Starch Hydrolyzing Enzyme Production from Fruit Peels using *Aspergillus Niger*

ABSTRACT

Amylase is one of the important industrial enzymes due to its application in detergents, foodandtextileindustries.In the presentstudy,theproductionofamylase,astarchhydrolysingenzyme,was optimized using *Aspergillus niger*under various conditions i.e., incubation time, carbonsource,pHandtemperature.Amylaseproductionby*Aspergillusniger*usingsubmergedfermentationofmixedfruitpeelwastesuchassweetlime,banana,cucumber,orange,pomegranate,pineapple,andwatermelonwasused.Theculturalandnutrientrequirementsof *Aspergillus niger*for the production of amylase in production media containing different pH,temperature, incubation period, metal ion concentrations, surfactants, carbon sources andnitrogen sources were quantified in the present study. The optimum temperature and incubationperiod for enzyme production was 37°C and 5th day, respectively. The main objectives of thepresentstudyweretouseasuitablefungalstrainfor the productionofextracellularalpha-amylaseand to determine the time course for the production of alpha-amylase. Thus, this enzyme canbe produced cost-effectively using low-cost fruit peel waste and could be utilized in the detergentortextile industry.

Keywords: Amylase, Aspergillus niger, mixed fruit peel waste, submerged fermentation,incubation

INTRODUCTION

Amylaseisan enzyme that catalysesthe hydrolysis of starchinto sugars.Amylaseis presentinthesalivaofhumansandsomeothermammals,whereitbeginsthechemicalprocessof digestion. Amylase generally breaks down starch or glycogen. Amylase can be obtained from numerous sourcessuchasplant, animal and microbes [33,34].Amylolytic enzymes are added to starch-based foods,

such as baking products, to retain moisture more efficiently and to increase softness, freshness, and shelf life [35].

Amylase is an enzyme that occurs naturally in the saliva of some mammals andhumans that aids in the process of digestion. It accelerates the breakdown or hydrolysis ofstarch into simple sugars. The pancreas and the salivary glands mainly synthesise amylase tohydrolyse dietary starch into disaccharides and trisaccharides that are converted into glucoseandusedasenergy.Amylasewasoneofthefirstenzymestobediscoveredinthe1800s.Itwasinitiallynameddiastasebutlaterrenamedamylaseinthelate20thcentury.Thefirstindustriallyproduced enzyme was an amylase from a fungal source in 1994, which was used for thetreatmentof digestive disorders (1).

Nowadays,Bacillus,Aspergillus,RhizopusandRhizobiaisolatesarespecifiedandconsideredtobethemostimportantsourcesofindustrialamylases.Nevertheless,variousothersourcesofmicrobial amylases have also been investigated throughout the world. Growth parameters andothernutrientsthatpromotehighyieldsofmicrobialamylaseswerealsostudiedextensively.The usual carbon sources such as dextrin, fructose, glucose, lactose, maltose and starch arevery expensive for commercial production of amylase. These expensive products can bereplaced in the medium with economically available agricultural by-products or industrialamylaceoussubstancesascarbonsubstrateswhich arecost-effective(2–4).

Thus,thepresentstudywasdesignedinsearchofcheapercarbonsourcesfortheproductionof alpha-amylase enzyme by using fungal strains. Amylase produced from the fungal cultureswas found to be more stable than that produced by bacteria on a commercial scale. Manysuitable strains of fungi have been optimized for their culture conditions. Moulds are capableofproducinghighamountsofamylase.Aspergillusnigerisusedforcommercialproductionoamylase. Studies on fungal amylases especially in developing countries have concentratedmainly on Aspergillus niger, probably because of their ubiquitous nature and non-fastidiousnutritionalrequirements(5,6).

Amylase constitutes an industrial enzyme that represents approximately 30% of worldwideproduction. It is also used in fields that are related to biotechnology such as: removing environmental pollutants, by using micro-organism conversion of starch into the desired substrate,infiltrationofwastecontainingstarchandproduction of biochemicalmaterialto help the starch substrate. The spectrum of amylase application has expanded into many other

fields due to the adventofbiotechnologysuchasmedical,clinicalandanalyticalchemistry.Generally,amylasecan be derived from various sources from plants, animals and various micro-organisms. Themicrobialenzymesgenerallymeetindustrialdemands.Thealpha-amylasesaremetalloenzymes means for their stability, activity and structural integrity they require calciumions(7,8).

Fermentation is the metabolic breakdown or biological conversion of complex substrates intosimplercompoundsbyusingvariousmicroorganismssuchasbacteriaandfungi.Fermentationha s been widely used for the production of a wide variety of substances that are highlybeneficial to individuals and industries. Over the years, this technique has gained immenseimportancedueto itseconomicand environmental advantages(9,10).

The main types of fermentation are solid state and submerged fermentation based on thesubstrate used during the fermentation process. The outcome of fermentation highly varies foreach substrate. Hence, it is extremely important to choose the right substrate. Fermentationtechniques have to be optimized for each substrate. This is primarily due to the reason that anorganismreactsdifferentlytoeachsubstrate.Theratesofutilizationofvariousnutrientsdifferineac hsubstrate,andsodoesproductivity.Fermentationisusedasaprimarytechniquefortheproductionof enzymes(11,12).

