UTILISATION OF AGRO WASTE FOR THE PRODUCTION OF PECTINASE BY MICROORGANISM

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LIST OF ABBREVIATIONS

g	-	Gram
µl	-	microliter
%	-	percentage
mM	-	Millimolar
OD	-	Optical Density
pH	-	Potential of hydrogen
ml	-	Millilitre
DNSA	-	di -nitrosalicylic acid
Hr	-.	Hour
IP	-	Incubation period
NaoH	-	sodium hydroxide
M	-	molarity
PST	-	sodium potassium tartarate
$NaHPo_4$	-	sodium hydrogen phosphate
DW	-	Distilled Water
KH2PO4	-	potassium dihydrogen phosphate
BWB	-	Boiling water bath
PG	-	Polygalacturonase
PE	-	Pectinesterase
PL	-	Pectatelyase
Ca	-	carbon
SSF	-	Solid state fermentation
N_2	-	Nitrogen
℃	-	degree celsius
Rpm	-	Revolutions per minute
w/v	-	Weight per volume

ABSTRACT

Pectinases are a big group of enzymes that break down pectic polysaccharides of plant tissues into simpler molecules like galacturonic pectin degrading enzymes were produced in solid state fermentation by Bacillus subtilis isolated from fruit and vegetable .Under optimized conditions, maximum Production of pectinase($3.190 \mu/ml$)was recorded in the presence of orange peel with a moisture content Of 70% at 35 °C and pH 6.0 after 48 hrs of incubation, respectively. Pectinase yield was enhanced upon supplementation with ammonium sulphate and yeast extract.Thus, B. subtilis exploited for cost- effective production of pectinase using agro-residues.

Keywords pectinase. Solid state fermentation. Optimization

Chapter 1

INTRODUCTION

Pectin is a heterogeneous structural polysaccharide present in primary cell wall and middle lamella of fruit and vegetable.it is a polymeric material having carbohydrategroup esterified with methanol . It is an important component of palnt cell wall. The highest concentration of pectin is present in middle lamella,where it act as a cementing substance between adjacent cells.pectincomprises of D – galacturonicacid occurred in -1,4 chain , naturally esterified with methoxygroup and natural sugars occupy the side chains.

Pectinase comprise a heterogeneous group of enzymes that catalyze the Breakdown of pectin containing substrates. Pectinase contribute to more than 25% of Global enzyme sales(Jayani et al.2005). pectinases are a group of enzymes involved in the deploymerisationof pectic polymers. Based on their mode of action, pectinases include polygalacturonase(PG) , pectin esterase(PE), pectin lyase (PL), and pectate lyase (Ahlawat et al .2009) . Pectinase is involved in the hydrolysis of pectin containing a certaindegree of esterified groups, while PG acts on unesterified polygalacturonic acid. Among all pectinases ,pectin lyasesare in the only enzymes capable of depolymerizing highly esterified pectin into small molecules without piror action of other enzymes.

Microorganism enzymes are involved In the production of pectinase by using pectin as a carbon source.pectinsare degraded by several microorganisms that produce a variety of compounds and enzymes which are involved in several industrial applications.Many important bacteria,fungi and test are skillful at degrading pectin substances to produce pectinases.

Pectinases are used in fruit juice extraction and clarification, Wastewater treatment, vegetable oil extraction,tea and coffee fermentations, alcoholic beverages and in food industries (Jayaniet al .2005). In order to meet this high demand,it is important to produce pectin – depolymerizing enzymes at a large scale in a cost effective manner. Solid state fermentation (SSF) has gained more popularity in recent years in the production of many enzymes due to its lower operation costs and energy requirements, higher enzyme production and lower effluent generation as compared to submerged fermentation (SmF)(Pandey et al

.1999;Couto and Sanroman 2006). The selection of substrate is important inSSF and depends on several factors, mainly the cost of availability.

Being both economical and eco-friendly, agro industrial residues are the prime choice of substrate for enzyme production. Orange peel was reported to contain 16.9% soluble sugar, 9.21% cellulose,10.5% hemicellulose and 42.5% pectin as the most important component (Rivas et

al .2008). Orange peel a rich source of pectin containing about 20- 30% pectic substances (May 1990), is a good inducer of pectinase.

Orange trash, which makes up around 10%w/w of the weight of the orange fruit, is a by-product of the cutting of oranges into slices. Its current disposal raises significant economic and environmental concerns. Additionally, B.subtilis uses the orange waste as a low-cost production medium for pectinase.

The environmental concern about the disposal of solid waste has contributed to the increased interest in producing pectinaseutilising trash. Biotechnology's main goal is to better manage and utilise the enormous amounts of domestic, industrial, and agricultural waste. Organic materials are frequently crucial for both economic and environmental reasons.Numerous by- products of the agro-industry have poor economic value and are frequently dumped into waterways, where they contribute significantly to environmental damage. The use of biotechnology in these by removing a source of pollution, wastes can turn some of them into beneficial byproducts.

The importance of agro-industrial leftovers in bioprocesses helps to address pollution issues while also supplying new substrates. Many new opportunities for their use have emerged with the introduction of

biotechnological advancements, particularly in the field of enzyme and fermentation technology. The following are some biotechnology usage techniques for acceptable organic waste materials:

- Improve the food waste's quality so that it can be consumed by people.
- Recondition the food waste either before or after processing to be fed to fish, fowl, pigs, or other single-toothed animals that can use it right away.
- Give cattle or other ruminants food waste as a supplement.
- Production of compost, biogas (methane), and other products of fermentation
- Fuel, construction materials, and chemical extraction are examples of direct uses
- For the production of biological fuel or bioenergy.

The use of agricultural and industrial products and their wastes, including wheat bran,rice bran, ground nut peel, lemon peel , pineapple peel, papaya peel, banana peel and orange processing waste, for the production of pectinase using B . subtilis under SSF has attracted considerable interest in recent years. With the help of some bacteria, nearly all fruit waste produces acertain quantity of pectinase.

AIM AND OBJECTIVE

AIM

To optimize the production of pectinase from cheap agro waste

OBJECTIVES

- Processing of agro waste
- Inoculum preparation for the fermentation
- Optimize the temperature,pH for the production of pectinase
- Perform enzyme assay
- Reduce the amount of agro waste

Chapter 2

REVIEW OF LITERATURE

Pectinases is responsible for catalysing the breakdown of pectic polymers found in plant cell walls. They are a member of the polysaccharidases family, which aids in the breakdown of Pectins come from many different plants and are also referred to asenzymes that break down or break up pectin (Prathyusha&Suneetha.(2011)In the current biotechnological era, pectinase are One of the forthcoming enzymes showing progressive Increase in their market. They maintained the average Annual growth rate of 2.86% from 27.6 million $ in 2013 To 30.0 million $ in 2016 and it is estimated that by 2021, The market size of the pectinase will reach 35.5 million $ (Global Pectinase Market Research Report, 2017).

