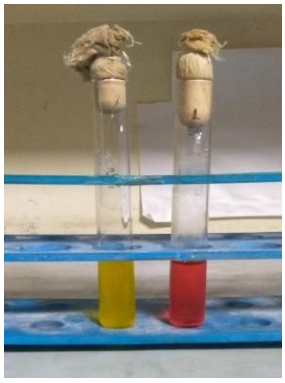
a. Gram's staining



b. Endospore staining

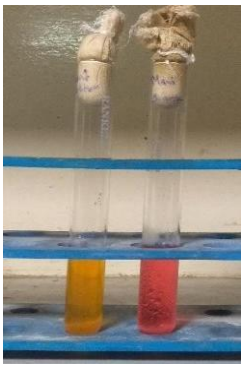


c. Catalase test



d. Mannitol test

e. MRVP test



f. Glucose fermentation test

Figure 2: Results of biochemical tests

ISSN: 2278-6252
Impact Factor: 7.388

3.4. Strain improvement:

a. Chemical mutation:

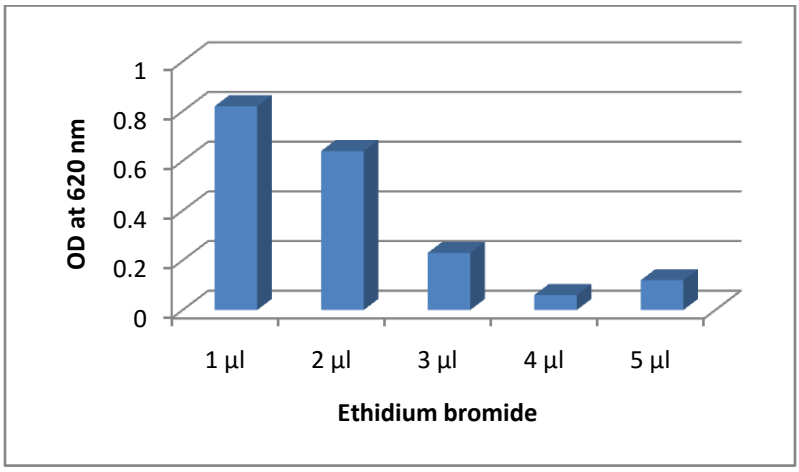


Figure 3: effect of EtBr on isolated strain CPVS19006,Maximum growth of strain CPVS19006 was seen in 1µl EtBr

b. Physical mutation:

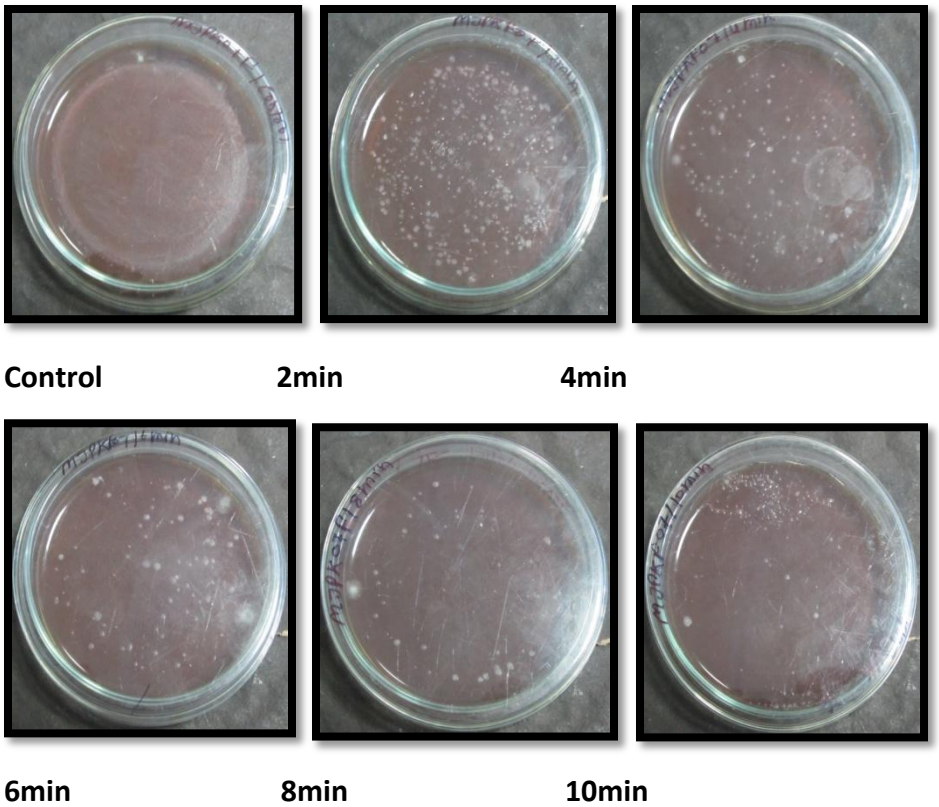
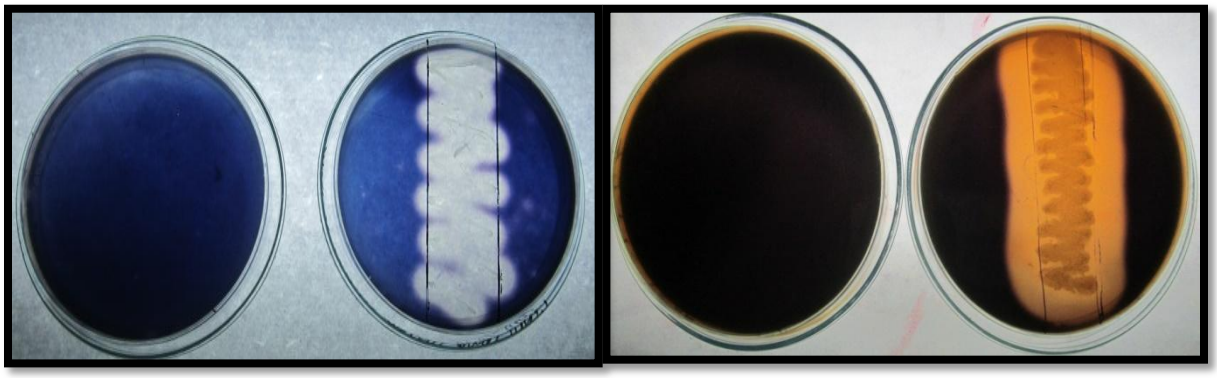


Figure 4: Effect of UV treatment

ISSN: 2278-6252
Impact Factor: 7.388

3.5. Screening of mutates strains:



a. Ethidium bromide mutant *b. 10 min. UV mutant*

Figure 5: Comparison between uv mutated (6min) and EtBr mutated (1µl) stain CPVS19006