Severalindustrialimportantenzymesareproducedfromfungibelongingtothegenus Aspergillus.As pergillus niger is the single largest fungal source of enzymes. Aspergillus is widespreadin the growing environment in soil, plants, decomposing organic matter and air. There are200 species of Aspergillus. The cell wall of Aspergillus niger composed of 6 sugars glucose,galactose, mannose, arabinose, glucosamine, galactosamine. Sixteen amino acids are found inthe cell wall of Aspergillus niger namely aspartic acid, glutamic acid, glycine, alanine, valine,tyrosine,phenylalanine,proline, cystine, arginine,lysine,histidine(13–15).

Agro-industrialwasteshavebeenreportedtobegoodsubstratesforthecost-effectiveproductionofalpha-amylasesandarethusattractingresearchersto useagro-industrialwasteas a substrate for alpha-amylase production. Fungal species have been studied a lot for theproduction of alpha-amylase because of the low cost of substrates used for the production ofalpha-amylases(16).

Aspergillus nigeris a mould classified within the*Nigri*section of the*Aspergillus*genus.The *Aspergillus* genus consists of common moulds found throughout the environment withinsoil and water, on vegetation, in faecal matter, on decomposing matter, and suspended in theair.Specieswithinthisgenusoftengrowquicklyandcansporulatewithinafewdaysof germination.Acombinationofcharacteristicsuniqueto *A.niger*makesthemicrobeinvaluabletotheproductionofmanyacids,proteinsandbioactivecompou nds.Characteristicsincluding extensive metabolic diversity, high production yield, secretion capability, and theabilitytoconduct post-translationalmodifications areresponsiblefor *A.niger'*srobustproductionofsecondarymetabolites.*A.niger'*scapabilitytowithstandextremelyaci dicconditionsmakesit especially important tothe industrial productionofcitricacid(17,18).

*A. niger*causes a disease known as "black mould" on certain fruits and vegetables such asgrapes, apricots, onions, and peanuts, and is a common contaminant of food. It is ubiquitousin soil and iscommonly found in indoor environments,where its blackcolonies can beconfusedwiththoseof*Stachybotrys*(species ofwhichhavealsobeencalled "blackmould")(19).

Therearetwowaysinwhich*Aspergillusniger*canbegrownforindustrialpurposes:solid-statefermentation (SSF) and submerged fermentation (SmF). SSF uses a solid substrate withnutrientsandminimalmoisturetogrowmicroorganisms.Nutrientssuchasnitrogenandcarbonc ome from agricultural by-products such as wheat bran, sugar pulp, rice husks, and corn flour.SSF gives a better yield of microbe products and is more cost-effective than SmF due to usingagriculturalby-products.SSFispredominantlyusedoverSmF.InSmF,microbesaregrowninа liquid medium inside large aseptic fermentation vessels. These vessels are expensive piecesof equipment that provide more water for growth and allow for tight control of environmentalfactors,suchastemperatureandpH, that affectmicrobialgrowth(20,21).

*A. niger*produces many useful enzymes for the catabolism of biopolymers to obtainnutrients from its environment. The production of specific enzymes can be increased forindustrial purposes. Another use for *A. niger*within the biotechnology industry is in theproduction of magnetic isotope-containing variants of biological macromolecules for NMRanalysis(22–24).

*Aspergillus*isagroupoffilamentousfunguscanbeeasilycultivatedinlaboratoryconditions –meaningthattheyare, therefore,oneofthemostextensivelystudiedgroupsof fungus.

The*Aspergillus* groupcan,oncecultivated,beusedtosynthesiseusefulindustrialcompoundssuch as 'glycoside hydrolases. These enzymes are used in the process of converting biomassinto biofuels – by breaking down cellulose and hemicellulose from plant cell walls, into asubstance that is later converted into second-generation ethanol. This species can also beusedto producebioactivemetabolites, aswell asotherpharmaceutical products(25,26).

Microscopicexaminationrevealedthedarkbrownrough-edgedconidiaspores and brownconidiophores.CulturalexaminationusingPotatodextroseAgarandMaltYeastAgar.Genomicsequencing of the fungi can be done for identification and differentiation from other fungi.Thin-layer chromatography can be used for the identification and quantification of ochratoxinmycotoxin(27,28).

*Aspergillus niger*causes a black mould of onions and ornamental plants by infecting onionseedlings.Itcandisseminateandbecomesystemicandmanifestwhenconditionsarefavourable.*A.niger*causesacommonpostharvestdiseaseofonions,inwhichtheblackconidiacan be observed between the scales of the bulb. It can also cause disease in peanuts and grapes(29).

MATERIALSANDMETHODS

1. SampleCollection:

- SampleiscollectedfromthelocalmarketofMohaan,KakoriandKrishnaNagar,Lucknow.
- Various fruit peels were collected
  fromBanana
  CucumberOr
  angePomegr
  anatePineap
  pleSweet
  LimeWater
  melon

2. SamplePreparation:

- Thecollected fruitpeelswerechoppedintosmallpiecesusingscissors.
- Thefinelychoppedpieceswerewashed thoroughlywithwaterforatleast5-6 times.
- Afterwashingtheywerekeptforair dryfor2-3 daysonblottingpaper.
- Thepeelswerethentransferred to a hot airoven for tray dryingat 60ºCfor2-3hours.
- Afterproperdrying,thepeelsweregroundinto finepowder.