Pectinases are a heterogeneous group of related enzymes That hydrolyze the pectin substances, present mostly in Plants .Pectic enzymes are widely distributed in nature and Are produced by bacteria, yeast, fungi and plants (Babu and Bayer 2014). In plants, pectic enzymes are very important Since they play a role in elongation and cellular growth as Well as in fruit ripening (Jansirani et al. 2014).

Pectolytic Activity of microorganisms plays a significant role, firstly, In the pathogenesis of plants since these enzymes are the First to attack the tissue (Ovodov 2009). In addition, they Are also involved in the process of

symbiosis and the decay Of vegetable residues (Hoondal et al. 2002). Thus by Breaking down pectin polymer for nutritional purposes, Microbial pectolytic enzymes play an important role in Nature (Yadav et al. 2009). These enzymes are inducible, produced only when needed and they contribute to the Natural carbon cycle (Hoondal et al. 2002).

Classification of Pectinases

According to the cleavage site, pectinases are divided into Three groups: (1) hydrolases consisting of Polygalacturonase, PG (EC 3.2.1.15); (2) lyase/transeliminases comprising pectinlyase, PNL (EC 4.2.2.10), and Pectate lyase, PL (EC 4.2.2.2); (3) pectin esterase, PE (EC 3.1.1.11) (Yadav et al. 2009; Osborne 2004)

1. Pectinesterase (PE)

Pectin methy esterase or pectinesterase (EC 3.1.1.11) Catalyzes deesterification of the methoxyl group of pectin Forming pectic acid and methanol. The enzyme acts preferentially on a methyl ester group of galacturonate unit Next to a non-esterified galacturonate unit. It acts before Polygalacturonase and pectate lyases which need non esterified substrates (Kashyap et al. 2001).

2. Polygalacturonase

Polygalacturonases (PGases) are the pectinolytic enzymes that catalyse the hydrolytic cleavage of the polygalacturonicAcid chain with the introduction of water across the oxygen Bridge (Kashyap et al. 2001)

3. Pectatelyase

Pectatelyase (PGL) cleaves glycosidic linkages preferentially on polygalacturonic acid forming unsaturated Product through transelimination reaction. PGL has an absolute requirement of Ca2+ ions. Hence it is strongly Inhibited by chelating agents as EDTA(Jayani, Saxena, and Gupta 2005a).

4. Pectin lyase

Pectin lyase catalyzes the random cleavage of pectin, preferentially high esterified pectin, producing unsaturated methyloligogalacturonates through transelimination of glyosidic linkages. PLs do not have an absoluteRequirement of Ca2+ but they are stimulated by this and Other cations (Jayani, Saxena, and Gupta 2005b).

PECTIN

Pectin, a polysaccharide, is composed primarily of Essentially linear polymers of D- galactopyranosyluronic acid units joined in α-D (1->4) glycosidic Linkages; the polymer chains are esterified to Various degrees with methanol. This regular Structure is interrupted, however, with L-rhamnopyranosyl units and with side chains Containing other neutral

sugars. The polymer chains May also be partially acetylated. The most Important physical property of pectin is its ability To form spreadable gels. Gel formation results when The polymer chains interact over a portion of their Length to form a three-dimensional network. This aggregation of chains occurs through hydrogen Bonding, divalent cation crossbridging, and/or Hydrophobic interactions. Pectin is not a homopolysaccharide however and has rhamnopyranosyl residues inserted in the galactosyluronic backbone at 1 to 4% substitution. The other major feature of these rhamnogalacturonan-I (RG-I) chains are large substituted side chains. Between 20 and 80% of the rhamnopyranosyl residues are, depending on plant source and method of isolation, substituted at C-4 with neutral and acidic oligosaccharide side chains. The predominant side chains contain large linear and branched α-L-arabinofuranosyl and /or β-D galactopyranosyl residues and their relative proportion and chain lengths may differ depending on plant source. Other rarer side chains are also present and generally shorter. The final and much more minor component of the backbone is rhamnogalacturonan-II (RG-II). This is not structurally related to RG-I since its backbone is composed of 1,4-linked α-D-galactosyluronic residues like HG. At approximately 30 glycosyl residues long it has a non-saccharide and an octasaccharide side chain attached to C-2 of some of the backbone residues and two structurally

different disaccharides attached to C-3 of the backbone. RG-II is of interest as it occurs in relatively high amounts in wine and other fruit juices and it has been demonstrated that it binds heavy metals and has immunomodulating activities.

It is possible to separate essentially pure galacturonan fractions from other high molecular weight pectin fractions by degrading purified pectins specifically in the galacturonan backbone either chemically or enzymatically . It appears that there is an intramolecular distribution in which the neutral sugars are concentrated in blocks of more highly substituted rhamnogalacturonan regions ('hairy' regions) which are separated in the polymer by D-galactosyluronic-rich regions ('smooth' regions). These smooth regions can be up to 100 units in length.

Physical Properties

Pectinsare soluble In pure water, but they are insoluble In aqueous solutions in which they would gel at the same temperature If dissolved at a higher temperature. Monovalent cation(alkali Metal) salts of pectinand pecticacids are usuallysolublein Water; di – and trivalent cation salts are weakly soluble or Insoluble .

Although pectin s are not employed as thickening agentsPectin solutionsexhibit the non-Newtonian, pseudoplastic behavior Characteristic of most polysaccharides . As with solubility , the Viscosity of a pectinsolution is relate d to the molecular weight, DE, and concentration of the preparation and the pH and presence of counter ionsin the solution .

These physical properties of pectinsare a function of theirStructure which Isthat of a linear poly anion (polycarboxylate) . As such, monovalent cation salt s of pectin s are highly ionizedin Solution , and the distribution of ionic charges along the molecule Tends t o keep it in an extended form by reason of coulombic Repulsion.

Chemical properties

Dissolved pectins undergo deesterification and polymerizationin Aqueous systems. The pH of greatest stability is about 4. At pH Values both above and below 4, deesterificationand Depolymerization occur concurrently, with the rate of Deesterification being greater than the rate of depolymerization.