ISSN: 2278-6252
Impact Factor: 7.388

3.6. Media selection and its optimization:

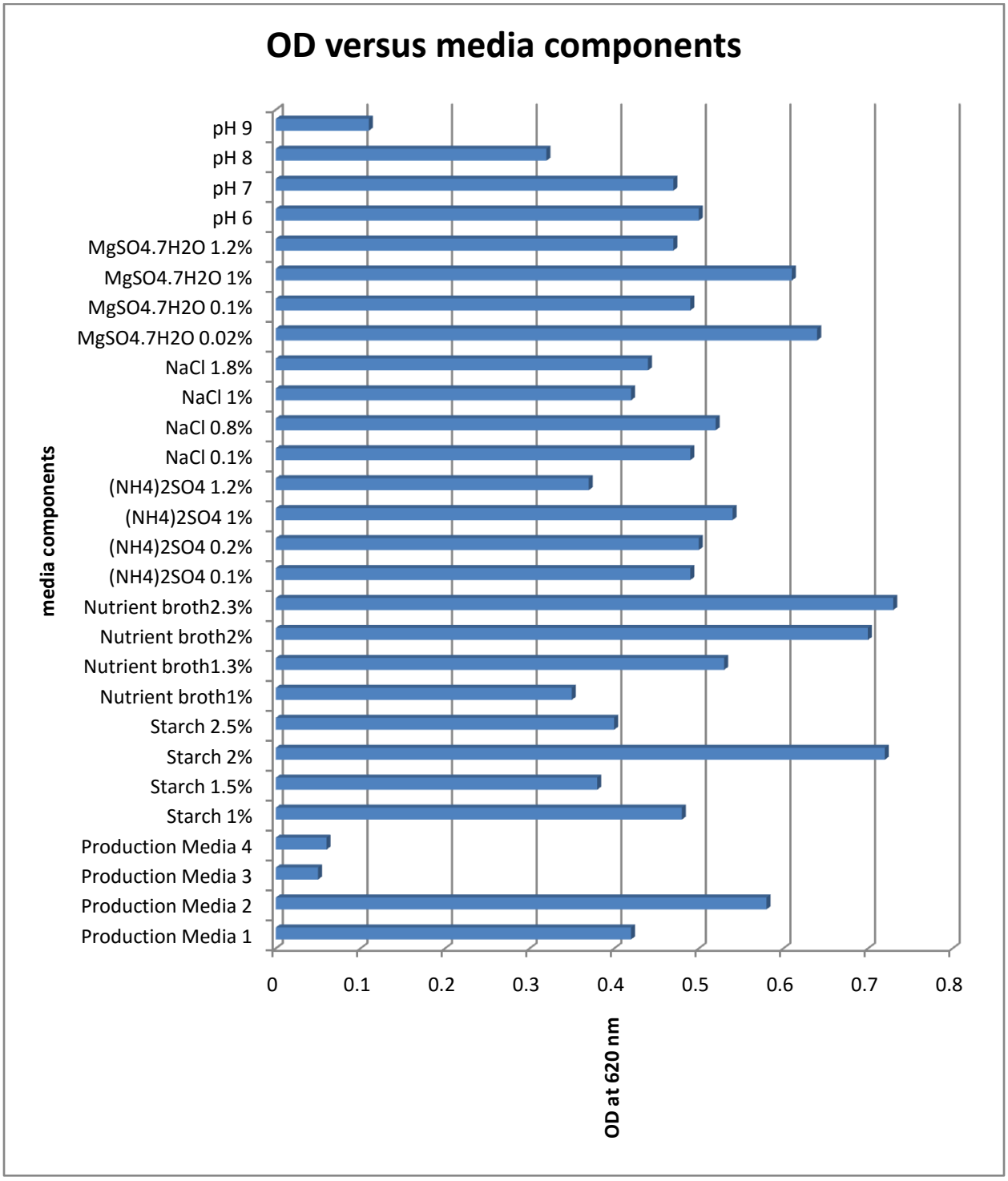


Figure 6: media optimization for production of amylase through uv mutated strain CPVS19006

3.6. Effect of temperature:

Table 4: Plates were incubated at different temperatures for overnight, Plate at 37^{o} C shows best result.

S.No.	Temperature	Growth
1.	4ºC	-
2.	Room temperature	+
3.	37 ºC	+++
4.	50 ºC	-

3.7. Bacterial growth curve:

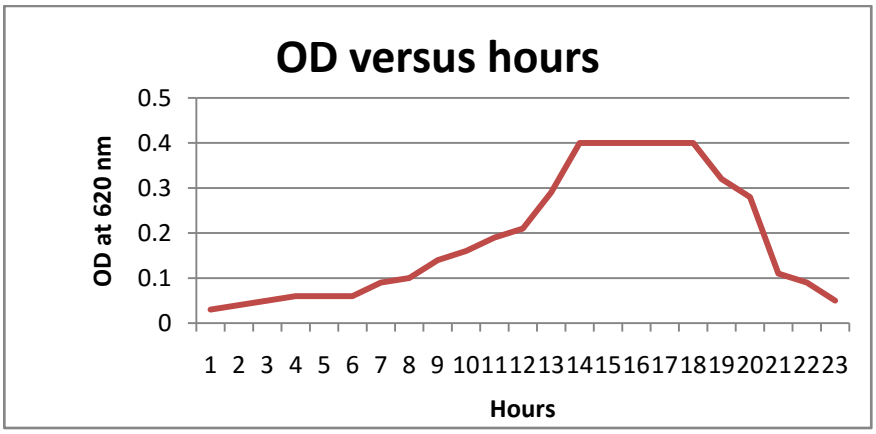


Figure 7: Growth curve of strain CPVS19006 detected by looking the OD with respect to time.

