EXTRACTION OF PECTIN FROM PINEAPPLE PEELS AND PRODUCTION OF PECTINASE BY*ASPERGILLUS NIGER*

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ABSTRACT

The agricultural wastes generated frompineapple (*Ananascosmosus*)represents about 35% of the entire fruit. These wastes can be converted to most useful products such as pectin. Pectin was extracted from pineapple peels with a percentage yield of 8.33% at pH2.2 and temperature of 70ºC. Three pectinolytic fungi: *Aspergillusniger*, *Aspergillusfumigatus*and *Aspergillusflavus*were isolated from natural sources and tested for their pectinolytic activity.In submerged fermentation system containing pineapple pectin broth, *Aspergillusniger*was inoculated and incubated for 4 days. The crude enzyme pectinase was harvested after fermentation by filteration process.

Keyword:-Pineapple peels, Pectin, Pectinase and*Aspergillusniger.*

Introduction:-

Pineapple (*Ananascosmosus*) belongs to Bromeliaceae family. This is a tropical plant and its edible fruit is a multiple fruit consisting of coalesced berries. However, processing and utilization of pineapple in to various products leads to generation of waste in the form of peels and pomace. Pineapple waste can be bio-transformed in to by-productssuch as pectin, dietary fibers and pectinases.

Pectin is one of the major components of the primary cellular walls in the middle lamella of plant tissues. Pectin was first isolated and described in 1825 by Henri Braconnot (Bracconot and Keppler, 1825.Pectinasescanbeproducedbybothsubmergedandsolidstatefermentation(SSF).Submergedfermentationisc ultivationofmicroorganismsinliquidbroth.Itrequireshighvolumesofwater,continuousagitationandgenerateslot ofeffluents.SSFincorporatesmicrobial growthandproductformationonorwithinparticlesofasolidsubstrate(Mudgett,1986)underaerobicconditions.

Pectinasesareagroupofenzymes,whichcausedegradationofpectinthat,arechainmoleculeswitharhamnogalactur onanbackbone;associatedwithotherpolymersandcarbohydrates.Thesepectinaseshavewideapplicationsinfruitj uiceindustryandwineindustry.Infruitjuiceindustry,itisusedforclarification;reductioninviscosityiscausedwhich ultimatelyleadstoformation ofclearjuice.

Abbreviations:-

UDP-D-Uridinediphosphate
PDA-PotatoDextroseAgar
SmF-Submerged fermentation
SSF-Solidstate fermentation

History and Description of Pineapple:-

Pineapple (Ananascosmosus) is the common name for a tropical plant and its edible fruit, which is actually a multiple fruit consisting of coalesced berries. It was given the name pine apple due to its resemblance to a pine cone. The pine apple is the most economically important plant in the Bromeliaceae family.The word "pineapple" in English was first recorded in 1398, when it was originally used to describe the reproductive organs of conifer trees. The term pine cone for the productive organ of conifer trees was first recorded in 1694. When European explorers discovered this tropical fruit, they called them pineapples (Wikipedia, 2011). The popularity of the pineapple is due to its sweet-sour taste. The core of the pineapple is continuous with the stem supporting the fruit and with the crown, a feature unique among cultivated fruits. The stems and leaves of the pineapple plant are sources of fiber, which can be processed in to paper and cloth. The cloth made from pineapple fiber is known as 'pinacloth'and was in use as early as 1571. Parts of the pineapple plant (Fig.1) are used as silage and hay for cattle feed such as the processed wastes in the form of pomace or centrifuged solids from juice production (Wikipedia, 2011).

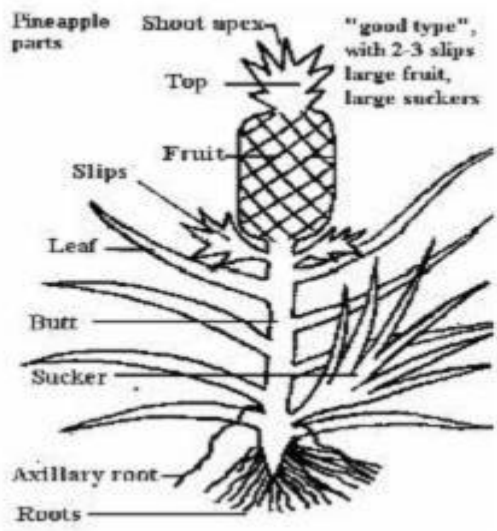

Figure.1: Parts of a pineapple fruit (Elfick, 2007).

