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Research Article

OPTIMIZATION AND PARTIAL CHARACTERIZATION OF PECTINASE PRODUCED BY BACILLUS SUBTILIS STRAIN ARIUM 1115 ISOLATED FROM SPOILT APPLE

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ABSTRACT

A pectinase producing bacterium was isolated from spoilt apple and was identified as *Bacillus subtilis* strain arium 1115 on the basis of morphological, cultural, biochemical and 16S rRNA sequence analysis using NCBI assessment with accession number NCBI- LC224327.1. Maximum production of pectinase (28.44 U/ml) was obtained by the 8% (v/v) of 0.2 O.D at 540 nm of *Bacillus subtilis* strain arium 1115 inoculated in an optimized medium containing Pectin (0.25%), Yeast extract (1%), Xylose (1.5%), pH 9 incubated on a shaker (120 rpm) at 45°C for 72 hrs. Maximum pectinase activity was observed at 45°C and pH 7.The residual enzyme activity in the presence of metal ions such as Mg^{2+} (76.9%), Na^{+} (34.8%), Cu^{2+} (30.5%), Mn^{2+} (26.4%), Ca^{2+} (25.8%), Ba^{2+} (22.9%), Zn^{2+} (21.4%), Fe^{2+} (16.6%), Ni^{2+} (14.1%), Li^{2+} (9.3%), Cd^{2+} (8.7%) and Hg^{2+} (5.5%) was reported. The residual enzyme activity in the presence of inhibitors such as Tween 80 (20.8%), β-Mercaptoethanol (20.1%), EDTA (15.5%) and SDS (6.2%) was also studied. The potential application of pectinase as a clarifying agent was demonstrated.

INTRODUCTION

Pectic substances are complex high molecular mass glycosidic macromolecules found in higher plants. They are present in the primary cell wall and are the major components of the middle lamellae, a thin extracellular adhesive layer formed between the walls of adjacent young cells. In short, they are largely responsible for the structural integrity and cohesion of plant tissues (Rombouts and Pilnik, 1980; Alkorta *et al*., 1998). Three major pectic polyssacharides groups are recognized. Homogalacturonan (HG) is a linear polymer formed by D-galacturonic acid which can be acetylated and/or methyl esterified. It can also be called as smooth regions of pectin (Jayani *et al*., 2005). Rhamnogalacturonan I (RGI) is composed of the repeating disaccharide rhamnose galacturonic acid and Rhamnogalacturonan II (RGII) is a homogalacturonan chain with complex side chains attached to the galacturonic residues (Willats *et al*., 2001).

Pectins are hydrolyzed by pectinases. Several types of pectinases can be found, including protopectinases, polygalacturonases (PG), pectin lyases (PL) and pectin esterases (PE). Polygalacturonases (PG) are considered to be the richest pectinases, they hydrolyze the main chain using water. Pectin lyase (PL) cleaves the chain by trans-elimination while pectinesterase (PE) promotes de-esterifying of the methyl ester linkages of the pectin backbone and is responsible for the release of pectins and methanol. Pectinolytic enzymes have wide application in many sectors such as fruit juice extraction, scouring of cotton, waste water treatment and vegetable oil extraction (Jayani *et al.,* 2005). Acid pectinases are widely used in extraction, clarification and removal of pectin in fruit juices, in maceration of vegetables to produce pastes, purees and in winemaking. They are often produced by fungi, especially *Aspergillus niger*. Pectinase addition in the extraction process improves the fruit juice yield through an easier process, decreases the juice viscosity and degrades the gel structure, thus improving the juice concentration capacity (Kashyap *et al*., 2001). Alkaline pectinases are generally produced by bacteria, particularly species of Bacillus, but are also made by some filamentous fungi and yeasts (Kashyap *et al*., 2000). They may be used in the pretreatment of waste water from vegetable food processing that contains pectin residues; the processing of textile fibers such as flax, jute and hemp, coffee and tea fermentation, vegetable oil extraction and the treatment of paper pulp (Kapoor *et al*., 2001; Zhang *et al*., 2000). Pectinase producing organisms are *Bacillus sp. TS47 (*Takao *et al.,* 2000), *Bacillus macerans* (Miyazaki, 1991), *Aspergillus niger* (Benkova and Slezarik, 1966), *Saccharomyces pastorianus* (Astapovich and Ryabaya, 1997). Most of the Bacterial isolates such as Bacillus sp and Pseudomonas.sp., *Pseudomonas*

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fluorescence and *Bacillus subtilis,* Bacillus sp. MFW7, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus cereus*, Bacillus sp. MBRL576, *Bacillus firmus* I-4071, *Enterobacter aerogenes* NBO2, *Pseudoalteromonas haloplanktis* strain ANT/505, *Paenibacillus xylanolyticus, Bacillus firmus*, *Bacillus firmus* (P1), *Bacillus coagulans* (P13), *Bacillus endophyticus* (P57) and *Bacillus vietnamensis* (P58), *Bacillus subtilis* BKDS1, Enterobacter sp. PSTB-1 and *Staphylococcus aureus* were reported as good pectinase producers (Geetha *et al*., 2012; Reetha *et al*., 2014; Mukesh kumar *et al*., 2012; Bhardwaj and Garg 2003; Bayoumi *et al*., 2008; Darah *et al*., 2013; Truong *et al*., 2001; Giacobbe *et al*., 2014; Roosdiana *et al*., 2013; Aaisha and Barate, 2016; Kavuthodi *et al*., 2015; Reddy *et al*., 2016; Raju and Divakar, 2013a).

MATERIALS AND METHODS

Enrichment, isolation and screening and identification

Spoilt apple sample was collected from a local fruit market at Grant road, Mumbai. Enrichment of pectinase producing organisms was done by inoculating 1ml of the sample (1g of spoilt apple sample in 10ml of sterile Phosphate buffered saline pH 7.2) into 50ml of sterile Vincent's Mineral Salt Broth (pH 7) (Vincent, 1970) on shaker (120 rpm) at 37°C for 7 days. The enriched broth was serially diluted using sterile Phosphate buffered saline pH 7.2 and plated on sterile Vincent's Mineral Salt agar (VAM) plates which were incubated at 37°C for 24 hrs and obtained colonies were further isolated on Vincent's Mineral Agar Medium (Lalitha *et al*., 2013).