3. RawMaterialPreparation:

- Brothwasmadeinaratioof 1:10bysoaking20 gramsof fruitpeelpowder into200millilitresof double distilled water.
- ThenitwaskeptovernightinaBODincubatorshakertoallowproperbrothformation.
- Thenthe brothformedwasfilteredusing a muslinclothtoremove anysolidthatmay bepresentin the broth.
- Thefilteredbroth wasmeasuredandautoclaved at121ºCfor15minutes.
- Thepreparedbrothwasusedfurther forthefermentationprocess.

4. MediaPreparation:

Media used for fungal growth is usually Potato Dextrose Agar (PDA). It contains all thenutritionalrequirementsrelevanttothegrowthoffungi.Thismediaismadefromextractsofinf usedpotatoandcontainslaboratory-gradedextroseandagar.Itisasolidmediathatisutilizedon petri plates.

Procedure:

- Usingaweighingbalance,3.9grams o f PotatoDextroseAgarpowderwasmeasuredinbutterpaper.
- Thiswasmixedinaconicalflaskusingdistilledwaterandthevolumewasmadeupto 100 millilitres.
- Thiswasthensealedwithacottonplugandplacedonahotplatetoallowthepreparedmedia todissolve.
- Oncethemediadissolvesthemediamustbeautoclavedat121ºCatapressureof15 pounds persquareinch,withaholding time of15minutes.
- ThesterilizedmediaisthentakentoaLaminarAirFlowcabinetandpouredintoPetriPlates .
- Themedia intheplates isallowedtosolidifybeforeinoculationisconducted.

5. Isolationoffungus*(Aspergillusniger)*:

The fungi species of *Aspergillus niger*is selected from the black gram mould disease thatoccurs in an onion bulb. Therefore, the spores of *Aspergillus niger*can be isolated from that.This can be done by suitable inoculating and the picking of a pure colony as mentioned in thestepsgivenbelow.

Procedure:

- AnonionbulbcontainingblackgrammoulddiseasewascollectedfromalocalvendorinBanth ara, Lucknow.
- Theonionbulbwastakeninthesterilizedenvironmentof theLaminarAirFlowCabinetand waspeeled to exposethe infected inner layers.
- 20microliteresofautoclaveddoubledistilledwaterweretakeninan Eppendorftube.
- Usingaflame-sterilizedinoculationloop,theblackmouldontheinnerlayersoftheonionislightly scraped.
- Thiswasthendissolvedinthewater containedwithintheEppendorftube.

6. MicrobialCulture:

Microbiologicalculturesaremultipliedmicrobialorganismsthataregrowninapre-setculturemediuminalaboratory.GrowingamicrobialculturerequiresanappropriatephysicalenvironmentwithcontrolledpH,atmosphericgases,temperature,andpressure.Othernutritional requirements of the microbes include carbohydrates, proteins, salts, vitamins andgrowthfactors.Microorganismsareculturedtodeterminethespeciesoftheorganism,todetectits presencein a sampleand for diagnostic purposes.

Procedure:

- PourtheautoclavedPDAmediaontosterilizedpetriplatesandleaveittosolidifyfor30minutes.
- Thenusingamicropipetteadd2-3dropsofthewatercontainedwithintheEppendorftubewhich hasscraps ofblackmould prepared earlier.
- Afterthat withthehelpofan L-shaped spreader, it isspread smoothly onplatecontainingsolidifiedPDAmedia. Then theplates weresealed usingparafilm tapes.
- Thenitwasincubatedfor2-3daysat 37ºC toachievepropergrowthof fungus.

7. Sub-Culturingforfungalgrowth:

This is done in order to obtain a pure culture that will ensure that a single strain of the fungalorganismhasbeenisolated.Thiswillhelpdeterminetheexactorganismusedfortheproduction ofthestarchhydrolysingenzyme.

Procedure:

- Preparedmediaispouredintopetriplatesasdescribedintheprocedureabove.Itwasthenallowedtosolidify.
- InaLaminarAirFlow,theplatesinitiallyinoculatedwereopenedinthevicinityofaspiritlamp.
- Fromhereanisolatedcolonywaspickedusing aflamesterilizedinoculationloop.
- Thestreak-platemethodwasusedtocreateastreakonthesolidifiedmediagentlyinS

shape.

- Theplateswerethensealedusingparafilmtapesandwereallowedtoincubateat37ºCfor 48hours.

Figure1: Sub-Culturingof*Aspergillusniger*

- Onceisolatedcolonieswereobtained,thecoloniesfromtheplateswerefurthersub-culturedusingthestreakingmethodwithasterilizedcottonbud.
- Thecolonieswereallowedtogrowat37ºCfor48hoursandeventually,pureisolatedcolonies of*Aspergillus niger*were isolated.

8. Detectionofisolated species*(Aspergillusniger)*:

Sincetheisolatingsourcecouldhavecontainedalargenumberoffungalspecies,itisnecessaryto determine which species has eventually been isolatedfrom the source. In order toperformthis, Lactophenol Blue staining is conducted. This can help to identify the morphology of thefungus. When this is studied under the microscope, it can help to identify the strain that hasactually been isolated. Lactophenol Blue contains phenol, lactic acid and methyl blue. Phenolkills the live microbes that are not fungal on the slide so that they can be properly stained.Lactic acid preserves the fungal strains. Methyl Blue satins the chitin on the cell walls of thefungusandhelpthembevisibleunderthemicroscope.

Procedure:

- Inthevicinityofalitspiritlamp,thePetriplatecontainingthegrowthculturewasopened.
- Thenmicroscopeslideandcoverslipwerethoroughlycleanedusing70percentethanol.
- Adropoflactophenolbluewasplacedonthe centreofthe slide using amicropipette.