Plant Cell Wall :-

Plant cell walls consist of plant middle lamella, primary cell wall and secondary cell wall as can be seen in Fig.2. The primary walls of enlarging plant cells are composed ofapproximately 30% cellulose, 30% hemicellulose and 35% pectin with about 1-5% structural protein (glycoprotein) on a dry weight basis (Cosgrove, 1997).

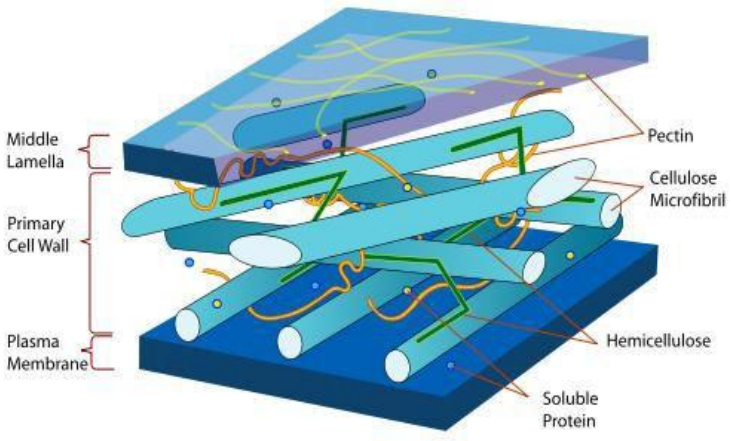

Figure.2: Structure of the Plant Cell Wall (Carpita and Gibeaut, 1993).

The Middle Lamella of the Fruit Cell :-

The middle lamella is the first layer formed during cell division, and can also be seen as the space between the cell walls, and as the connecting region between adjacent cells, binding cells together. The highest concentrations of pectin are found in the middle lamella of cell walls, with a gradual decrease as one passes through the primary wall toward the plasma membrane (Kertesz, 1951).

Pectic Substances:-

Pecticsubstance is the generic name used for the compounds that are acted up on by the pectinolytic enzymes. They are high molecular weight, negatively charged, acidic, complex glycosidicmacromolecules (polysaccharides) that are present in the plant kingdom. They are present as the major components of middle lamella between the cells in the form of calcium pectateand magnesium pectate(Rastogi, 1998). The synthesis ofpecticsubstances occurs in the Golgi apparatus from UDP-D-galcturonic acid during early stages of growth in young enlarging cell walls (Sakai*etal*., 1993).Compared with young, actively growing tissues, lignified tissues have a low content of pectic substances. The content of the pecticsubstances is very low in higher plants usually less than 1%. They are mainly found in fruits and vegetables, constitute a large part of some algal biomass (upto 30%) and occur in low concentration inagricultural residues(Table:1). Pectic substances account for 0.5–4.0% of the fresh weight of plant material (Kashyap*etal*., 2001; Sakai*etal*., 1993). Contrary to the proteins, lipids and nucleic acids, which are polysaccharides, pecticsubstances do not have defined molecular masses.

Table1:Compositionofpecticsubstancesin differentfruitsandvegetables

Fruit/vegetable	Tissue	Pecticsubstance(%)
Apple	Fresh	0.5-1.6
Bannana	Fresh	0.7-102
Peaches	Fresh	0.1-0.9
Strawberries	Fresh	0.6-0.7
Cherries	Fresh	0.2-0.5
Peas	Fresh	0.9-1.4
Carrots	Dry matter	6.9-18.6
Orange pulp	Dry matter	12.4-28.0
Potatoes	Dry matter	1.8-3.3
Tomatoes	Dry matter	2.4-4.6
Sugar beet pulp	Dry matter	10.0-30.0

Source:Kashyap*etal*.,2001.