The presence of solutes, which lowers water activity, reduces the Rates of both reactions.

There are several types of enzymes that act on pectin Molecules. Those enzymes produced by the higher plants themselves play a significant role in

the processes resulting textural Changes in fruits and vegetables during ripening, storage, and Processing. Fungal enzyme preparations are used by the Fruit juice e industry to improve the clarity of juices and the Yield from processing.

Production of Microbial Pectinase

Microorganisms are currently the primary source of industrial enzymes: 50% originate from fungi and yeast; 35% from bacteria, while the remaining 15% are either of plant origin. The microbial world has shown to be very heterogeneous in its ability to synthesize different types of pectolytic enzymes with different mechanisms of action and biochemical properties (Gummadi and Panda 2003). There were two fermentation techniques we can use for pectinases production, as many other enzymes. These techniques are Solid State Fermentation (SSF) SMF.

Solid state fermentation is defined as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can be used as carbon and energy source. This process occurs in the absence or near absence of free water in the space between substrate particles. In this system, water is present in the solid substrate whose capacity for liquid retention varies with the type of material (Pandey, Soccol, Nigam, Soccol, et al. 2000). In

contrast, in submerged fermentation (SmF) the nutrients and microorganisms are both submerged in water.

Approximately 90% of all industrial enzymes are produced in SmF, frequently using specifically optimized, genetically manipulated microorganisms. In this respect SmF processing offers an insurmountable advantage over SSF. SSF has several advantages over SmF system such as higher concentration of products, less effluent generation, requirement for simple equipments etc (Pandey et al. 1999). The price of commercially available enzymes which are produced mostly by submerged fermentation is usually too high for agro-biotechnological applications. An alternative technique of enzyme production is solid state cultures (Kawano et al. 1999).Microbial production of pectinases has been studied during recent years (Kashyap et al. 2001). Pectinase production has been reported from bacteria including actinomycetes (Beg et al. 2000), yeast (Reid and Ricard 2000) and fungi. However, almost all the commercial preparations of pectinases are produced from fungal sources (Singh, Ramakrishna, and Appu Rao 1999). *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Gummadi and Panda 2003; Murad and Azzaz 2011; Jayani, Saxena, and Gupta 2005b).

Most extracellularly induced enzymes are known to be synthesized in higher quantities when inducers are present in the cultivation medium (Alkorta et al. 1998). The production of pectolytic enzymes using different sources and the effect of physical parameters such as temperature, aeration rate and type of fermentation were investigated and reported in literature(Naidu and Panda 1998). Pectolytic enzymes have been reported to be induced by several substances. In many cases pectin itself has been used. Many investigators had used complex media such as beet sugar, wheat bran, ground nut meal, citrus fruit peels etc(Hoondal et al. 2002).

Higher cost of the production is perhaps the major constraint in commercialization of new sources of enzymes. Though, using high yielding strains, optimal fermentation conditions and cheap raw materials as a carbon source can reduce the cost of enzyme production for subsequent applications in industrial processes (Murad and Azzaz 2011).

There are many studies that have been conducted related to the characterization of different microbial pectic enzymes concerning their mechanisms of action and biochemical properties. The optimal pHs that these enzymes may act range between 3.5-11, while the optimal temperatures vary between 40-75 °C (Gummadi and Panda 2003; Kashyap et al. 2001).

Microbial sources

Different microorganisms are involved in the production of pectinase by using pectin as a carbon source. Pectins are degraded by several microorganisms that produce a variety of compounds and enzymes which are involved in several industrial applications. Many important bacteria, fungi and yeasts are skillful at degrading pectins substances to produce pectinases.

- Pectinolytic Fungi

Several fungal species can degrade pectic substances by producing pectinolytic enzymes. The most popular and more efficient fungi in the pectinase production are Aspergillus Niger, Aspergillus awamori, Penicillium restrictum, Trichoderma viride, Mucor piriformis and Yarrowia lipolytica have a great role in both submerged as well as solid-state fermentation for the production of various industrially important products. Aspergillus Niger, Aspergillus oryzae ,and Penicillium expansum are the types of fungi that are generally considered safe by the United States Food and Drugs Administration are put to use in the food industry [16]. Kumari et al. [49] isolate pectinase-producing strain Penicilliumjanthinellum from the soil and has been found to produce significant amounts of an extracellular pectinase subsequently characterized as exo- polygalacturonase. The different fungal strains from vegetable wastes and screened them for their

pectinolytic activity. Among them, Tetracoccosporium species was found to be good producers of pectinase and it showed a clearance zone of 20 mm pectinolytic activity around the colonies . Khairnar et al. studied the pectinase production of different strains of Aspergillus Niger.They observed the highest zone of clearance of pectin hydrolysis in Aspergillus Niger is 4.5 mm. Ten fungal isolates were isolated from municipal solid waste. Among them, a maximum zone of clearance of above 3.0 mm for pectinolytic activity was exhibited by Penicilliumchrysogenum and Aspergillus Niger .

- Pectinolytic Yeasts

Kavuthodi and Sebastian [78] reported that Saccharomyces fragilis, Saccharomyces thermantitonum, Torulopsis kefyr, Candida pseudotropicalis var, lactosa, and Candida pseudotropicalis are types of yeast that can degrade pectin substances in the pectinase production processes. The other report also indicates additional yeast species for pectinase production, these species include Saccharomyces species, Cryptococcus species, Aureobasidium pullulans, Rhodotoruladairenens is, Kluyveromyces marxianus, Geotrichum klebahnii, and Wickerhanomyces anomalus, [13, 79].

Wickerhamomyces anomalous one of the classification of species Pichia anomala produced pectinolytic enzymes in liquid medium containing glucose

and citrus pectin as carbon and energy sources. In the current studies, enzymes made by this wild yeast strain were characterized, and physicochemical properties of polygalacturonase were determined by the study of the influence of temperature and pH on its activity and stability to evaluate the application of the supernatant in the maceration of potato tissues [80].The different investigations identified different yeast species and characterized in molecular method to the production of pectinolytic enzymes from grapes peel. Based on that identification, several species have a good potential to degrade pectin substance these species include: Hanseniaspora species, Saccharomyces cerevisiae, Rhodotorula dairenensis, Candida zemplinina, Metschnikowia species, Aureobasidium pullulans, and Cryptococcus saitoi [81].