- Then aflame-sterilizedinoculationloopwasusedtolightlypickupafungalspore.
- Thesporewasplacedonthedropoflactophenolblueontheslideanditwascoveredwithacover slipgently,avoiding airbubbles.
- Thiswasthenstudiedunderthemicroscopeto viewmorphology.

9. SuspensionofthefungalCells:

A suspension is formed using the Tween 80 solution in order to count the spores using ahemocytometer.Tween80isadetergentthathelpsdetachthesporesfromthemediaontheplate.It dislodgesthespores.Thissolutioncanbeusedforthecountingofthesporeswhichcanbedoneusin gahemocytometer.

Procedure:

- AdilutionofTween80waspreparedin1percent-whichwasdoneby putting100microlitersin10milliliters ofdistilledwater.
- Thissolutionwasthenpouredontoaplatecontainingisolatedcoloniesof*Aspergillusniger*.
- Thatsolutionwasallowedtositontopofthe Petriplateforabout5minutes.
- Usingaflame-sterilizedinoculationloop,thefungalcolonieswereg entlyscrapedandasuspensionofthe sporeswasformedalong withtheTween80solution.
- Thissolutionwasthenusedtocountthesporesormeasureconcentration.

10. Countingofthefungalspores:

Ahemocytometerisasuitablydividedchamberthatcanbeusedto countthesporesthatare present in the solution. The volume of the chamber is known which is 0.1 cubicmillimeters. The spores are counted in boxes containing 16 squares each and an average istaken to establish whether a suitable count of spores is present to proceed for fermentation ornot.

Procedure:

- Thehemocytometerandcoverslipwerewipedwith70percentethanol.
- Using amicropipette,thechamberswerefilledwiththesuspensionofthespores.
- Thiswasthenplacedundera microscopeforvisualization.

- Thenthesporeswerecounted.

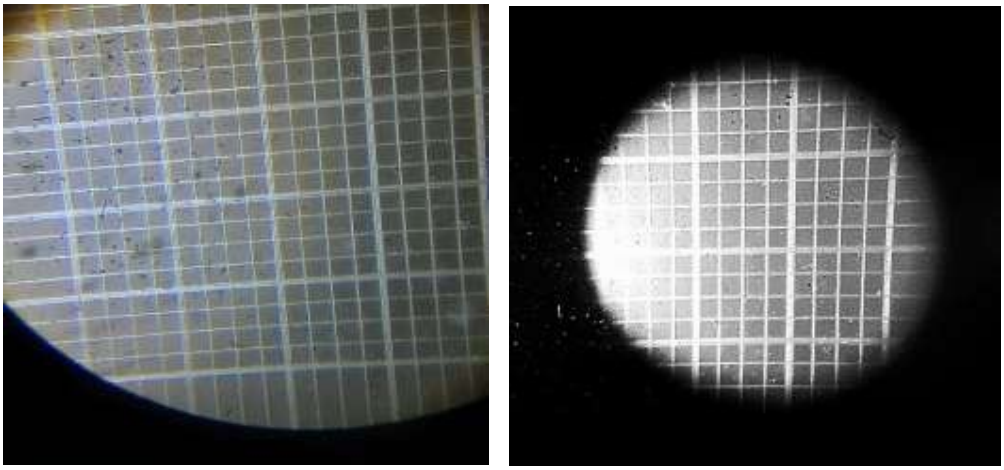

Figure2: Sporesvisualizedonthehaemocytometerusingamicroscope.

11. Establishmentofsuitabledilutionforfermentation:

Inordertoperformfermentationforadesiredvolume,asuitablevolumeofinoculumsolution is added.Inorderto estimatethisquantity,opticaldensityisperformedtomeasure theappropriateconcentrationofthesporesolutionusedforgrowth.Inordertocreatethissuspension Tween 80 is utilized. Tween 80 dilutions are used. According to the Clinical andLaboratoryStandardsInstitute,anabsorbanceof0.08to0.1atawavelengthof530nanometersequ atesto 10^6 spores.Thisamountissaid tobesuitablefor inoculation.

Procedure:

- Thedilutionpreparedearlierisusedintheamountsof300microlitresand500microlitres.
- Thenvolume ismadeupto4millilitresineachtube.
- Eachofthesewere usedtomeasuretheopticaldensityoftheformedsolutionat530nano meters.
- Thendesiredconcentrationwaschosenastheinoculum.

12. Fermentation:

Thisoutlinestheentireprocessutilizedforthepreparationofthesuitablefermentationbrothforthe productionofthestarchhydrolyzingenzyme andthe fermentationprocess.

12.1 Preparationofthefermentationbroth:

Thisincludesthecollection,washing,drying,grindinganddesolvationoffruitpeelstoforma suitable broth for the fermentation. Fruit peels were taken from different sources as thatwouldresultinabettersubstratefor *Aspergillusniger*toproducethedesiredenzymes.

Procedure:

- Fruitpeelswerecollectedfrombanana,cucumber,orange,pomegranate,pineapple,sweetli me, and watermelon.
- Thosewerethenthoroughlywashedwithwater.
- Thepeelswerecutintosmallpiecesandwereallowedtoairdryfor24hoursonablottingpape r.
- Thentheyweretransferredtoahotairovenwheretheyunderwentfurtherdryingat60degre es Celsiusfor48hours.
- Oncecompletelydriedthefruitpeelsweregrinded toforma finepowder.
- Ofthis20gramswereusedtomakeabrothbyadding200 millilitresofdistilledwatertoit.