ISSN: 2278-6252
Impact Factor: 7.388

3.8. Bradford's Standard:

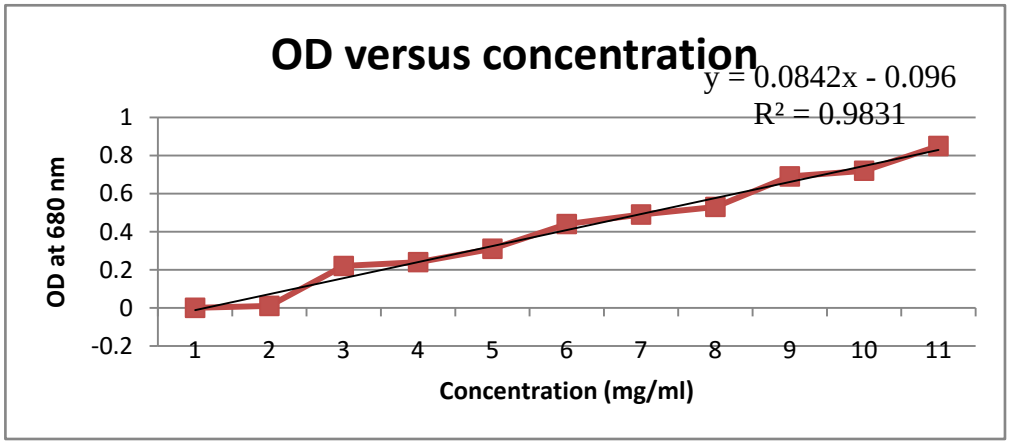


Figure 8: Standard graph between OD and known protein concentration

3.9. Fermentation and downstream processing:

3.9.1- Estimation of enzyme by Bradford's method:

Table 5: Concentration of protein (Enzyme) after fermentation, detected by Bradford's method.

S. no.	Sample	OD at 680nm	Concentration (mg/ml)
1.	Blank	0	0
2.	Amylase	0.40	0.14

ISSN: 2278-6252
Impact Factor: 7.388

3.11. DNS Standard:

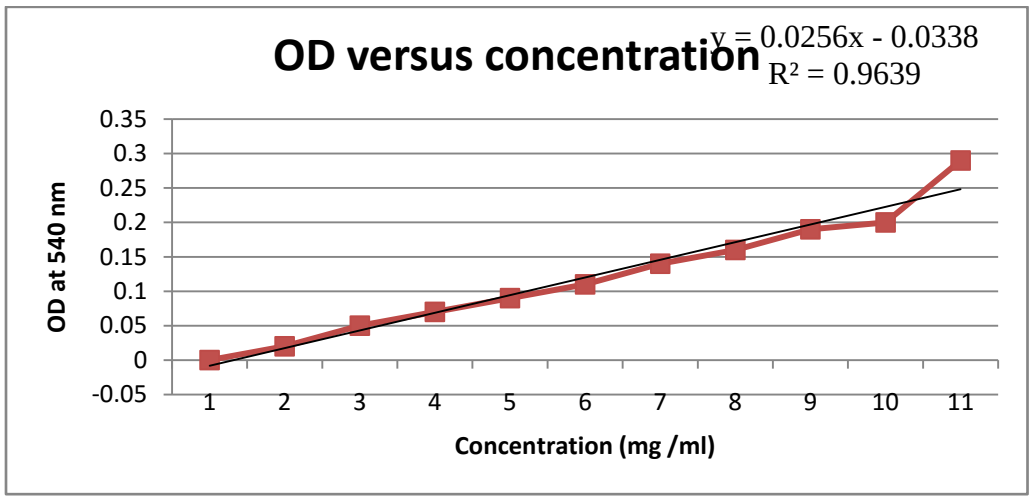


Figure 9: Standard graph between known concentration of maltose and OD at 540 nm.

3.12. Estimation of enzyme activity by DNS method:

Table 6: Activity of extracted Amylase enzyme.

S. no.	Sample	OD at 680nm	Concentration	Activity U/ML
1.	Blank	0	0	00
2.	Amylase	0.59	0.14	5.41 U/mL

4. DISCUSSION:

Microorganism was isolated from the soil containing rotting fruits by serial dilution and agar plating methods KHAN J.A. 2015. Isolated bacteria were further purified which were named as **CPVS19006.** The culture were grown on minimal agar media (MAM) at pH 7 supplement with 1% starch. The culture grown on MAM were flooded with iodine solution and zone of hydrolysis were obtained in the plate showing starch hydrolysis method has been used earlier by **Gupta, S.K., 2015** in order to screen the microorganism for amylase production.

Morphological properties and the taxonomical characteristics of the isolated

bacteria were studied according to **Bergey's manual** as earlier done by the ***Khan, et al. 2015*** and the isolated bacteria were identified as ***Staphylococcus aureus.*** Strain improvement was done to develop high amylase producing bacteria by treatment with uv and EtBr and media optimization and **CPVS19006** was improved by UV mutation. The bacterial strain was identified by the help of various physical characteristics, staining and biochemical activities.