- Pectinolytic Bacteria

Erwinia species, Pseudomonas fluorescens, Bacillus, Pseudomonas, and Micrococcus have a good potential to degrade pectin in the production of pectinase [20, 56, 78]. Other such as Streptomyces bacteria also has pectinolytic properties as reported by Ramirez-Tapias et al. [82]. Bacillus licheniformis has been reported as pectinolytic bacteria that were isolated from the rotten vegetable. The efficiency of Bacillus licheniformis to pectinase production was determined by the primary and secondary

screening methods. The primary screening was carried out by the potassium–iodide flooding method and the secondary screening was carried out by fermentation. The efficiency of Bacillus licheniformis on the pectinase activity was recorded as 341 U/ml [83]. A newly isolated Brevibacillus borstelensis reported good pectinase (pectin lyase) production and characterization. The enzyme activities of Brevibacillus borstelensis were reported as 5.25 U/ml [84]. Soil is collected from different villages of Guntur District (Duggirala and Burripalem) from a depth of 1–15 inches to isolate desired pectinase producing Bacteria.

Bacteria	Bacillus sterothermophilus Bacillus cereus Bacillus mojavensis Erwinia app Bacillus subtilis Bacillustropicus Erwiniacarotovora Erwiniachrysanthemi
Fungi	Aspergillusoryzae Aspergillusflavus Aspergillussojae AspergillusNiger Penicilliumchrysogenum Trichodermaharzianum Schizophyllum commune
Yeast	Wickerhanomycesanomalus Saccharomyces cerevisiae Kluyveromycesmarxianus Filobasidiumcap suligenum

Substrates used for pectinase enzyme production.

The petinase enzyme production from various substrates have been identified and listed in the following table.

Substrate	Organism
Wheat bran	Bacillus subtilis
Date syrup	Bacillus subtilis
Agriculture waste	Bacillus pumilus
Cassava waste	Bacillus sp
Sugar beet pulp	Aspergillus Niger
Mango peel	Aspergillusfoetidus
Lemon peel	Aspergillusoryzae
Banana peel	Aspergillusterrus
Wheat	Aspergillusawamori
Soy & wheat bran	Aspergillus Niger
Pectin	Bacillus subtilis
Citrus pectin	Bacillus sphaericus
Sucrose	Aspergillus Niger

Orange pomace	Aspergillus Niger

Pectinase production by Bacillus species

Pectic enzymes are of functional relevance in the retting process and evidence regarding pectinolytic properties of Bacillus spp. Was recorded years ago. Different species of the genus Bacillus have been reported to be retting agents and Active against pectic materials (Potter & McCoy, 1955). Nortje and Vaughn, 1953 tested the pectinolytic activity Of B. subtilis and B. pumilus in relation to the softening Of olives and pickles (Nortje & Vaughn, 1953). The first in Vitro fermentation studies of pectin and pectic acid was Reported in 1955 using B. polymyxa strain 30 (Potter & McCoy, 1955). Over the past few years,pectinolytic properties have been described in several Bacillus species .

It is evident from many research works that, among different bacterial isolates screened for pectinolytic properties Bacillus strains were selected as the most potent enzyme producers (Soares et al., 1999; Jayani et al., 2010; Rehman et al., 2012; Kavuthodi et al., 2015; Sohail& Latif, 2016). As mentioned in the introduction, alkaline pectinases have a wide variety of industrial applications and, bacteria mainly Bacillus spp are the chief producers. Apart from this fact, there are also some other

reasons for researchers to focus on pectinase from Bacillus spp.These include; (i) they produce all class of pectic enzymes, (ii) have short fermentation period for enzyme production, (iii) can produce enzymes very economically by using different agro-wastes as cheap substrates, (iv) fermentation can be attained byeither SSF, Smf and (v) genetic information regarding pectinase genes of many Bacillus spp. Are available in various nucleotide sequence databases. Thus it supports successful cloning and expression of pectinase gene in other organisms.

Applications of Microbial Pectinases

Application of enzymes in biotechnological process has expanded considerably in recent years. In food and related Industry, major importance was being attached to the use of Enzymes in upgrading quality, increasing yields ofExtractive processes, product stabilization, and Improvement of flavor and by product utilization (Patil and Dayanand 2006). Pectinases or pectinolytic enzymes are Today one of the upcoming enzymes of the commercial sector.

It has been reported that microbial pectinases Account for 25% of the global food enzymes sales ((Jayani, Saxena, and Gupta 2005b). On the bases of theirApplications, pectinases are mainly of two types: acidic Pectinases and alkaline pectinases(Jayani, et al., 2005; Murad and Azzaz 2011).

Acidic Pectinases

Acidic pectic enzymes used in the fruit juice industries and Wine making often come from fungal sources, especially From Aspergillusniger(Kashyap et al. 2001). Potential applications of acidic pectinase are briefly described Below.

Fruit juice clarification/extraction

Fruit juice clarification/extraction Is one among the Important applications of acidic pectinases Fruit juices contain colloids that may lead to fouling problem during filtration process and these colloids are basically polysaccharides such as pectin and starch (Rai et al. 2004). Pre-treatment of juices with pectinases is performed to lower the amount of pectin present and to decrease the viscosity of the juice, which in turn accelerates the subsequent filtration process. Also, it helps to increase the clarity of the juice.

Wine processing

Wine processing industry also recognizes the importance of Acidic pectinases (Roldán et al. 2010), where the enzyme Can be applied at different stages. The addition of pectinases during crushing of the fruits increases the juice yield and Also accelerates the release of anthocyanins into the juice. Pectinase treatment at the pre-fermentation or fermentation Stage,

settles out suspended particles. After fermentation, Enzyme is added to the wine to increase its clarity and Filtration rate (Kashyap et al. 2001).

Tissue maceration

Tissue maceration is another important application of acidic pectinases in which organized tissue is transformed into a suspension of intact cells and it is significant in the food industry as well as in the field of biotechnology. The process can be applied for the liquefaction and saccharification of biomass, isolation of protoplasts.

Alkaline pectinases

Alkaline pectinases are mainly used in the degumming and Retting of fibeber crops and pretreatment of pectic Wastewater from fruit juice industries. These enzymes come Mostly from bacterial sources (Kashyap et al.2001).In the Industrial sector, alkaline pectinases, mainly from Bacillus Spp. Are applied for the following purposes.

Paper and pulp industry

During papermaking, pectinase can depolymerisepectins And subsequently lower the cationic demand of pectin Solutions and the filtrate from peroxide bleaching (Reid and Ricard 2000).