Pectin :-

Through various studies, it has been brought in notice that the structure of pectin is difficult to determine because pectin subunit composition can change during isolation from plants, storage and processing of plant material (Novosd'skaya, 2002). Pectin was first isolated and described in 1825 by Henri Braconnot(Braconnotand Keppler., 1825). At present, pectin is thought to consist mainly of D-galacturonic acid (Gal A) units (Sriamornsak, 2002), joined in chains by means of α(1-4) glycosidic linkage (Fig.3).

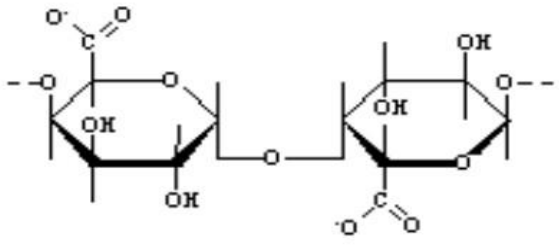

Figure.3: Structure of Galacturonic Acid (Pilnikand Voragen, 1993)

GeneralProperties ofPectins :-

Pectinissolubleinpurewaterasmonovalentcation(alkalimetal)saltsofpectinicandpecticacids;areusuallysolubleinwaterunlikedi-andtrivalentcationsaltsthatareweaklysolubleorinsoluble(Sriamornsak,1998).DilutepectinsolutionsareNewtonianbutatamoderateconcentration,theyexhibitthenon-Newtonian,pseudoplasticbehaviour and characteristics.

Applications of Pectin:-

- MucoadhesivePolymer.
- Gellingagent,ThickenerandWaterBinder.
- MedicineandPharmaceuticalIndustry.

BiotechnologicalApplications of MicrobialPectinases:-

- FruitJuice Extraction.
- CoffeeandTeaFermentation.
- TextileProcessingandBioscouringof CottonFibers.
- Degummingof PlantBastFibers.
- WasteWaterTreatment.
- PaperandPulpIndustry.
- AnimalFeed.
- Improving theStabilityof RedWines.

Substrates fortheProductionof Pectinases:-

Substratesthatareemployedintheproductionofenzymeshouldbesolid,assolidsubstratecanencouragethegrowingcells.Substratesshouldprovideallneedednutrientstothemicroorganismsforitsgrowth.Otherfactorslikeparticlesize,moisturelevelsarealsotobetakenforconsideration.Generallyagro-industrialwastesareemployedforthepectinaseproduction.Varioussubstratesthatarebeingusedaresugarcanebagasse,wheatbran,ricebran,wheatstraw,ricestraw,sawdust,bananawaste,teawaste,sugarbeetpulp,applepomace,orangepeel,etc(Pilar*etal*., 1999).

FermentationConditions:-

Pectinasesareconstitutiveorinducibleenzymesthatcanbeproducedeitherbysubmerged(AquilarandHuitron,1999)orsolidstatefermentation(Acuna-arguelles*etal*.,1995).Variousfactorsaffectingtheproductionofpectinaseareconcentrationofnutrients,pH,temperature,moisturecontent,influenceofextractionparametersonrecoveryofpectinasesandtheeffectsplayedbytheinducers.Bothcarbonandnitrogensourcesshowoveralleffectontheproductivityofpectinases(Catarina*etal*.,2003;AlmeidaandHuber,2011).Pectin,glucoseandsucrosewhenaddedtothemediainhigherconcentrationhavearepressioneffecton thestudiedenzymeactivity(Maria*etal*.,2000)ofthevariousnitrogenousmatterthatcanbeused.Optimumsourcesare$(NH_4)_2SO_4$,yeastextract,soyabeanpulppowder,soyapeptone.

TemperatureandpHarealsoimportantparameters, wherepHis regulatedusingamixtureofsourcesofnitrogenwhen*Aspergillusniger*isbeingused,pHturnstobeacidic.Moisturecontentinthesubstratealsoplaysasignificantrole(Martin*etal*.,2004).Thepreviousstudies showthatitwasgenerallymaintainedaround50-55%fortheproductionofpectinasesbymicrobialmeans(Leda*etal*., 2000).