All the isolates were checked qualitatively for the pectinase activity by spot inoculating on VAM plates. Plates were incubated at 37°C for 24 hrs. Pectinase activity was detected by adding a solution of Lugol's iodine to the Vincent agar medium. Isolates that showed a yellow coloured zone around the spot indicated hydrolysis of pectin (Dharmik and Gomashe, 2013). Isolate giving maximum pectinase production was identified by using morphological, cultural and biochemical tests as per Bergey's Manual of Determinative Bacteriology, 8th edition. Further confirmation of the strain's identity by 16S rRNA sequence analysis was carried out at Sai Biosystems Private Limited, Nagpur for its identification.

Extraction and assay of pectinase

Pectinase activity was assayed by DNSA method (Miller, 1959). Briefly, the fermented broth was centrifuged at 5000 rpm at 4ºC for 30 mins. The clear supernatant was used as crude enzyme for enzyme assay (Lalitha *et al*., 2013). 0.5 ml of pectin dissolved in 50mM sodium phosphate buffer (pH 7) was mixed with 0.5 ml of crude pectinase enzyme incubated at 37ºC for 25 mins. After incubation 1 ml of DNSA was added, mixed and kept on a boiling water bath for 5 mins. The tubes were cooled in cold water and the absorbance was recorded at 540 nm. Reaction mixture containing heat-inactivated enzyme was used as a Blank. The absorbance of the test supernatant against the blank was obtained and plotted on the standard graph of Glucose (400 - 2000 mcg/ml) to obtain the amount of product formed. One unit of pectinase activity is the amount of pectinase enzyme which liberates 1µmole of glucose per min under standard assay conditions (Mukesh kumar *et al*., 2012).

Optimization of culture conditions for maximum pectinase production

Media screening for optimum pectinase production: The yeild of pectinase was studied using 10 different media. The media used were: M-1 (Aguilar and Huitron, 1990), M-2 (Fonseca and Said, 1995), M-3 (Ikura and Horikoshi, 1987), M-4 (Contreras and Esquivel, 1999), M-5 (Poondla *et al*., 2015), M-6 (Torimiro and Okonji, 2013), M-7 (Sandhya and Kurup, 2013), M-8 (Raju and Divakar, 2013b), M-9 (Hitha and Girija, 2014) and M-10 (Vincent's Mineral salt broth Medium) (Vincent, 1970).

Effect of different carbon sources on pectinase production: The effect of different carbon sources on pectinase production was studied by adding 1% each of dextrose, maltose, sucrose, lactose, xylose, arabinose, sorbitol, mannitol and galactose in the optimized production medium which were incubated at 37°C for 48 hours under shaker conditions (Kaur and Kaur, 2014).

Effect of different concentrations of the optimized carbon source on pectinase production: Different concentrations of the optimized carbon source in the optimized production medium used were 0.5%, 1%, 1.5%, 2%, 2.5% and 3%.

Effect of different nitrogen sources on pectinase production: The effect of different nitrogen source on pectinase production was studied by adding 1% of different organic and inorganic nitrogen sources in the optimized production medium (Kaur and Kaur, 2014).The different nitrogen sources used were peptone, beef extract, yeast extract, urea, NH_4NO_3, $(NH_4)_2SO_4$ and NH_4Cl.

Effect of different concentration of optimized nitrogen source on pectinase production: Different concentrations of the optimized nitrogen source in the optimized production medium used were 0.5%, 1%, 1.5%, 2%, 2.5% and 3%.

Effect of variable pH on pectinase production: The range of pH used for the study was pH3 to pH10. The pH of the optimized production medium was adjusted using 1N HCl (3-7) and 1N NaOH (8-10) (El-sayed, 2015).

Effect of the incubation period on pectinase production: The time required for pectinase production was studied over 24, 48, 72, 96, 120, 144 and 168 hours. The incubation period giving maximum pectinase yield was selected for further studies (Singh and Mandal, 2012).

Effect of aeration on pectinase production: The effect of aeration on pectinase production was studied by incubating inoculated flasks, one under static condition and the other flask under shaker conditions (120rpm) at 37°C for 72 hrs respectively (Kouhoundé *et al*., 2015).

Effect of variable temperatures on pectinase production: The temperatures used for the study were 30°C, 37°C, 45°C and 55°C (Aaisha and Barate, 2016).

Effect of optical density of a culure on pectinase production: The effect of O.D on the production of pectinase was studied by using different O.D at 540 nm such as 0.2, 0.4, 0.6, 0.8 and 1 of the culture (Bhardwaj and Garg, 2014).

Effect of inoculum size of a culture on pectinase production: The effect of inoculum size on the production of pectinase was studied by using different inoculum size such as 2%, 4%, 6%, 8% and 10% (v/v) of culture of O.D. 0.2 at 540 nm (Bhardwaj and Garg, 2014).

Effect of different physicochemical parameters on pectinase activity

The characterization of pectinase activity was carried out based on stepwise modification of the parameters. For each experiment of optimization, the crude enzyme extract was incubated with pure pectin, after adjusting the parameters. The enzyme activity was detected by DNSA method (Miller, 1959). The experiments were carried out in triplicates. The following parameters were studied for obtaining maximum activity of the enzyme:

Effect of different temperatures on pectinase activity: Different temperatures used were 30°C, 37°C, 45°C and 55°C. The assay was carried out by incubating enzyme-substrate mixtures at different temperatures. The activity was then determined (Kaur *et al*., 2016).

Effect of different pH on pectinase activity: The effect of pH for pectinase activity was determined by incubating crude enzyme and buffers, adjusted to pH 3.0 -10, with 1% pectin at optimum temperature. The activity was then determined. The following buffer systems was used for obtaining a range of pH: sodium-acetate buffer (pH .0-6.0), sodium-phosphate buffer (pH 7.0-8.0) (Gummadi and Panda, 2003) and glycine-NaOH buffer (pH 9.0-11.0) (Rehman *et al*., 2014).