Animal feed

Pectinases are used in the enzyme cocktail, used for the Production of animal feeds. This reduces the feed viscosity, Which increases absorption of nutrients, liberates nutrients, Either by hydrolysis of non-biodegradable fibers or by Liberating nutrients blocked by these fibers, and reduces the Amount of faeces(Jayani, Saxena, and Gupta 2005b).

Coffee and Tea Fermentation

Fermentation of coffee using pectinolytic microorganisms is Done to remove the mucilage coat from the coffeebeans and To enhance the tea fermentation and foam forming property Of tea. Fungal pectinases are also used in the manufacture of Tea. Enzyme treatment accelerates tea fermentation, Although the enzyme dose must be adjusted carefully to Avoid damage to the tea leaf. Large-scale treatment of Coffee with commercial pectinases is costly and Uneconomical, inoculated waste mucilage is used as a Source of microbial pectin enzymes. The fermentation Liquid is washed, filtered and then sprayed on to the beans (Pandey, Soccol, Nigam, Brand, et al. 2000).

Textile processing and bio-scouring of cotton fibers

Pectinases have been used in conjunction with amylases, lipases, cellulases and hemi-cellulases to remove sizing agents from cotton in a safe

and eco-friendly manner, replacing toxic caustic soda used for the purpose earlier. Bio-scouring is a novel process for removal of noncellulosic impurities from the fiber with specific enzymes. Pectinases have been used for this purpose without any negative side effect on cellulose (Hoondal et al. 2002).

Pectic waste water treatment

The wastewater from the citrus-processing industry contains pectinaceous materials that are barely decomposed by microbes during the activated-sludge treatment have tried to develop a new wastewater treatment process by using an alkalophillic microorganism. Pretreatment of these wastewaters with pectinolytic enzymes facilitates removal of pectinaceous material and renders it suitable for decomposition by activated sludge treatment (Beg et al.2000).

Oil Extraction

Citrus oils such as lemon oil can be extracted with pectinases. They destroy the emulsifying properties of pectin, which interferes with the collection of oils from citrus peel extracts(Mohnen 2008).

Purification of plant viruses

Pectinases have also been reported to work on purification of viruses. But they are yet to be commercialized. When virus particle is restricted to phloem, to

release the virus from the tissues, alkaline pectinases and cellulases are used. This gives very pure preparations of the virus (Reid and Ricard 2000).

Retting and degumming of plant bast fiber

Bast fibers are the soft fibers formed in groups outside the xylem, phloem or pericycle, e.g. Ramie and sun hemp. The fibers contain gum, which must be removed before its use for textile making. The chemical degumming treatment is polluting, toxic and non-biodegradable. Biotechnological degumming using pectinases in combination with xylanasepresents an eco-friendly and economic alternative to the above problem (Kapoor et al. 2001). Pectinases have been used in retting of flax to separate the fibers and eliminate pectins (Hoondal et al. 2002).

Chapter 3

MATERIALS AND METHODS

Materials

Instruments

- Laminar air flow
- Spectrophotometer
- Centrifuge
- Incubator
- Rotary shaker

Chemicals

- Nutrient broth
- Peptone
- Magnesium sulphate
- Sodium nitrate
- Pottasium dihydrogen
- Sodium hydrogen phosphate
- Glycine
- Sodium hydroxide
- Dinitrosalicyclic acid
- Crystal phenol

- Sodiumsulphite
- PST
- Yeast extact
- Ammonium chloride
- Ammonium sulphate

APPARATUS

- Test tubes
- Conical flask
- Micropipette
- Funnel
- Filter paper
- Glass rod
- Inoculation loop
- Test tube rack
- Measuring cylinder
- Glass beakers

Methods

PROCESSING OF AGRO- RESIDUE AS SUBSTRATE

Collection of agro waste

The waste from orange is chosen as the source of solid substrate for the production of pectinase. Orange fruits were collected from local fruit and vegetable market in kayamkulam . In a single batch, enough solid substrate were obtained for all the eexperiments.

Pretreatment of substrate

Orange peel was removed and washed several times with tap water to remove water soluble compounds.Then it is dried in aluminium trays under sunlight for1- 2 days.Thesubstrate was dried untill they were completely dehydrated. The dried orange peel were grounded to 2-3 mm particle size in a laboratory grinder and used for the production of pectinase.The powdered samples are gathered in sized vials and stored until they are needed.

MICROORGANISM

Bacillus subtilis was obtained from stock colure in the laboratory.

Inoculum preparation

The inoculum was prepared by transferring a loop full of B.subtilis culture into 50 ml autoclaved nutrient broth taken in 250 ml conical flask.which was then incubated at370c under agitation at 100 rpm on a rotary shaker for 18 hrs.

Preparation of nutrient broth

- Weigh 0.65 g nutrient broth powder

- Take 49 ml distilled water in a measuring cylinder
- Mix the accurately weighed nutrient broth powder in DW
- Nutrient broth is prepared

Solid state fermentation for the production of pectinase

SSF was performed by Erlenmeyer flasks (250ml) .Each flask carrying 5 g of orange peel as substrate that is moistened with 70% distilled water before sterilization.The basal production medium in flasks was autoclaved,cooled and inoculated with 1.0ml of 18hr- old inoculum of B.subtilis.Flasks were then incubated at 370c for 48 hrs in a bacterial incubator.

Basal medium Preparation (100 ml)

Component	Quantity g/ml
Peptone	0.1
KH2po4	0.104
NaNo3	0.4
MgSo4	0.01
NaH2PO4	0.2
DW	93

Enzyme extraction

Pectinase were extracted from the fermented substrate by constant shaking in an orbital shaker at 100 rpm for 20 min at 300c.The extract was squeezed through filter paper and centrifuged at 10000rpm for 5 min. The clear supernatant was collected as the crude enzyme.

Assay of pectinase

Pectinase activity was assayed by measuring the amount of D-galacturonic acid liberated from pectin. The sample mixture contains 50 µL of diluted enzyme and 450 µL 0.5% pectin was incubated for 20 min at 300c and the end product was quantified by 3ml DNSA reagent and 1ml of PST . Then the sample place in boiling water bath for 5 min. After that cool the sample and measure the OD value at 510 nm.

REAGENT PREPARATION FOR ASSAY

1. 0.5 % pectin

Component	Quantity g/ml
Pectin	0.005
NaoH	0.4
Glycine (0.1M)	0.7507
DW	100 ml

DNSA REAGENT (20 ml)

Component	Quantity g/ml
DNS	0.2
Crystal phenol	0.04
Sodium sulphite	0.01
NaoH	0.2
DW	20

- Dissolve by stirring 0.2 g DNS in 0.04 g crystalline phenol.