Twotypesoffermentationscanbecarriedoutforpectinaseproduction,theyaresolidstatefermentationandsubmergedfermentation.Thegrowthoforganismsisveryhighwithlargequantities ofenzymebeingproducedinsolid-statefermentation(RamanujamandSaritha,2008).Howeverintheproductionofextracellularpectinases,submergedfermentationispreferableastheextracellularpectinasesareeasierandcheapertouseingreatquantities.Submergedorsolidstate mediumsareusedforproducing ofthepectinoliticenzymesbyfungi(Bali,2003).

Types of Fermentation:-

i) SolidStateFermentation(SSF)

ii)SubmergedFermentation(SmF)

Solidstatefermentationisdefinedasthecultivationofmicroorganismsonmoistsolidsupports,eitheroninertcarriers oroninsolublesubstratesthatcanbeusedascarbonandenergysource.Thisprocessoccursintheabsenceornearabsen ceoffreewaterinthespacebetweensubstrateparticles.Inthissystem,waterispresentin thesolidsubstratewhosecapacityforliquidretentionvaries withthetypeof material(Lonsane*etal*., 1985;Pandey*etal*.,2001).
Submergedliquidfermentationisthecultivationofmicroorganismsinliquidnutrientbroth.Industrialenzymescanb eproducedusingthisprocess.Thisinvolvesgrowingcarefullyselectedmicroorganismsinclosedvesselscontaining arichbrothofnutrientandahighconcentrationofoxygen(Grigelmo-MigeulandMartin-Belloso,1998).
ThereareseveraldisadvantagesofSSFwhichhavediscouragedtheuseofthistechniqueforindustrialproductionand thereforehavemadeSmFmoreapplicableintheproductionofenzymes.Theseinclude:thebuildupofgradientsofte mperature,pH,moisture,substrateconcentrationorCO_2duringcultivationwhicharedifficulttocontrolunderlimite dwateravailability(Holker*etal*.,2004).

Aim and Objectives of the Study:-

- Toextractpectinfrompineapplepeels.
- Toisolate*Aspergillusniger*fromsoilcontainingdecomposingpineapplepeels.
- Toproduceextracellularpectinasebyinducing*Aspergillusniger*insubmergedfermentationwithpectinextract s frompineapplepeels.

Equipments:-
Autoclave, Centrifuge, Magneticstirrer, Microscope, Millingmachine, Oven, pHmeter, Waterbath, Weighingbalance.

Collectionof PineappleSamples:-
Pineapple(*Ananascosmosus*)peelswereobtainedfromfruitmarket.

Collectionof Micro-organisms:-
Mixedcoloniesofmicroorganismswereobtainedfromadumpcontainingdecayingpineapplepeelsand*Aspergillusn iger*wasisolatedusingmorphologicalcharacteristics.

Methods:-

Preparationof GroundPineapplePeels:-

Thepineapplepeelswerewashedandcutintosmallbitsandthentreatedwithhot96%ethanolinordertoreduce themicrobialload.Theethanoltreatedpeelswere washedwithwaterandsun driedfor7days.Thedriedpeelswerethengroundtopowderusingamillingmachine.

Extractionof Pectinfrom PineapplePeels:-

Pectinwasextractedbythemethodof McCready,(1970).100gofgroundpineapplepeelswaspouredintoa20 00mlbeakercontaining800mlofdistilledwater,then12goffreshlygroundsodiumhexa-metaphosphatewasaddedtothemixtureandtheinitialpHwasadjustedwith3N HClto2.2±0.Themixturewasheatedupinawaterbathat70ºCfor1hourandstirredcontinuouslyusingapropellertype dstirrer.

ThepHwascheckedatanintervalof15minutes.Thewaterlostbyevaporationwasreplacedexceptinthelast2 0minutesoftheextractiontime.Theextractwasvacuum-

filteredusingamuslinclothandtheresiduewaswashedwith200mlofdistilledwater.Thewashingswerehoweveradd edtothefiltrateandthenconcentratedbyevaporationonahotplatetoapproximatelyone-fifthofitsinitialvolume.