Effect of different metal ion concentration on pectinase activity: The effect of various metal ions (5mM) such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Na^{+}, Cd^{2+}, Li^{2+}, Ni^{2+}, Ba^{2+} and Hg^{2+}on pectinase activity was investigated. The metal ions used were in the form of $CaCl_2$, $MgCl_2$, $FeCl_3$, $CuCl_2$, $MnCl_2$, $ZnCl_2$, NaCl, CdCl2, LiCl2, NiCl2, BaCl2 and HgCl2 (Khatri *et al*., 2015).The crude enzyme was pre-incubated with 5 mM solutions of the above mentioned metal ions for 1 hour at 45°C. The residual activity (%) was measured by standard pectinase assay.

Effect of different inhibitors on pectinase activity: The effect of inhibitors such as β-mercaptoethanol, EDTA, SDS and Tween 80 on pectinase activity was investigated (Mei *et al*., 2013). The crude enzyme was pre-incubated with 1% solutions of the above mentioned compounds for 1 hour at 45°C. The residual activity (%) was measured by standard pectinase assay.

Purification of pectinase enzyme: The crude enzyme was subjected to precipitation by various saturated concentration of ammonium sulphate (30% - 80% saturation) to precipitate the enzyme. Different amounts of solid ammonium sulphate were added to each part to attain different levels of saturation i.e., 30%, 40%, 50%, 60%, 70% and 80%. The addition of salt was done with constant stirring in an ice bath. The precipitate was collected by centrifugation at 10,000 rpm for 10 mins at 4°C. The precipitate pellet was dissolved in 1ml of 50mM sodium phosphate buffer (pH 7) and its enzyme activity was determined. It was further purified by dialysis.

Dialysis: Dialysis tubing with pore size 2.4nm was cut and boiled for 10 minutes in 2% Na_2CO_3 and 1mM EDTA. It was rinsed with distilled water and boiled in1mM EDTA for 10 mins. The tubing was allowed to cool and stored at 4°C. Prior to use it was washed thoroughly with distilled water. One end of the tubing was tied and it was filled with enzyme precipitate dissolved in buffer. The other end was tied. The tubing was then suspended in sodium-phosphate buffer (pH 7) to dialyse. The assembly was kept in ice bath overnight on a magnetic stirrer. The tubing was punctured and the dialysed enzyme was collected and assayed for its enzyme activity.

Application of pectinase as a juice clarifier: The banana fruits were washed, peeled manually and cut into small pieces. Juice of 10ml from these were taken after heating at 85°C for 3 min to inactivate the natural fruit enzymes or microbes present and then cooled to 40ºC. 2.5 ml of crude enzyme was added. The samples were incubated for 4 hours and then treated at 85ºC for 3 min to inactivate the enzymes. Centrifuged at 3000rpm for 20 min and the supernatant were filtered out using Whatman's filterpaper. Control was treated in the same way except addition of enzyme. Clarity of the juice was determined by measuring the absorbance at 660 nm using a UV–Vis spectrophotometer, distilled water was used as blank. The clarity was expressed in terms of percentage by using Absorbance value (Abs) (Hitha and Girija, 2014; Joshi *et al*., 2011) % CLARITY = {(Control – Test) / Control} × 100

RESULTS AND DISCUSSION

Enrichment, isolation, screening and identification of pectinase producing bacterium

The samples were enriched in Vincent's mineral medium with 1% pectin for 1 week at 37°C under shaker conditions (120 rpm). 51 isolates were isolated from the enriched medium and were maintained on Vincent's mineral agar slants at 4°C. They were spot inoculated on Vincent's agar plates with 1% pectin and incubated at 37°C for 48 hours. The isolates were subjected to Lugol's Iodine test (Dharmik and Gomashe, 2013). Out of 51, 5 isolates showed pectinase production on Vincent's mineral agar plates, characterized by a zone of hydrolysis around the spot inoculated culture (fig.1).

Fig 1 Detection of pectinase activity on Vincent's mineral Agar plate by the isolate AM-8

Out of 5 isolates, AM-8 was selected for further studies as it showed maximum pectinase activity (12.83U/ml) as shown in figure 2.

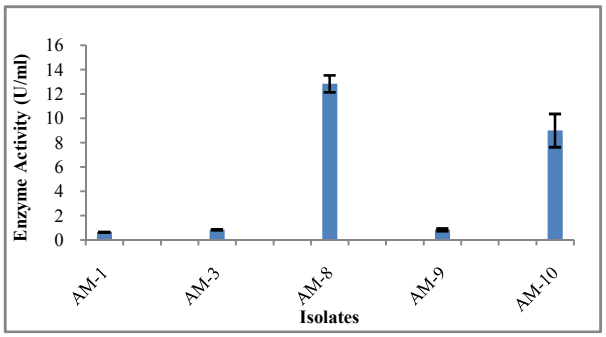

Fig 2 Pectinase activity of 5 different isolates.

On the basis of morphological, cultural and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology, 8th Edition and by 16S rRNA gene sequencing analysis, isolate AM-8 was identified as *Bacillus subtilis* strain arium 1115 by the NCBI assessment with accession number NCBI- LC224327.1. Many of the researchers have also used apple wastes as their sample in order to get pectinase producing micro-organisms (Nahar *et al*., 2015; Hitha and Girija, 2014; Namasivayam *et al*., 2011). Many strains of Bacillus have been previously reported to produce extracellular pectinases, for eg., *Bacillus subtilis* Strain NVFO 19 (El-sayed *et al*., 2015), marine *Bacillus subtilis* (Joshi *et al*., 2013), *Bacillus subtilis BKDS1 (*Kavuthodi, 2015), *Bacillus sphaericus* MTCC 7542 (Jayani *et al*., 2010),*Bacillus macerans* (Miyazaki, 1991), *Bacillus sp.* TS47 (Takao *et al*., 2000), *Bacillus sp.*MBRL576 (Bhardwaj and Garg, 2014), *Bacillus licheniformis* KIBGE-IB21(Rehman *et al*., 2014), *B. cereus*, *B. subtilis*, *B.stearothermophilus* (Torimiro and Okonji, 2013), *Bacillus firmus* (Roosdiana *et al*., 2013), *Bacillus gibsonii* (Li *et al*., 2005) and *Bacillus cereus* (Namasivayam *et al*., 2011). Other pectinase producing microorganisms are *Aspergillus niger* (Benkova and Slezarik, 1966), *Rhizopus stolonifer* (Manachini *et al*., 1987), Amycolata *sp* (Bruhlmann, 1995) and *Saccharomyces pastorianus* (Astapovich *et al*., 1997).