Figure 3: Broth kept in overnight BOD incubator shaker and filtered using muslincloth.

- ThatwaskeptovernightinaBODincubatorshakerandallowedfortheproperformationofb roth.
- Thenthebrothformedwasfilteredusingamuslinclothtwicetoremoveanysolidsthatmayb epresent in that broth.
- Thefilteredbrothwasmeasuredandthenautoclaved at121degreesCelsiusfor15minutes
- Thisbrothwasusedfurtherforthe fermentationprocess.

12.2. FermentationProcess:

The fermentation process lasts for 5 days at a temperature of 25 degrees Celsius at anrpm of 120. Fermentation is the chemical breakdown of a substrate by an organism which is*Aspergillus niger*in this case. The optical density is measured at regular intervals in order tocheckthegrowthofthemicrobes.

Procedure:

- The fermentation broth prepared using the fruit peels was divided into twosterilizedconicalflasksonecontaining50millilitresandtheother150millilitres.
- Thepreparеddilutionofthe1percentsporecontainingsolutionexplainedearlierwasusedfor theinoculation.
- Intheflaskthatcontained150millilitresoffermentationbroth,500microlitresoftheprepared dilutionofinoculumwereaddedusingamicropipette.
- Thisflaskwasthensealedwithcottonplugsandplacedinanincubatorshakerat25degreesCel sius atrpmof120foraperiodof5-6days.
- Theflaskcontaining50millilitresoffermentationbrothwasalsoplacedinthesamecondition as acontrol.
- Everyalternateday,theopticaldensityofthemediawastakenat540nanometresusing the controlasablank.

13. MicrobialCount:

The microbial count is performed on fermentation broth in order to estimate the amount of*Aspergillus niger*grown. This aids in the estimation of the production of the enzyme infermentationbroth.Thisisconductedbyestimatingthewetanddrymassofthecells.Itisalsoastepint hepurificationprocessofstarchhydrolysingenzymewhichisproducedinthebroth.

Procedure:

- Whilefilteringthefermentationmedia,thefungalmasswasweighed.Thatwasthewetm ass.
- The fungalmasswasplacedinahotair ovenat60degreesCelsiusfor12to16hours.
- Thatwasthenrecoveredand thedrymasswastaken.

14. CrudeEnzyme:

The liquid fermentation broth left behind after the filtration of microbial growth is collectedand considered as Crude Enzyme. In order to purify it, the crude enzyme was centrifuged at1000 rpm for 5 min. After centrifugation supernatant part was collected and the pellet part whichcontained celldebris was discarded and further assays were performed on the resulting solutioninorder to detect theactivity of theenzyme.

15. EnzymeAssay:

Enzymeassaysarelaboratorytechniquesthatmeasureenzymeactivitywithinasample.Theyare used for a variety of purposes, which include identifying the presence of an enzyme, studyofenzyme kineticsand enzyme inhibition.

15.1 Starchassayofstarchhydrolysingenzyme:

Thisincludestheuseof citratebuffer andstarchsolutioninordertomodulatetheactivityofthe produced starch hydrolyzing enzyme. The amylase acts on the starch solution under thesuitable conditions that are maintained by the citrate buffer. The resultant activity can bemeasuredbytakingthe opticaldensityat540nanometers.

Procedure:

- Starchsolutionwaspreparedbyadding0.25gramsofstarchto25millilitresof

distilledwaterandwasallowedtodissolve evenly by placingit onahotplate.

- A citrate buffer of normality 0.1 was prepared by adding 1.34 grams of Sodium Citrateand 1.095 grams of Citric acid in 100 millilitresof distilled water. The pH mustbemaintainedat4.5.

Figure4: FreshlypreparedCitratebufferandStarchsolution.

- The enzyme assay was performed by adding 2 millilitres of the prepared starchsolution into two cuvettes in which one acted as control or blank and the other assample.
- Then200microlitresofthepreparedCitratebufferwasadded toeachcuvette.
- Intheblank,200microlitresofdistilledwater wasadded.
- Intheothercuvette,whichwasforsampleenzymefromfermentationmediawasadded.
- Theopticaldensitiesweremeasuredat540nanometres.

15.2. AssayofEnzymeactivitybyusingDNSmethod:

Enzyme activity is the process used to determine activity for enzyme by DNS method. In thismethod 3,5 Di-nitro salicylic acid is reduced to 3amino 5 nitro salicylic acid and this is donein the presence of reducing sugars such as maltose. The activity of amylase on starch can bemeasuredusingthismethodbycheckingtheabsorbanceofthesolutionat540nanometres.

PreparationofrequiredreagentsforDNS:

- Potassiumsodiumtartrate:Dissolve9gofPotassiumsodiumtartratein15mldistilledwater.
- NaOHSolution:Take1.6gofNaOHpelletsandproperlydissolvein200mldistilledwater.
- 3,5-DNSsolution:Dissolve0.3gofDNSreagentin6mldistilledwater.

DNSReagent:

- PrepareFreshracemicsolutionbymixingreagentsPotassiumsodiumtartrateand3,5-DNSand makeup the volumeup to 150mlNaOH.

PreparationofStandardSugarsolution:

- Stockstandardsugarsolution:250gofGlucosein100mlofdistilledwater.
- Workingstandardsolution: Take10mlfromstockandmakevolumeupto 100ml.