- Weigh 0.2 g NaoH and mix it with DW.

- Then weigh 0.01 g of sodium sulphite and add to DW

- Mix the DNS to the DW mixture

40% Rochelle salt solution

PST – 2g

DW - 5ml

OPTIMIZATION

Effect of temperature

The effect of cultivation of temperature were studied by incubating the three flask containing 5.0 g of orange peel at different temperature 25˚c,35˚c,40°c.The common procedure follows by enzyme extraction and pectinase assay.

Effect of pH

The common procedure follows by enzyme extraction and pectinase assay for pH 5,6,&9

Effect of incubation period

- Take 3 autoclaved conical flask

- Three conical flask contains 5 g of orange peel and moistened with 70% distilled water
- Add autoclaved basal medium to each flask
- Add inoculum to each flask within the LAF
- Kept the properly sealed flasks with time period of 12hrs ,24hrs, 48hrs,&72 hrs
- The common procedure follows by enzyme extraction and pectinase assay

Effect of nitrogen source

The effect of nitrogen source for the production of pectinase by supplymenting different organic and inorganic source of nitrogen in solid media. This is carried out by four flask contains moistened orange peel ,basal medium and inoculum . Allow flask mixture to ferment. After fermentation, enzyme is extracted .The supernatant was collected as source of crude enzyme.Then it follows the pectinase assay.

Chapter 4

RESULTS AND DISCUSSION

1.POWERED SUBSTRATE

Dried orange peel

Powdered orange peel

2. BACILLUS SUBTILIS CULTURED IN NUTRIENT BROTH

3.BACILLUS SUBTILIS CULTURED IN LOW COST SUBSTRATE

CRUDE ENZYME FROM PECTINASE ASSAY

Assay of pectinase activity

Pectinase activity was assayed by measuring the amount of D – galacturonic acid liberated from pectin. For enzyme assay, 1.0 ml of Bacillus culture was inoculated into flask containing basal medium. flask were incubated for 48 hrs.The fermented extract was filtered and centrifuged at 10000 rpm for 15 min .The supernatant is enzyme source.The enzyme unit was defined as the amount of enzyme that catalyzes μ/ mol of galacturonic acid per minute (μ/mol min−1) under the assay conditions.

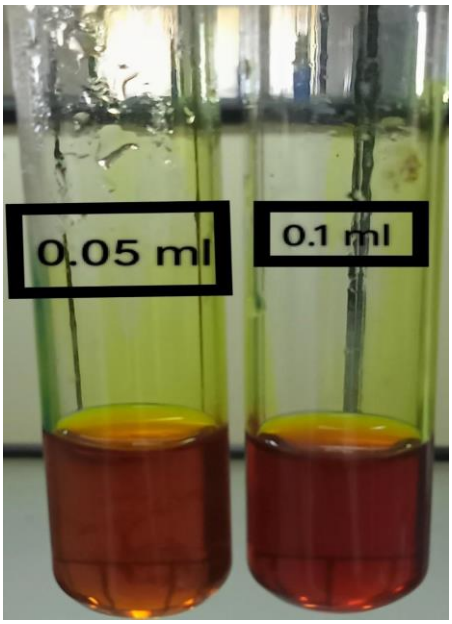
The enzyme activity was calculated thus the orange peel with 0.05 ml enzyme volume liberate 1.09 pectinase than orange peel with 0.1 ml enzyme volume liberate 3 .19 Therefore the Orange peel with 0.1ml of enzyme volume taken as standard for further experiments.

Enzyme activity expressed in

$$= \frac{\text{Con of galacturonic acid x 1000x dilution factor}}{\text{Molecular weight of galacturonic acid x incubation time}}$$

Substrate	Volume of enzyme ml	Concentration	OD at 510nm	Pectinase activity
Orange peel	0.05 ml	0.425	0.314	1.09
Orange peel	0.1 ml	0.620	0.352	3.19

Assay of pectinase



Pectinase activity (0.05ml)

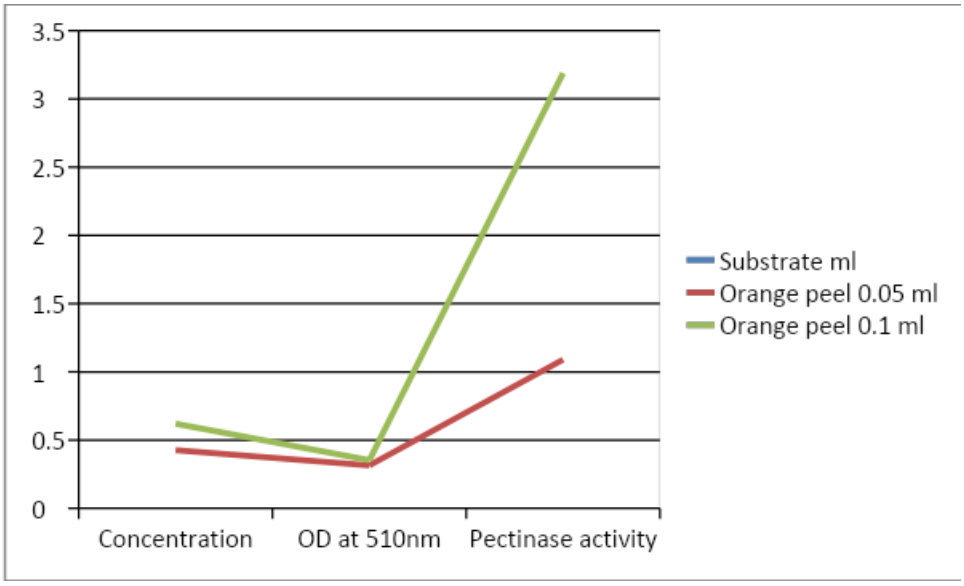
$$= \frac{0.425 \times 1000 \times 0.05}{194.139 \times 10}$$

$$= 1.09$$

Pectinase activity (0.1ml)

$$= \frac{0.620 \times 1000 \times 0.1}{194.139 \times 10}$$

$$= 3.19$$



EFFECT OF TEMPERATURE

The orange peel with appropriate moisture and were inoculated and incubated at various temperatures.The maximum pectinase production attained at 35˚c in fermentation technique. The succeeding studies were performed at 35˚ c.