Optimization of culture conditions for maximum pectinase production

Media screening for optimum pectinase production

It was found that out of ten media studied, medium-5 facilitated maximum pectinase yield (16.21U/ml) by *Bacillus subtilis* strain arium 1115 and hence medium-5 containing commercial pectin and yeast extract was used throughout the study (Fig. 3). Pectin serves as the substrate for the induction of pectinase enzyme in the organism, whereas Yeast extract serves as the source of carbon, nitrogen and different growth factors. Similar medium was also used by many researchers for pectinase production with a little modification or change in the composition (Kavuthodi *et al*., 2015; Rani *et al*., 2012; Alhijjaji, 2015; Roosdiana *et al*., 2013; Joshi *et al*., 2013).

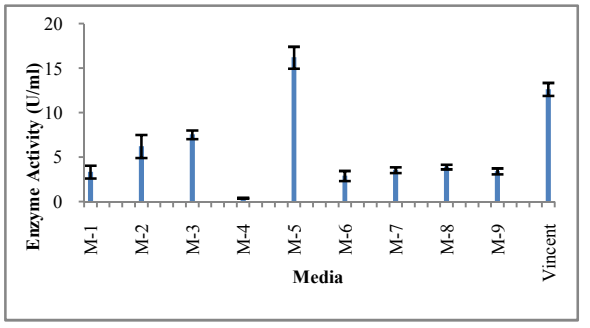

Fig 3 Effect of different media on Pectinase production by *Bacillus subtilis* strain arium1115

Effect of carbon source on pectinase production

The present study was aimed at optimization of medium components which have been predicted to play a significant role in enhancing pectinase production. Pectin can be used as the source of carbon for pectinase but addition of carbohydrate further enhances pectinase production. As shown in Figure 4, maximum pectinase yield by *Bacillus subtilis* strain arium 1115 was observed in Medium-5 with 1% Carbon sources like Xylose (17.28 U/ml), followed by Dextrose (15.52 U/ml), Sucrose (15 U/ml), Lactose (9.49 U/ml), Mannitol (7.24 U/ml), Galactose (6.96 U/ml), Arabinose (6.61 U/ml), Maltose (3.95 U/ml), Sorbitol (2.91 U/ml) and Starch (2 U/ml). When different concentrations of xylose was used, *Bacillus subtilis* strain arium 1115 showed best pectinase production in the presence of 1.5% Xylose along with 0.25% pectin (19.40 U/ml) (Fig.5).

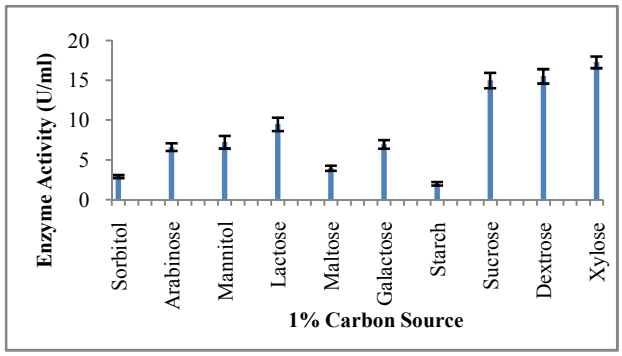

Fig 4 Effect of different carbon sources on pectinase production by *Bacillus subtilis* strain arium1115

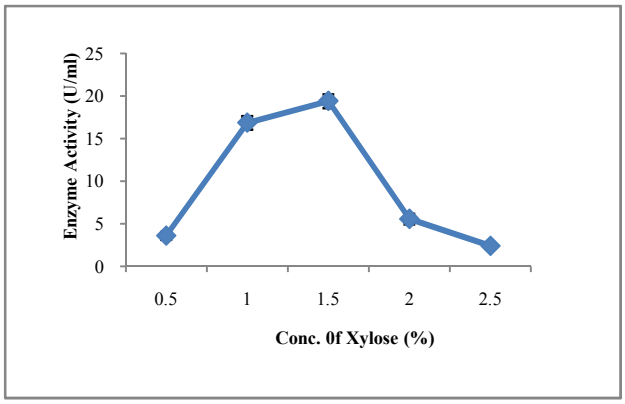

Fig 5 Effect of different concentrations of Xylose on pectinase production by *Bacillus subtilis* strain arium1115

Different carbon sources have different impacts on pectinase production. In our studies with *Bacillus subtilis* Strain arium 1115, highest pectinase production was observed in presence of 1.5% Xylose and also with the increase in the concentration of the carbon source (xylose), production of the pectinase enzyme had decreased. However, earlier studies in case of Bacillus sp.

MFW7, Bacillus sp. MBRL576, Bacillus *circulans* and *Acinetobacter oleivorans* DR1 showed that lactose, sucrose, galactose and cellulose supported maximum pectinase production respectively (Mukesh Kumar *et al*., 2012; Bhardwaj and Garg, 2014; Raju and Divakar, 2013a; Bhardwaj and Garg, 2003). There are reports which mentioned that different carbon sources have varied influence such as catabolite repression on the production of extracellular pectinase enzyme (Kilara, 1982; Ward and Fogarty, 2010). There has been observations that many micro-organisms demonstrate better pectinase production in the presence of crude carbon sources like citrus powder, wheat bran, apple pectin, orange waste peel and molasses (Joshi *et al*., 2013; Torimiro and Okonji, 2013; Rehman *et al*., 2015; Soares *et al*., 1999; Sughra *et al*., 2013; Ahmed *et al*., 2015; Patil and Dayanand, 2006; Jaradat *et al*., 2008).