Production media for the amylase production was optimized according to the isolated requirements. The optimized media contains 2% starch, Nutrient Broth 2.3%, AmmoniumSulfate1%,Sodium Chloride 0.8%andMagnesiumSulfate0.02%.The selected PM applies for the production of amylase under submerged fermentation. Partial purification of the crude amylase was done by ammonium Sulfate precipitation and dialysis technique as used earlier by **Yandri*et al., 2010.***

The enzyme activity was assayed by incubating the 1 ml of the enzyme with 1% starch in the 100mM tris of the pH5 at 37^{o}C for 15 min after the incubation the reaction was stopped by adding 1 ml of DNS reagent and reducing sugar were assayed using colorimeter. Protein concentration was measured by Bradford method using BSA as done earlier by **Khan, *et al.,* 2015** and total protein content of pure enzyme was 0.14mg/ml.

5.CONCLUSION:

This research shows that the isolated and uv mutated strain CPVS19006 can be a fine source for amylase production for commercial uses. The amylase produced here was found to be steady in the pH range of 6 to 8 and temperature range of 30^{o}C to 40^{o}C. The efficiency of amylase produced here was similar to that of diverse researchers. This study can prove to be a foundation to the future commercial production of amylase from bacterial source. This study can help relieve the stress over fungal source for the commercial amylase and enhance the production through different optimization techniques at the same time.

6. ACKNOWLEDGEMENT:

I wish to express my immense gratitude to Mr. Manoj Verma, Director, MRD LifeSciences Pvt. Ltd. Lucknow. I am very grateful and my heartiest thanks to Ms. Shraddha Prakash (Research Scientist), Mr. Raj Shekhar Mishra (Research Assistant) and Ms. Pragya Srivastava (Research Assistant) MRDLS, Lucknow, for their kind support throughout the research work, I am also thankful to the almighty without whose blessings nothing was possible.

7. REFERENCES:

- ***Yao et al. 2019*** Enhanced extracellular expression of *Bacillus stearothermophilus*α-amylase in *bacillus subtilis*through signal peptide optimization, chaperone over expression and α-amylasemutant selection.

  Microbial cell factories (2019) 18:69

- ***Altaf Ahmed Simair et al. 2017*** production and partial characterization of α-Amylase enzyme from *Bacillus* sp. BCC 01-50 and potential applications.

  BioMed Research nternational, volume 2017, Article ID 9173040, 9 pages.

- ***PadmaSingh,PallaviSingh2016***I solation and characterization of amylase

  producing*Bacillus*spp. from selected soil sample. *International Journal of Research in Biosciences, vol. 5, issue 2, pp. (24-29) April-2016*

- ***YingfangMaetal.2016*** Significantly enhancing recombinan talk alineamylase

  production in *Bacillus subtilis*by integration of a novel mutagenesis-screening strategy with system-level fermentation optimization. *Journal of Biological Engineering (2016) 10:13 DOI 10.1186/s13036-016-0035-2*

- ***JingqiChenetal.2015***Enhancedextracellularproductionofα-amylasein

  *Bacillus subtilis*by optimization of regulatory elements and over-expression of Prs Alipoprotein.*S pringer, April 2015, volume 37, issue 4, pp 899-906*

- ***Roy et al. 2013*** Cloning and over expression of raw starch digestingα-amylase

gene from *Bacillus subtilis*strain AS01a in *Escherichia coli* and application of the purified recombinant α-amylase (AmyBS-I) in raw starch digestion and baking industry. *Journal of Molecular Catalysis B: Enzymatic 97, 118-129, 2013*