Figure5:DNSReagent andWorkingGlucose

Procedure:

PreparationofStandardSolution:

- Toatesttube500microlitresofdistilledwaterwasadded.
- Then500microlitresofworkingglucosesolutionwasadded.
- Thiswasthenincubatedat25degreesCelsius for3minutes.
- DNSreagentof1millilitrewasaddedtothetesttube.

- Thiswasthenplacedinboilingwaterbathfor5minutes.
- Then10millilitresofdistilledwaterwereaddedtothetesttube.

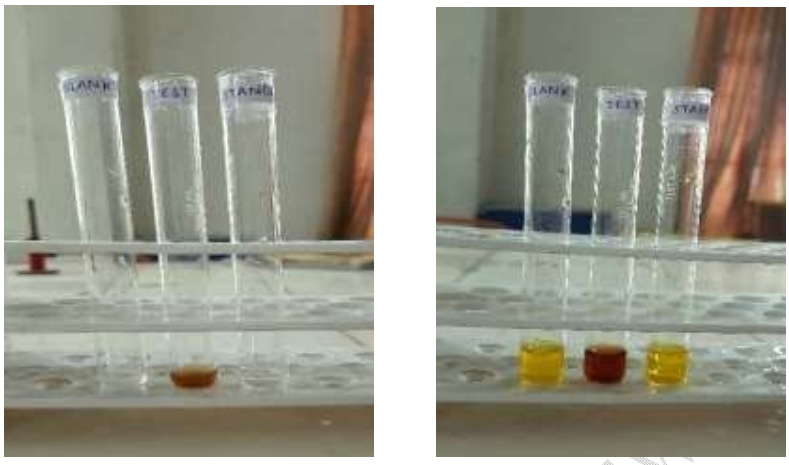

Figure6: Blank,TestandStandardtesttubesafterincubationandaddition ofDNSreagent

PreparationofBlankSolution:

- Toatesttube500microlitresofstarchsolution was added.
- Then500microlitresofworkingglucosesolutionwasadded.
- Thiswasthenincubatedat25degreesCelsius for3minutes.
- DNSreagentof1millilitrewasaddedtothetesttube.
- Thiswasthenplacedinboilingwaterbathfor5minutes.
- Then10 millilitresofdistilledwaterwereaddedtothetesttube.

Figure7: Blank,TestandStandardtesttubesplacedinboilingwaterbathandafterthat.

PreparationofTestSolution:

- Toatesttube500microlitresofstarchsolution was added.
- Then500 microlitresofworkingglucosesolutionwasadded.
- Thiswasthenincubatedat25degreesCelsius for3minutes.
- DNSreagentof1millilitrewasaddedtothetesttube.
- Thiswasplacedinboilingwaterbathfor 5minutes.
- Then10 millilitresofdistilledwaterwereaddedtothetesttube.

16. PurificationandDownstreamProcessingofEnzyme:

16.1 ProteinprecipitationusingAmmoniumSulfate:

The addition of ammonium sulphate results in the salting in and salting out of the solution.Initially, the protein is suspended in the solution. In addition to ammonium sulfate the proteinsolubility increases at first due to the addition of the salt. This is the saltingin process.However, after certain concentrations of the added salt the salting outbegins to occur wherethe proteins, precipitate as the solubility of the salt increases in the solvent. The precipitatedproteinscanthenbeextractedbycentrifugation.

PreparationofTrisbuffer: Take2.428g ofTrisandaddinto100mlof distilledwater.Procedure:

- 25millilitresofthecentrifuged enzymeweretakeninabeaker.
- Thiswasplacedon amagneticstirrer andthetemperaturewasmaintainedat4ºC.

Figure8: CentrifugedenzymeonmagneticstirrerandAmmoniumsulfateadded

- Tothis18gofAmmoniumsulfatewasaddedgraduallypinchbypinchandwasmixed thoroughly.
- Thesolutionwasthencentrifugedat10000rpmfor10minutes.
- ThesupernatantwasremovedandabOut500microlitresofTrisbufferisaddedtoit.
- Thiswasthoroughlymixedwiththepelletsandwasstoredforfurtherpurificationat4ºC.

16.2. DialysisofStarch Hydrolysingenzyme:

Dialysis process work on the principle of diffusion and size exclusion. It is a process bywhich a membrane is used to allow the retention of the enzyme and then allowfurtherdiffusion of the buffer outside. This is based on the principle of diffusion in which theenzymeremainsinsidethedialysisbagonthebasisoftheconcentrationgradient.Thishelpsisolatethepure enzyme.

Procedure:

- Adialysisbagwastakenanddipped inSDS solution3-4times.
- Thenitwasdippedinaboilingwaterbathat100degreesCelsiusaround5-6times.
- Afterthis, aknotwastiedatoneend ofthebag.
- Theenzymepelletswithbufferwereplacedinsidethe bag.
- Thebagwasthenknottedattheotherend.

Figure9: ConicalflaskcontainingTris buffer anddialysisbagwithenzymeinside.

- InaconicalflaskcontainingTrisbuffer,thedialysisbagcontainingenzymewasdippeda ndmaintainedonamagneticstirrerat 4 degreesCelsiusfor48 hours.
- Thus,thepureenzymewasretainedinsidethebagwhilethebufferwasdiffusedout.