Effect of nitrogen source on pectinase production

Besides the carbon source, the type of nitrogen source in the medium also influences the pectinase yield in the production broth. The highest pectinase production by *Bacillus subtilis* strain arium 1115 was observed in presence of 1% Yeast extract (19.18 U/ml) followed by Peptone (19.02 U/ml), Beef extract (13.58 U/ml), $(NH_4)_2SO_4$ (11.31 U/ml), Urea (5.39 U/ml), NH_4Cl (4.27 U/ml) and NH_4NO_3 (3.3 U/ml) (Fig.6).

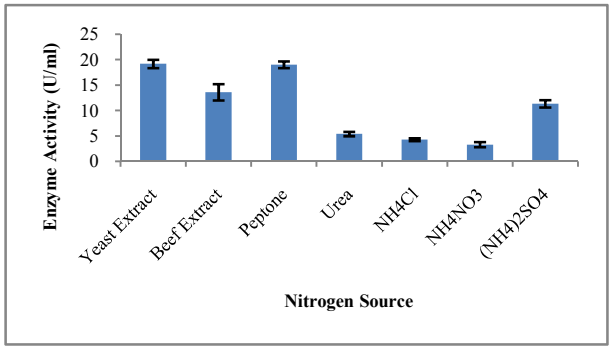

Fig 6 Effect of different Nitrogen sources on pectinase production by *Bacillus subtilis* strain arium1115

When different concentrations of Yeast extract were studied, the best pectinase production by *Bacillus subtilis* strain arium 1115 was seen in the presence of 1% yeast extract (Fig.7).

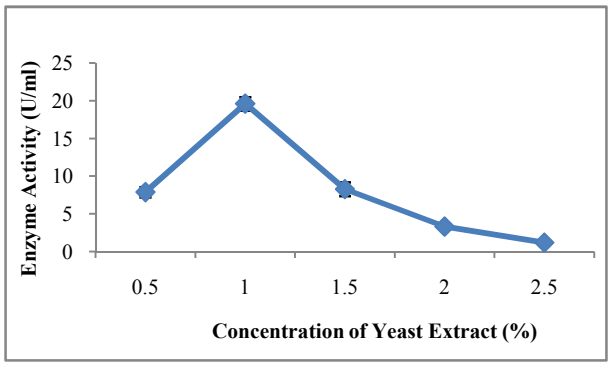

Fig 7 Effect of different concentrations of Yeast Extract on pectinase production by *Bacillus subtilis* strain arium1115

It can be inferred that organic nitrogen sources can induce pectinase production much better than the inorganic nitrogen sources. Yeast extract is a complex nutrient source with unknown composition containing essential amino acids, vitamins and other trace elements which may be responsible for enhancing pectinase production (Rehman *et al*., 2015). Similar results were observed in case of marine *Bacillus Subtilis* (Joshi *et al*., 2013), *Bacillus cereus* (Namasivayam *et al*., 2011), *B. licheniformis* KIBGE-IB21 (Rehman *et al*., 2015), *Bacillus subtilis* EFRL 01 (Qureshi *et al*., 2012), *Bacillus circulans* (Raju and Divakar, 2013a) and *Enterobacter aerogenes* NBO2 (Darah *et al*., 2013). However, Bacillus sp. MFW7, Bacillus sp. MBRL576 and *Acinetobacter oleivorans* DR1 exhibited maximum pectinase in presence of peptone, sodium nitrate (0.2%) and potassium nitrate (0.2%) respectively (Bhardwaj and Garg, 2014; Mukesh Kumar *et al*., 2012; Bhardwaj and Garg, 2003). However, in some cases of fungi, pectinase production was better in presence of inorganic nitrogen sources (Ibrahim *et al*., 2013; Tariq and Latif, 2012). Lonsane and Ramesh (1990) reported that ammonium salts enhanced the enzyme production and Sodium nitrate exhibited steep decrease in pectinase production.

Effect of pH on pectinase production: Pectinase production was tested when the organism was cultured at different pH ranging from 3-10. As shown in Figure 8 the optimum pectinase production by *Bacillus subtilis* strain arium 1115 was observed at pH 9 (21.44 U/ml) and minimum pectinase production was exhibited at pH 3 (0.61 U/ml).

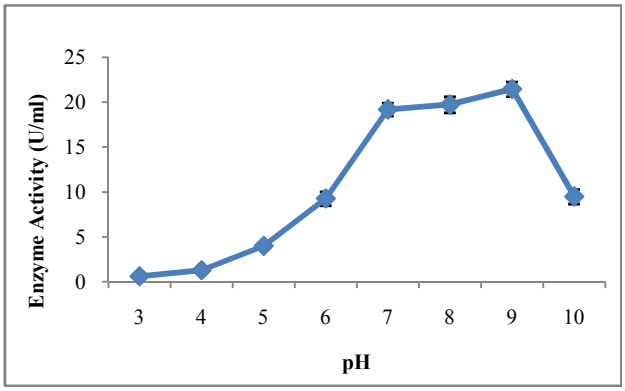

Fig 8 Effect of different pH values on pectinase production by *Bacillus subtilis* strain arium1115

This can be an indication that the isolate requires alkaline conditions for its optimum enzyme production. Similarly optimum alkaline pH requirement for pectinase production was exhibited by many bacterial species such as *Bacillus* sp. RCPTM1 (Patil *et al*., 2012), *Bacillus licheniformis* KIBGE-IB21 (Rehman *et al*., 2015), *Paenibacillus xylanolyticus* (Giacobbe *et al*., 2014), *Bacillus subtilis* EFRL 01 (Qureshi *et al*., 2012), *Bacillus pumilus* (Sharma and Satyanarayana, 2006), *Bacillus coagulans* and *Bacillus endophyticus* (Aaisha and Barate, 2016). However, marine *Bacillus subtilis,* Bacillus sp, *Bacillus firmus,* Bacillus sp isolate FW2, Bacillus sp. MFW7, *Bacillus coagulans* and *Bacillus sphaericus* demonstrated optimum pH between 5 and 6 for pectinase production (Joshi *et al*., 2013; Kaur *et al*., 2016; Roosdiana *et al*., 2013; Reda *et al*., 2008; Mehta *et al*., 2013; Mukesh Kumar *et al*., 2012; Odeniyi *et al*., 2009; Jayani *et al*., 2010). But some species like Bacillus sp. MBRL576 and *Bacillus circulans* have optimum pH like pH 4 and pH 7 for pectinase production respectively (Bhardwaj and Garg, 2014; Raju and Divakar, 2013a).