SL no	Temperature	OD at 510 nm	Concentration	Pectinase activity
1	35	0.2	0.306	1.57
2	40	0.176	0.282	1.45
3	25	0.136	0.242	1.24

Temperature at 35°c

$$= \frac{0.306 \times 1000 \times 0.1}{194.139 \times 10}$$

$$= 1.57$$

Temperature at 40°c

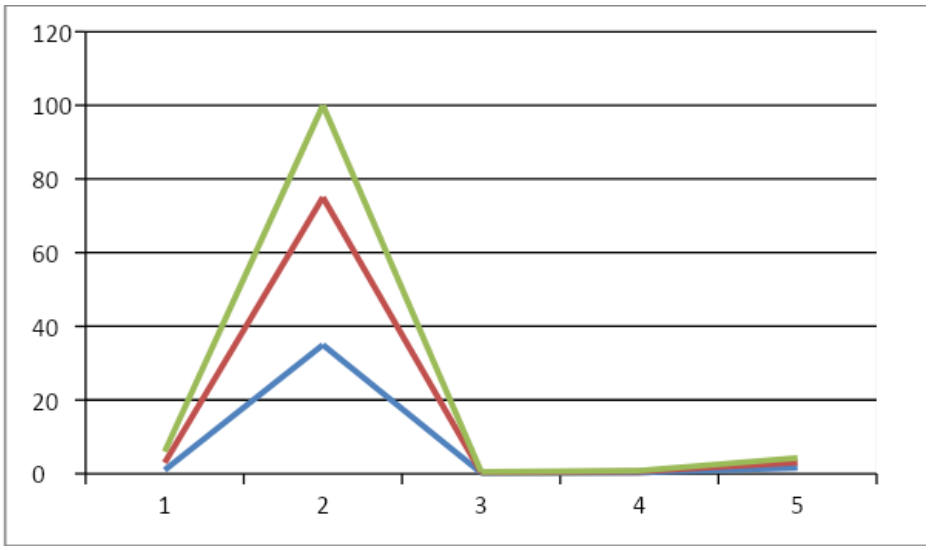
$$= \frac{0.282 \times 1000 \times 0.1}{194.139 \times 10}$$

$$= 1.45$$

Temperature at 25 °c

$$= \frac{0.242 \times 1000 \times .01}{194.139 \times 10}$$

$$= 1.24$$



EFFECT OF pH

The effect of pectinase activity at different pH like pH5 ,pH 6 , pH 9 is evaluated . The maximum amount of pectinase activity , Bacillus shows in pH 6 (1.31).In acidic pH , pectinase activity is high therefore the medium with pH 6 is selected for
Further experiments.

SL No	PH	Concentration	OD at 510 nm	Pectinase activity
1	5	0.245	0.139	1.26
2	6	0.256	0.15	1.32
3	9	0.247	0.141	1.27

pH 5 = $\frac{0.245 \times 1000 \times 0.1}{194.139 \times 10}$

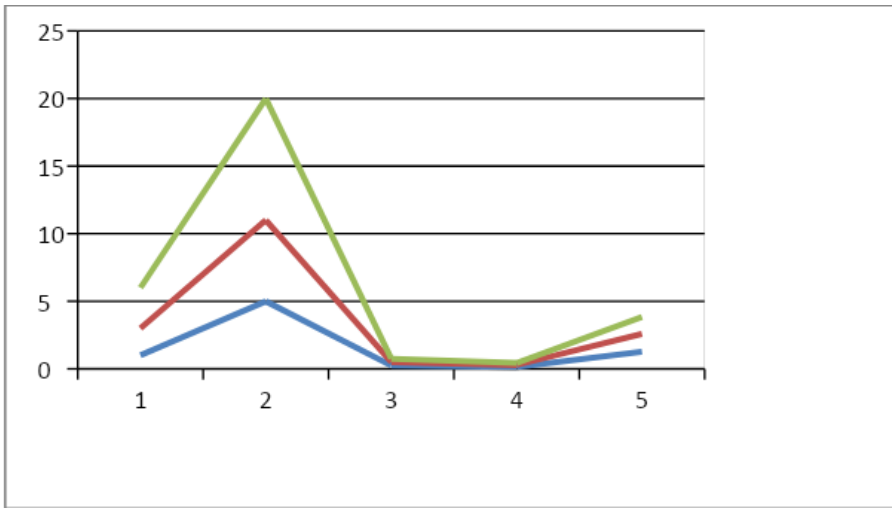
= 1.26

pH 6 = $\frac{0.256 \times 1000 \times 0.1}{194.139 \times 10}$

= 1.31

pH 9 = $\frac{0.247 \times 1000 \times 0.1}{194.139 \times 10}$

= 1.27



EFFECT OF INCUBATION PERIOD

The effect of incubation period , the orange peel as solid substrate mixed with basal medium inoculated and incubated were assayed for pectinase activity with different time period of incubation like 24,48,72,96 hrs. The highest pectinase enzyme production was achieved at 48 hrs of incubation (3.19). So 48 hrs of incubation period is taken for the further experiments.

SlNo	Incubation period	Concentration	OD at 510 nm	Pectinase activity
1	24	0.448	0.18	2.30
2	48	0.620	0.352	3.19
3	72	0.419	0.319	2.15
4	96	0.398	0.298	2.05