Figure10: Undergoingdialysisprocess

Theproperdiffusionprocesstookwithin4-5daysandthen,pureenzymewastransferredfromthedialysisbag into asterileconditionin abeaker.

RESULTSANDDISCUSSION

1. SampleCollection:

Samplecollectedfromlocalmarketsof Mohaan,Kakori,andKrishnaNagar,Lucknow.

Figure11: Fruitpeelswashedandcutintosmallpieces.

2. SamplePreparation:

After collection, sample was chopped into small pieces, thoroughly washed, air dried andovendried.

Figure12: Samplebeingoven-dried

Then,afterdryingthesamplewasgrindedtomakefinepowder asshownbelow

Figure13: MixedFruitPeelPowder

3. Rawmaterialpreparation:

Thepreparedbrothwasusedfurtherforthefermentationprocessbysoakingfruitpeelpowderintodoubl e distilled water.

Figure14: Brothprepared forfermentation

4. Isolationoffungus(*Aspergillusniger*):

Funguswasisolatedfromanonionbulbcontainingblackgrammoulddiseasewascollectedfroma local vendorin Banthara, Lucknow.

Figure15: Onionbulbwithblackmoulddisease

5. MicrobialCulture:

For the growth of fungus, a swab has been taken from the diseased onion and grown onPDAmediaasshownbelow.

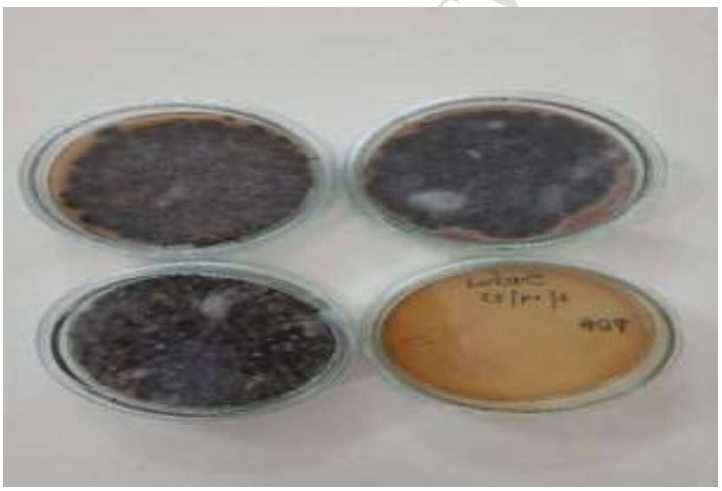

Figure 16: Plates containing solidified PDA media and fungus growth using the spreadplatemethod

6. Sub-culturingforfungalgrowth:

This is done to obtain a pure culture that will ensure that a single strain of the fungalorganism (*Aspergillus niger*)hasbeen isolated.

Figure17: Pureculture of fungus

7. Detectionofisolated species*(Aspergillusniger)*:

The stainingtechniquehasbeenusedby usinglactophenolbluedyeforidentificationand *Aspergillusniger*funguswasidentifiedasshown below.

Figure18: Stained*Aspergillus niger*

8. SuspensionofthefungalCells:

AsuspensionisformedusingtheTween80solutiontocountthesporesusingahemacytometer.

Figure19: Tween80solutioncontainingfungalspores

9. Countingofthefungalspores:

Ahemacytometeris asuitablydividedchamber thatcanbeusedtocountthesporesthat arepresentin the solution.

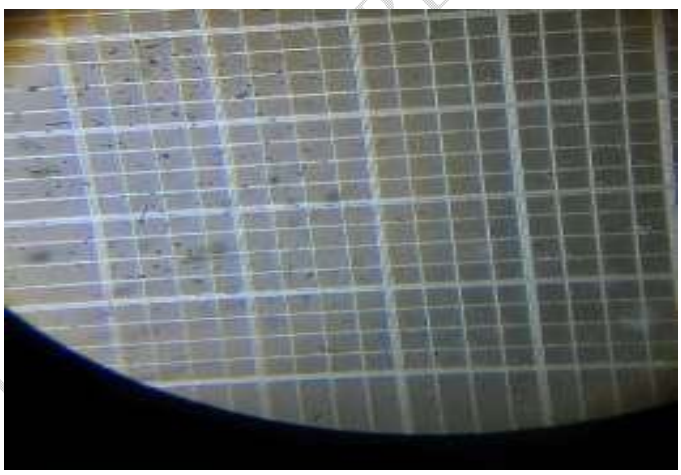

Figure20: Sporesvisualizedon thehaemocytometerusing amicroscope.

Result: Theaveragenumberofspores estimatedwas:2980sporespermillilitre

10. Establishmentofsuitabledilutionforfermentation:

To perform fermentation for a desired volume, a suitable volume of inoculumsolution is added. To create this suspension Tween 80 is utilized. According to the ClinicalandLaboratoryStandardsInstitute,anabsorbance of 0.08to0.1 issaidtobesuitableforinoculation.

Figure21: Cuvettecontainblanksampleand Tween80suspension

MicrolitersofTween80suspension	Opticaldensityat530nm
Blank	0
300	0.079
500	0.164

Table1: Demonstratingtheopticaldensityofdilutionusedforfermentation

Result: Therefore,thedilutionof300microlitreswasselectedforfermentation.

11. Fermentationprocess:

Inoculum was added in a conical flask containing fruit peel waste broth and kept forthefermentationprocessfor5-6 days.