Effect of incubation period on pectinase production: As shown in Figure 9 pectinase production by *Bacillus subtilis*

strain arium 1115 started from 24 hrs of growth (7.18 U/ml) and maximum amount was produced in 72 hrs (23.54 U/ml) and then declined with further increase in duration of incubation. Further increase of incubation period decreases the rate of pectinase production, sugar and protein concentration (Sughra *et al.*, 2013).

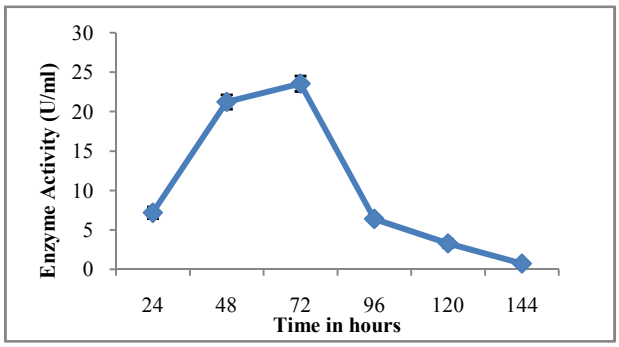

Fig 9 Effect of different incubation period (hrs) on pectinase production by *Bacillus subtilis* strain arium1115

In our case, *Bacillus subtilis* strain arium 1115 showed best pectinase production in the late log phase indicating that the organism takes time to produce its inducible enzyme pectinase. Similar results were found in the case of *Bacillus subtilis* BKDS1 (Kavuthodi *et al.*, 2015), *Bacillus sphaericus* (MTCC 7542) (Jayani *et al.*, 2010) and Bacillus sp. MFW7 (Mukesh kumar *et al.*, 2012). Different species of Bacillus gave maximum pectinase production at different incubation time; eg., *Bacillus firmus* at 18 hrs (Roosdiana *et al.*, 2013), *Enterobacter aerogenes* NBO2 and *Bacillus* sp. MBRL 576 at 24 hours (Darah *et al.*, 2013; Bhardwaj and Garg, 2014), *Bacillus cereus* at 36 hrs (Namasivayam *et al.*, 2011), *Bacillus subtilis* EFRL 01 and *Bacillus circulans* at 48hrs (Raju and Divakar, 2013a; Qureshi *et al.*, 2012), Bacillus sp and *Bacillus subtilis* at 96 hrs (Kaur *et al.*, 2016; Lalitha *et al.*, 2014). However, the time taken by the bacillus species to produce pectinase is comparitively less than the time taken by the fungal species. Therefore, for the faster production of pectinase, Bacillus species are preferred over the fungus.

Effect of aeration on pectinase production: Different types of physicochemical conditions could affect the production of pectinase enzyme. As shown in figure 10, the shaker culture of *Bacillus subtilis* strain arium 1115 at 120 rpm gave optimum pectinase yield (23.08 U/ml) than the culture incubated at static conditions (4.34 U/ml). Shaker conditions are usually used for the production of extracellular enzymes by aerobic organisms, as it enhances the aeration rate.

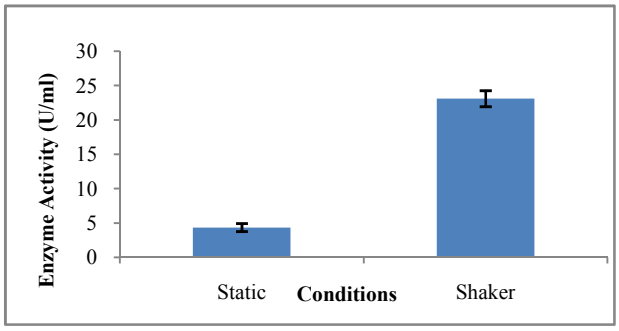

Fig 10 Effect of aeration conditions on pectinase production by *Bacillus subtilis* strain arium1115

Aeration in culture medium could lead to sufficient supply of dissolved oxygen in the production medium. This shows that shaker conditions provides excess amount of dissolved oxygen for cell growth as compared to the static condition which leads to improper mixing of media constituents and clumping of cells. Many bacterial pectinase producers such as *Bacillus sphaericus* (MTCC 7542) (Jayani *et al.*, 2010), *Bacilus firmus* (Roosdiana *et al.*, 2013), *Bacillus subtilis* (Joshi *et al.*, 2013), *Bacillus subtilis* Strain NVFO 19 (El-Sayed, 2015), Bacillus sp.(Patil *et al.*, 2012), *Bacillus subtilis* BKDS1(Kavuthodi *et al.*, 2015), *Paenibacillus xylanolyticus* (Giacobbe *et al.*, 2014), *Acinetobacter oleivorans* DR1, Bacillus sp. MBRL576 (Bhardwaj and Garg, 2003; Bhardwaj and Garg, 2014), Bacillus sp. MFW7 (Mukesh Kumar *et al.*, 2013), Pseudomonas sp. (Sivasakthivelan *et al.*, 2014) and *Bacillus cereus* (Namasivayam *et al.*, 2011) also need aeration for maximum pectinase production. However, there are anaerobic and facultative anaerobic bacteria such as *Erwinia carotovora*, *Clostridium butyricum* and *Clostridium felsineum* which produce pectinolytic enzyme (Obi, 1981).

Effect of incubation temperature on pectinase production: *Bacillus subtilis* strain arium1115 showed different pectinase yield at different incubation temperatures with the best pectinase production at 45°C (26.71 U/ml) (Fig.11).