- ***Chaietal.2012*** cloning and characterization of two new thermo stable and alkalitolerant α-amylases from *Anoxybacillus*species that produce high levels of maltose. *J Ind Micriobiol Biotechnol (2012) 39:731-741.DOI 10.1007/s10295-011-1074-9.*
- ***GargiDey et al 2001*** Enhanced production of amylase by optimization of nutritional constituents using response surface methodology. *Biochemical Engineering Journal 2001, volume 7, issue 3, May 2001, pages 227-231.*
- ***Jayi et al. 2003*** utilization of corn starch as substrate for β-amylase from bacillus supp. *African journal of biomedical research, 6(1),37-42.2.*
- ***Baysal et al. 2008*** production of extracellular alkaline α-amylase by SSF with a newly isolated bacillus sp. *Prep. Biochem. Biotechnol. 38, 184-190,7.*
- ***Aneja, K.R. (2002)***.Experiments in Microbiology, Plant Pathology, Tissue culture and Mushroom Production Technology. *New Age International (P). Ltd Publishers, New Delhi, India,Pp169-171.3.*
- ***Anto, H.; Trivedi, U.; Patel, K (2006)***. Alpha Amylase production by Bacillus cereus MTCC 1305 Using Solid-State Fermentation. *Food Technology. Biotechnology. 44 (2), 241-A245.4.*
- ***Balkan, B.; Ertan, F. (2007).*** Production of a-Amylase from *P. chrysogenum*, under SSF by using some agricultural by-products. *Food Technology Biotechnology 45 (4), 439-442.5.*
- ***Basal,Z.;Uyar,F.;Aytekin,C.(2003)***.Solid state fermentation for production of a-amylase by a the rmotolerant *B. Subtilis* from hot-spring water. *Process Biochemistry 38, 1665-1668.6.*
- ***Bernhardsdotter, E.C.M.J.; Ng. J.D.; Garriott, O.K.; Pusey, M.L. (2005)***. Enzymic properties of an alkaline chelator-resistant α-amylase from an

alkaliphilic*Bacillus sp.* isolate L 1711*. Process Biochemistry 40,2401-2408.8.*

- ***Chung, Y.C.; Kobayashi, T.; Kanai, T.; Kudo, T. (1995)***. Purification and properties of extracellular amylase from the hyper thermo philic Archaeon *The rmococcusprofundus*DT 5432*. Applied Environmental Microbiology. 61.(4): 1502-15069.*
- ***Coronado, M.; Vargas. C.; Hofemeister, J.; Ventosa, A.; Nieto, J.J. (2000)***. Productionandbiochemicalcharacterizationofanα-amylasefromthemoderate halophile Halomonas, meridian*. FEMS Microbiology. Lett. 183, 67-71.10.*
- ***Hagihara, H.; Igarashi, K.; Hayashi, Y.; Endo, K.; Ikawa-Kitayama, K.; Ozaki, K.; Kawai, S.; Ho, S (2001)***. Novel α-amylase that is highly resistant to chelating reagents and chemical oxidants from the alkaliphilic Bacillus isolate KSM.K.38*. Applied Environmental. Microbiology. 67,1744-1750.*

Ikram-ul-Haq, H.; Ashraf, J.; Iqbal, M.A.; Qadeer. (2003). Production of alpha amylase by *Bacillus lichen iform i*susing an economical medium. *Biore source. Technology 87, 57-61.1*

ISSN: 2278-6252
Impact Factor: 7.388

- ***Kathiresan, K.; Manivannan, S. (2006)***. α-amylase production by *Penicilliumfellutanum*isolatedfrommangroverhizospheresoil.*AfricanJournalBiotech.5 (10), 829-832.13.*
- ***Kashyap, P.; Sabu, A.; Szakass, G.; Soccol, C.R. (2002).*** Extracellular L-glutaminase production by *Zygosaccharomycesrouxii*under SSF. *Process. Biochemistry 38, 307-312.14.*
- ***Kokab, S.; Ashgar, M.; Rehman, K.; Asad, M.J.; Aedeyo, O. (2003*)**. Bio-Processing of banana peel for a-Amylase production by *B. subtilis. International. Journal of Agricultural Biology. 1560-8530, 36-39. 15.*
- ***Konsula, Z.; Liakopoulou-Kyr4iakides, M. (2004*)**. Hydrolysis of starches by the action of an α-amylase from Bacillus subtilis*. Process Biochemistry 39, 1745-1749. 16.*
- ***Krishnan, T.; Chandra, A.K. (1982)***. Effect of Oilseed Cakes on α-Amylase Production by Bacillus licheniformis CUMC 305. *Applied Environmental. Microbiology 44: 270-274.17.*
- ***Kunamneni, A.; Perumal, K.; Singh, S. (2005)***. Amylase production in solid state fermentation by the thermophilic fungus *Thermomyceslanuginosus. Journal of Biosciences and Bioengineering. 100(2):168-171*