Theconcentratedpectinmixturewascooledto50ºCandavolumeofethanolcontaining0.5MHClintheratioo f3:1tothepectinmixturewasadded,itwasstirredcontinuouslyfor30minutesandallowedtostandfor1hour.Thepreci pitatewasvacuumfilteredandwashedwithacetoneinordertoremovetracesof HClandethanol.Theextractwasdriedi nanovenat40ºCforafewhours andgroundtopowder.

IsolationofPectinolyticFungi:-

CollectionofSoilSamples:-

Soilsampleswerecollectedfromsitecontainingdecomposingpineapplepeels.Thesoilsampleswerecollect edin acleandryplasticcontainer.

PreparationofSoil SampleExtracts forMicrobialIsolation:-

Soilsamplesfromsiteofdecomposingpineapplepeelswerehomogenizedinsterilemediumcontaining1%p ineapplepectin,0.14% of$(NH_4)_2SO_4$,0.2%ofK_2HPO_4,0.02%of$MgSO_4.7H_2O$&0.1%of nutrientsolutioncontaining5mg/Lof$FeSO_4.7H_2O$,1.6mg/Lof$MnSO_4.H_2O$,1.4mg/Lof$ZnSO_4.7H_2O$,2.0mg/Lof $CoCl_2$.Themixturewasincubatedat30ºC for24 hours.

PreparationoftheSolidMedium:-

Themixturecontained1%pineapplepectin,0.14%of$(NH_4)_2SO_4$,0.2%ofK_2HPO_4,0.02%of$MgSO_4.7H_2O$ &0.1%ofnutrientsolutioncontaining5mg/Lof$FeSO_4.7H_2O$,1.6mg/Lof$MnSO_4.H_2O$,1.4mg/Lof$ZnSO_4.7H_2O$,2 .0mg/Lof$CoCl_2$and3%agar-agar(thegellingagent)(w/v).

Themediumwasautoclavedat121^{o}Cfor15min.Itwasallowedtocool toabout45^{o}CandthenpouredintoPetridishesandallowedtogel.Theplateswerethenincubatedinaincubatorat37^{o}C overnighttocheckforsterility.

Sub-culturingintoSolidMedium:-

Aloopofhomogenizedextractfromthesampleextractswasstreakedontothesolidmediumunder theflameo fBunsenburner.Theplateswereincubatedat35^{o}C(roomtemperature)tillvisiblecolonies wereobserved.Allmorphologicalcontrastingcolonies werepurifiedbyrepeatedstreakingandsub-culturingonseparateplates.Thisprocesswascontinuedtillpurefungalcultureswereobtained.

Storageof Micro-organisms onPotatoDextroseAgar(PDA):-

Purefungalisolateswereмaintainedon PDAslantsasstockcultures.ThePDAmediumwaspreparedaccordin gtothe manufacturer's instructions.

MicroscopicFeaturesoftheIsolatedFungi:-

Threedaysoldpurecultureswereexamined;thecolor,texture,natureofmyceliaorsporesandgrowthpattern swerealsoobserved.Photographs oftheculturewerealsotaken.

FungalIdentification:-

Threedaysoldcultureswereusedinpreparingmicroscopicslides.Atuftofthemyceliawasdroppedonthesli deandadropoflacto-phenolbluewasaddedtoit.Acoverslipwasplacedoveritandviewedunderalightmicroscopeat×40magnification.Id entificationwascarriedoutbyrelatingthemicroscopicfeaturesandthemicrographstoAtlasofMycologybyBarnett andHunter(1972).

Preparationofthefermentationmedium:-

Thesubstrateforfermentationconsistedofgroundpineapplepectinextracts.Submergedfermentationwasc arriedoutusing10250mlErlenmeyerflaskcontaining200mlofthesterile cultivationmedium.Themediumwasoptimizedforpectinaseproductionwith0.1%NH_4NO_3,0.1%$NH_4H_2PO_4$,0.1 %$MgSO_4.7H_2O$and20gofpowderedpineapplepectinextracts.Theflaskwascoveredwithaluminiumfoilandautoc lavedat121ºC for20 minutes.