$$IP\ (24) = \frac{0.448 \times 1000 \times 0.1}{194.138 \times 10}$$

$$= 2.30$$

$$IP\ (48) = \frac{0.620 \times 1000 \times 0.1}{194.139 \times 10}$$

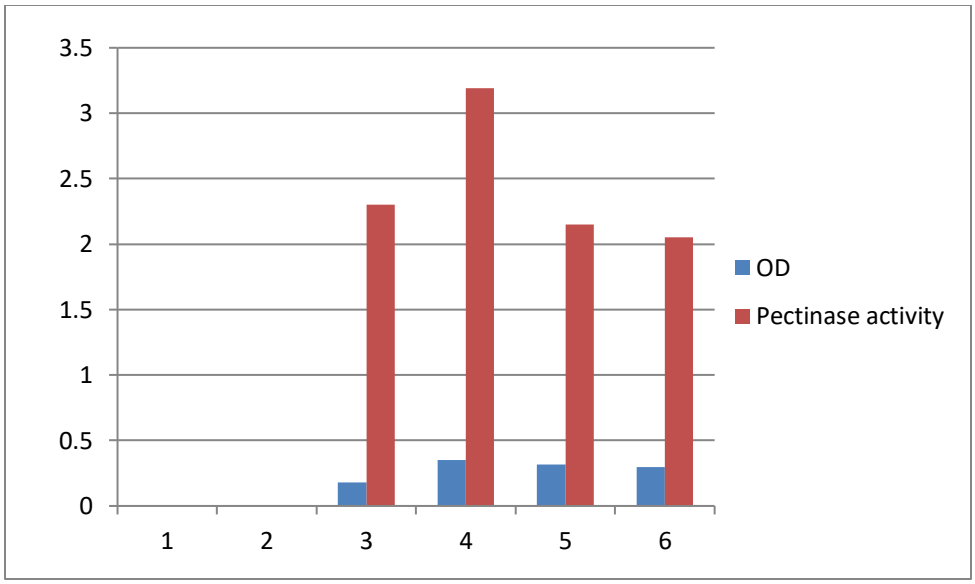
$$= 3.19$$

$$IP\ (72) = \frac{0.419 \times 1000 \times 0.1}{194.139 \times 10}$$

$$= 2.15$$

$$IP\ (96) = \frac{0.398 \times 1000 \times 0.1}{194.139 \times 10}$$

$$= 2.05$$



EFFECT OF NITROGEN SOURCE

The effect of nitrogen source on pectinase production the solid substrate supplemented with organic and inorganic sources of nitrogen 1 % (w/v).The highest pectinase production attained at supplementing yeast extract (1.85) as organic nitrogen source. The highest pectinase production attained at supplementing Ammonium sulphate (1.83) as inorganic nitrogen source.so 1% yeast extract and 1% ammonium sulphate is the best source of nitrogen for pectinase production.

SlNo	N source	Concentration	OD at 510nm	Pectinase activity
1	Yeast extract	0.361	0.255	1.85

2	Peptone	0.251	0.145	1.29
3	NH_4Cl	0.293	0.187	1.50
4	$(NH_4)_2So_4$	0.356	0.25	1.83

Yeast extract $= \frac{0.361 \times 1000 \times 0.1}{194.139 \times 10}$

$= 1.85$

Peptone $= \frac{0.251 \times 1000 \times 0.1}{194.139x\ 10}$

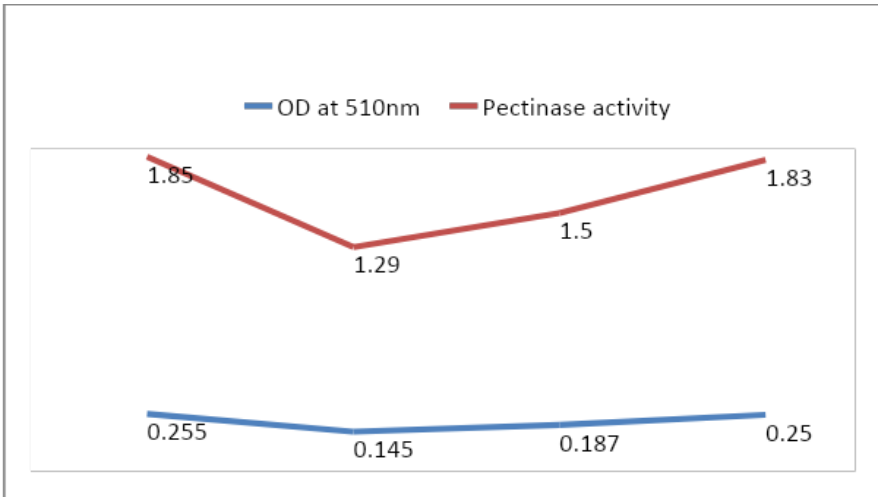
$= 1.29$

$NH_4Cl = \frac{0.293 \times 1000 \times 0.1}{194.139 \times 10}$

$= 1.50$

$(NH_4)So_4 = \frac{0.356 \times 1000 \times 0.1}{194.13 \times 10}$

$= 1.83$


OD at 510nm
Pectinase activity
1.85
1.29
1.5
1.83
0.255
0.145
0.187
0.25

Chapter 5

SUMMERY AND CONCLUSION

Emerging pectinase applications highlight the value of selecting pectinase-producing microorganisms with unique characteristics, higher enzyme activity, and widespread production of these enzymes.Environmental factors like temperature, pH, incubation period, nitrogen source and the presence of substrates have an impact on microbes' ability to synthesise extracellular enzymes.

In this study, parameters that affect the pectinase production have been standardized and diligent optimization steps were carried out to make the production of pectinase enzyme to be cost effective and commercially viable.

Since, to meet the growing industrial demands for pectinase, it is necessary to improve yield without increasing the cost of production. Thus, in this study the biotechnological capacities of agricultural wastes are considered for economical production of pectinase.

This study aimed evaluvating the production and activity of pectinase from agro waste by using Bacillus subtilis.The results from the study showing that we can produce pectinolytic enzyme using agro waste as substrate, bacillus as Microorganism, allow this set up for solid state fermentation. After fermentation the extracted enzyme under assayed condition we can obtain the amount of pectinase liberated. The parametric optimization of temperature,pH, incubation period, nitrogen source enhanced the petinaseproduction.The production of pectinase was enhanced more than a 6-fold in solid state fermentation.

The potential of agricultural wastes for the production of pectinase using solid state fermentation is highlighted in this study. This result conveys the very economized production of pectinase.This is an efficient method for cost reduction in the production of enzymes.

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APPENDICES

- **Basal medium Preparation (100 ml)**

It is prepared by dissolving basal medium in 100ml distilled water

Component	Quantity g/ml

Peptone	0.1
KH2po4	0.104
NaNo3	0.4
MgSo4	0.01
NaH2PO4	0.2
DW	93

- **0.5 % pectin preparation**

It is prepared by dissolving 0.5% pectin in 100ml distilled water

Component	Quantity g/ml
Pectin	0.005
NaoH	0.4
Glycine (0.1M)	0.7507
DW	100 ml

DNSA REAGENT PREPARATION (20 ml)

Component	Quantity g/ml
DNS	0.2

Crystal phenol	0.04
Sodium sulphite	0.01
NaoH	0.2
DW	20

- Dissolve by stirring 0.2 g DNS in 0.04 g crystalline phenol.
- Weigh 0.2 g NaoH and mix it with DW.
- Then weigh 0.01 g of sodium sulphite and add to DW
- Mix the DNS to the DW mixture

40% ROCHELLE SALT SOLUTION PREPARATION

It is prepared by dissolving 2g PST in 5 ml of distilled water

PST – 2g

DW - 5ml