Figure22: BrothkeptforfermentationinBODincubatorshaker

Theopticaldensityismeasuredatregularintervalstocheckthegrowthofthemicrobes.Theopticaldens ityofthemediawastakenat540nanometres.

Figure23: Cuvettecontainingfermentation broth

Results:

NumberofDays	**OpticalDensityoffermentation**
Day1	0.349
Day3	1.354
Day5	0.561

Table2: Demonstratingtheopticaldensityoffermentationmedia

Therefore,the opticaldensitywasgreaterinthefirsttwomeasurementsbutlater decreasedasthe nutrients from themediabegan to deplete by Day5.

12. MicrobialCount:

Themicrobialcountisperformedonfermentationbrothtoestimate theamountof *Aspergillusniger*grown.

Whilefilteringthefermentationmedia,thefungalmasswasweighedwhichwaswetmass.

Figure24: Wetmass

Results:

Wetmass(g)	Drymass(g)
3.34	0.69

Table3: Demonstratingtheweightoffungalmass.

Themicrobialcountismeasuredbysubtractingthedrymassfrom the wetmasswhichis**2.65.** Thiscanbeusedto quantifythenumberofcellsgenerated.

13. CrudeEnzyme:

Theliquidfermentationbrothleftbehindafterthe filtrationofmicrobialgrowthwascollectedandconsidered aCrude Enzyme.

Figure25: Crudeenzyme

14. Starchassayofstarchhydrolysingenzyme:

Thisincludestheuseofcitratebufferandstarchsolutiontomodulatetheactivity oftheproduced starch hydrolyzing enzyme.

Figure26: FreshlypreparedCitratebufferandStarchsolution.

Theresultantactivitywasmeasuredbytakingtheopticaldensityat540 nanometers.

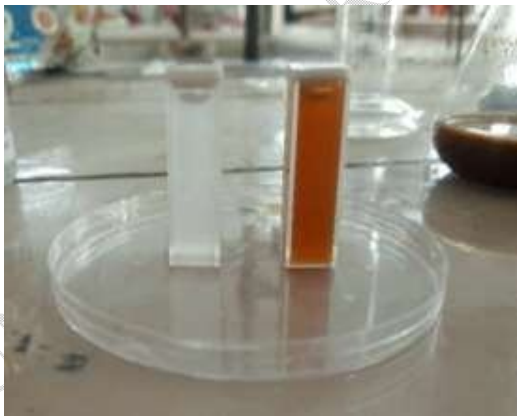

Figure27: Cuvettecontainingblankandsamplesolutions

Results:

Solution	Opticaldensityat540nanometres
Blank	0.508
Sample	1.868

Table4: DemonstratingStarchassayofcrudeenzyme

Theincreaseinopticaldensityofthecuvette-containingenzymeshowstheeffectoftheamylaseenzymeonthestarchsolution.Hence,theabsorbanc

eis greater.

15. PreparationofStockSolution:

- 1.34gsodiumcitrateand1.095gcitricacidaddedin100mldistilledwater.
- 0.25gstarchaddedin 25mldistilledwater.
- Crudeenzyme.
- 1.6gNaOHpellets added in200mldistilledwater.
- 25gglucoseaddedin100mldistilled water.
- Potassiumsodiumtartrate,NaOHSolution, and 3,5-DNSsolutionaddedtogethertoforma DNSreagent.

Figure28: Stock Solution

16. AssayofEnzymeactivitybyusing the DNSmethod:

Enzymeactivity istheprocessusedto determine the activityofenzymes by the DNSmethod.

Theactivityofamylase onstarchcanbemeasuredusingthismethodbycheckingtheabsorbanceof thesolutionat540nanometres.

Forthis,threetesttubesofblank,test,andstandardweremaderespectivelybydifferentreagentsand the optical densities weremeasured.

Figure29: Blank,Test,andStandardtesttubesafteradditionof10mldistilledwater

After that all the test tubes were kept at room temperature and the optical densities weremeasuredaccording totheirmentioned name.

Results:

Solution	**Opticaldensityat540nanometres**
Blank	0.588
Standard	0.413
Test	0.330

Table5: DemonstratingOpticaldensitiesofdifferenttesttubes

Thus,thedecreasedabsorbanceshowstheactivityoftheenzymeonthestarchsolution.

17. PurificationandDownstreamProcessingofEnzyme:

Figure30: Pureenzyme

CONCLUSION

Based on the performed experiment, we can conclude that amylase can be produced fromfruit peel waste as substrate by using submerged fermentation which was carried out for 5-6daysin a B.O.Dincubatorshaker machine at 37°C.

Enzyme activity was performed to know the activity of the enzyme by using the DNS method. AfterperformingtheenzymeactivitybyusingextractedcrudeenzymesolutiontheobtainedO.D.ofam ylaseenzyme-0.504.

This project was carried out on a laboratory scale. If we want to enhance the production of theseenzymes,wehavetocarryoutalltheprocessesat an industrialscale.Asthepopulationoftheworldis rapidly increasing and approaching the exhaustion of various natural resources, the productionof enzymes by using mixed fruit peel offers great potential for many industries to help meetthechallenges(30–32).

CONFLICT OF INTEREST

The authors attest that they are free of any known financial or personal conflicts of interest that would taint the findings of this study.

Disclaimer (Artificial intelligence)

The authors hereby certify that neither the drafting nor editing of any papers has involved the use of generative AI tools, such as text-to-image generators or large language models (ChatGPT, COPILOT, etc.).

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