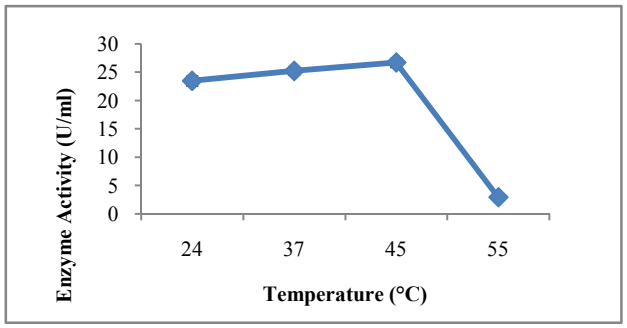

Fig 11 Effect of different incubation temperatures on pectinase production by *Bacillus subtilis* strain arium1115

This can be due to reduced growth rate, lower survival or the inactivity of the pectinase enzyme or suppression of cell viability at a temperature higher than 45°C. Similar results were observed in case of Bacillus sp. MBRL576 which showed best results at a temperature of 45°C (Bhardwaj and Garg, 2014). Other Bacillus species demonstrated slightly higher temperature for pectinase production such as *Bacillus pumilus* dcsr1 at 50°C (Sharma and Satyanarayana, 2006), marine *Bacillus subtilis* and *Bacillus circulans* at 40°C (Joshi *et al.*, 2013; Raju and Divakar, 2013a). However, *Bacillus cereus*, *Bacillus firmus* I-10104, *Bacillus cereus* and *Bacillus endophyticus* and *Bacillus coagulans* exhibited maximum pectinase production at 37°C (Namasivayam *et al.*, 2011; Aaisha and Barate, 2016; Reda *et al.*, 2008). Temperature of 30°C was observed to yield maximum pectinase production in case of *Bacillus sphaericus* (MTCC 7542), *Aspergillus niger, S. cerevisiae, Aspergillus foetidus* and *Kluyveromyces wickerhamii* (Jayani *et al.*, 2010; Ahmed *et al.*, 2015; Kumar *et al.*, 2012; Moyo *et al.*, 2003).

Effect of optical density and inoculum size of culure on pectinase production: Figure 12 exhibited the effect of different optical density of *Bacillus subtilis* strain arium 1115

on pectinase production where the culture gave highest pectinase production at an Optical Density of 0.2 at 540nm (27.99 U/ml). Of all the inoculum size of *Bacillus subtilis* strain arium 1115 studied, an inoculum size of 8% of 0.2 O.D at 540 nm demonstrated the maximum pectinase yield (28.44U/ml) (Fig.13).

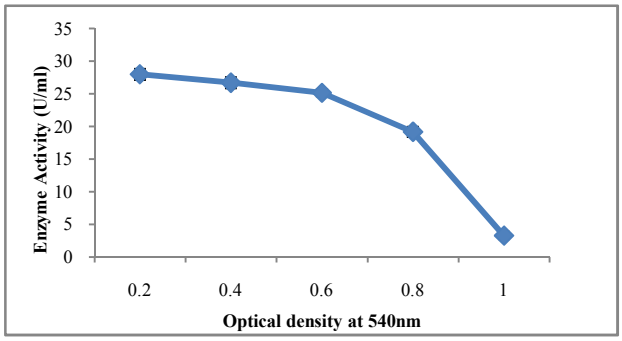

Fig 12 Effect of different Optical Densities of *Bacillus subtilis* strain arium1115 on pectinase production

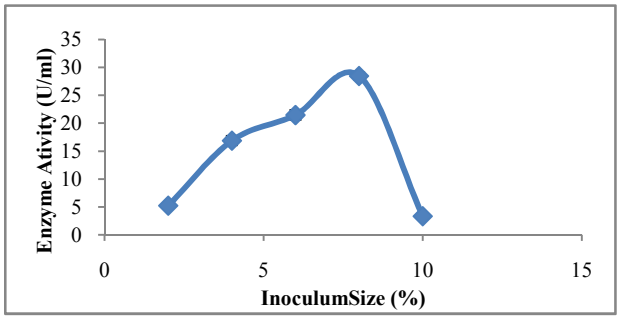

Fig 13 Effect of different inoculums size of *Bacillus subtilis* strain arium1115on pectinase production

The pectinase production decreased with further increase in inoculum size, which caused a decline in the enzyme activity. An optimal inoculum level is necessary for maintaining the balance between proliferating biomass and available nutrients to obtain maximum enzyme yield. A lower enzyme yield at higher inoculum level could result from faster consumption of nutrients. And also lower inoculums levels may give insufficient biomass and allow the growth of undesirable organisms in the production medium (Kuhad *et al.,* 2004). Similar results were observed where higher inoculum size was necessary in many Bacillus species such as Bacillus sp. MBRL576 (Bhardwaj and Garg, 2014), *Bacillus circulans* (Raju and Divakar, 2013a), *Bacillus subtilis* (Ahlawat *et al.*, 2009), *Bacillus pumilus* (Sharma and Satyanarayana, 2006) and *Bacillus sphaericus* (MTCC 7542) (Jayani *et al.*, 2010). However, the best inoculum size for *Enterobacter aerogenes* NBO2 and *Acinetobacter oleivorans* DR1 was found to be 3% (v/v)) and 2.5% (v/v) respectively (Darah *et al.*, 2013; Bhardwaj and Garg, 2003).

Effect of different physicochemical parameters on crude pectinase activity

Effect of different temperatures on pectinase activity

Effect of different temperature on the pectinase activity was studied of which the pectinase enzyme showed maximum activity at 45°C (32.76U/ml) (Fig.14). Similar result was reported in case of *Bacillus licheniformis* (Rehman *et al.*, 2013). However, there are reports where many Bacillus species showed different temperatures for pectinase activity such as Bacillus sp. strain BR1390 (60°C) (Rastegari and Karbalaei-Heidari, 2014), Bacillus sp. TS47 (70°C) (Takao *et al.*, 2000), Bacillus sp. HD2 (40°C) (Paudel *et al.*, 2015), *Bacillus firmus* (50°C) (Roosdiana *et al.*, 2013), Bacillus spp KSM-P15 B. (50-55°C) (Kobayashi *et al.*, 1999), *Bacillus cereus* (37°C) (Namasivayam *et al.*, 2011), *Bacillus stearothermophilus* (60°C), *Bacillus cereus* and *Bacillus subtilis* (50°C) (Torimiro and Okonji, 2013).