Innoculatingwith*Aspergillusniger*:-

Ineverysterileflask,twodiscof *Aspergillusniger*obtainedfromthefreshlypreparedplatesareaddedusingacorkborerofdiameter10mmundersterilconditions.Theflasksarepluggedfirmlyandincubatedforfourdaysatroomtemperature.

Harvestingthecrudeenzyme:-

Attheendofday4 onwhichthehighestenzymeactivityhasbeendetected,themycelialbiomasswasfilteredusingfilterpaper.2.0litresof filtraterecoveredwasusedas thecrudeenzyme,whiletheresiduewas treatedwithlimeanddiscardedproperly.

Result and Analysis:-

PineapplePectinExtraction:-

Pectinextractionyieldwasfoundtobe8.33%atpH2.2,temperatureof70^{o}Candextractiontimeof1hour.

Photographof PineapplePectinExtract:-

Fig.4shows aphotographofthepineapplepectinafterextraction.

Figure.4: PhotographofPineapplePectin

SelectionofPectinolyticFungi:-

Threespeciesoffunginamely:*Aspergillusniger*,*Aspergillusfumigatus*and*Aspergillusflavus*wereisolated fromnaturalsourceofsoilcontainingdecayingpineapplepeels.Theseorganismswerequalitativelyscreenedforpectinolyticactivityonselectivemediaandtheirisolationwasbasedonthesimilaritiesoftheirmorphologicalfeaturesin bothtestculturescontainingpineapplepectinandthestandardculturecontainingapplepectinascarbonrespectively.

MacroscopicandMicroscopicFeaturesof FungalIsolates:-

Genusidentificationwasbyexaminingbothmacroscopicandmicroscopicfeaturesofathree dayoldpureculture.Color,texture,natureofmyceliaand/orsporesproduced,growthpatterninadditiontomicroscopicfeaturessuchasseparationandsporeshapeswereexamined.Basedonthesecharacteristics,*Aspergillusniger*,*Aspergillusfumigatus*and*Aspergillusflavus*wereconfirmedasthethreepectinolyticfungalisolates,respectively.However,*Aspergillusniger*showedrelativelyhigherpectinaseactivityandwasselectedforfurtherstudies.Fig.5 showsaphotographofthepureculture of*Aspergillusniger*.

Figure.5: PureCultureof*Aspergillusniger*

PectinaseProductionunderSubmergedFermentationSystem:-

Avolumeof2litresofcrudeenzymewasharvestedafter4daysofsubmergedfermentationusing*Aspergillusniger.*

Discussion:-

Pineapple (*Ananascosmosus*) peels as agricultural wastes represent about 35% of the fruit mass. During extraction of pectin from pineapple peels, the yield of pectin extracted was 8.33% at pH2.2, temperature of 70ºC and extraction time of 1hour using the method as described by Mc.Cready (1970). The yield could be affected by the pH of the extraction medium and extraction time.

Three fungal species *Aspergillusniger* was isolated from natural waste source selected including 2 other spsie.,*Aspergillusfumigatus*and *Aspergillusflavus* which showed low pectinase activity in the fermentation process when compared to *A.niger.*In a fermentation process substrate should provide all nutrients needed to the microorganisms for its growth. The accumulation of maximum extracellular pectinase was observed after 96 hours of fermentation. The period of fermentation depends on the nature of medium, fermenting organisms, concentration of nutrients and the process physiological conditions (Patiland Dayanand, 2006).

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. In a submerged fermentation microorganisms are grown inclosed vessels containing a rich broth of nutrientsand a high concentration of oxygen (Grigelmo - Migeul and Martin-Belloso, 1998).

Conclusion:-

From these investigations it is evidenced that the pineapple peels with 8.33% pectin content were successfully used to induce the production of pectinase under submerged fermentation process. Thus the Pectinase enzymes obtained using natural raw materials with biologically natural methods can be further characterized for its purity and activity at various physiological conditions for the benefit of food industries.

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