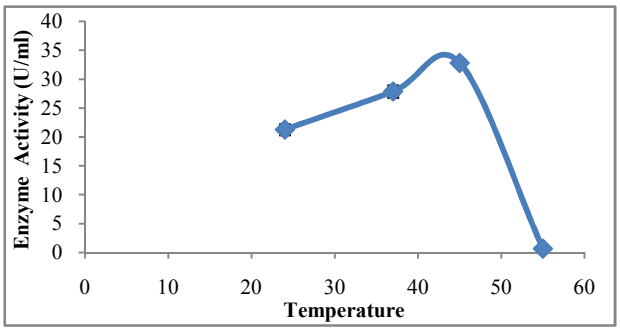

Fig 14 Effect of different Temperature (°C) on activity of pectinase produced by *Bacillus subtilis* strain arium1115

Effect of pH on pectinase activity

Of all different pH studied, the pectinase enzyme produced by *Bacillus subtilis* strain arium1115 exhibited highest pectinase activity at pH 7(32.50U/ml) (Fig.15).

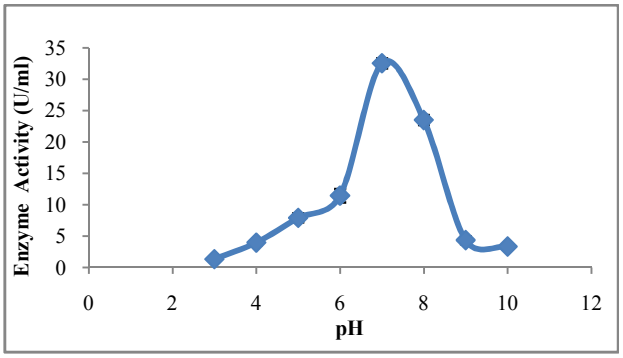

Fig 15 Effect of different pH on activity of pectinase produced by *Bacillus subtilis* strain arium1115

Similar results were shown by Bacillus sp and *Bacillus firmus* (Soares *et al.*, 1999; Roosdiana *et al.*, 2013; Torimiro and Okonji, 2013). However, pectinase from most of the Bacillus species such as *Bacillus coagulans*, *Bacillus endophyticus, Bacillus vietnamensis, Paenibacillus xylanolyticus,* Bacillus sp. TS47, Bacillus spp KSM-P15, *Bacillus licheniformis*, *Bacillus halodurans* M29, *Bacillus pumilus* dcsr1, *Bacillus macerans* and Bacillus sp. RN1 show activity at alkaline pH range (pH8 - pH11) (Aaisha and Barate, 2016; Sharma and Satyanarayana, 2006; Kobayashi *et al.*, 1999; Miyazaki, 1991; Takao *et al.*, 2000; Giacobbe *et al.*, 2014; Rehman *et al.*, 2015; Mei *et al.*, 2013; Sukhumsiirchart *et al.*, 2009; Soares *et al.*, 1999). Most of the fungal pectinases have optimal activity at acidic pH range (Kant *et al.,* 2013; Kaur *et al.,* 2016; Rani *et al.*, 2013; Poondla *et al.*, 2015).

Effect of different metal ions on pectinase activity

In general, the pectinase activity can be inhibited or activated by addition of metal ions. The presence of metal ions theoretically affects the bonding between active site of pectinase and substrates (Roosdiana *et al.*, 2013). The effect of

few metal ions was assessed by using various metal ions in 5mM concentration. The residual enzyme activity of *Bacillus subtilis* strain arium 1115 in the presence of metal ions such as Mg^{2+}(76.9%), Na^{+}(34.8%), Cu^{2+}(30.5%), Mn^{2+}(26.4%), Ca^{2+}(25.8%), Ba^{2+}(22.9%), Zn^{2+}(21.4%), Fe^{2+}(16.6%), Ni^{2+}(14.1%), Li^{2+}(9.3%), Cd^{2+}(8.7%) and Hg^{2+}(5.5%) was observed. Among the metal ions tested, Mg^{2+} showed minimum inhibition with 76.93% residual activity and Hg^{2+} demonstrated maximum inhibition with 5.53% residual activity (Fig.16).

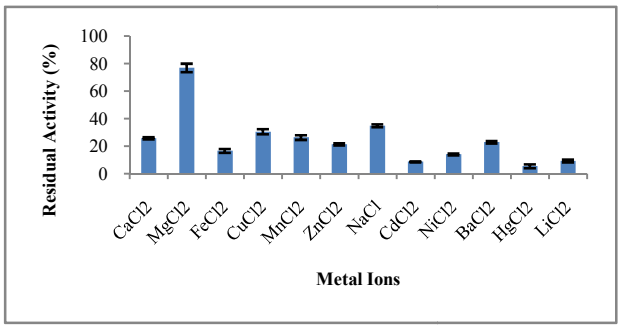

Fig 16 Effect of different metal ions on the activity of pectinase produced by *Bacillus subtilis* strain arium1115

In our studies pectinase from *Bacillus subtilis* strain arium 1115 was inhibited by most of the divalent ions. However, the enzyme from *Bacillus macerans, Bacillus cereus, Bacillus halodurans* M29, *B. pumilus* DKS1, *Bacillus polymyxa, B. xylophilus* and Bacillus sp. HD2 was reported to have enhancement of activity in the presence of Ca^{2+} (Gummadi and Panda, 2003; Namasivayam *et al*., 2011; Mei *et al*., 2013; Dave and Vaughn, 1971; Nagel and Vaughn, 1961; Kikuchi *et al*., 2006; Sukhumsiirchart *et al*., 2009; Paudel *et al*., 2015). There are reports of pectinase activity from many Bacillus sp. significantly enhanced in the presence of most divalent cations such as Ca^{2+} and Mg^{2+}, but Hg^{2+} and Fe^{3+} served as strong inhibitor (Rastegari and Karbalaei-Heidari, 2014; Roosdiana *et al*., 2013; Torimiro and Okonji, 2013). Similarly, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} were reported to activate pectinase from fungal species like *Penicillin italicum* but was inhibited by Cu^{2+} and Fe^{2+} (Alana *et al*., 1990). Banu *et al*. (2010) observed little effect of Mg^{2+} and Ca^{2+} on pectinase from *P. chrysogenum.*

Effect of different inhibitors on pectinase activity

In an attempt to characterize the pectinase enzyme; different inhibitors were tested at 1% (w/v) concentrations. Results presented in Figure 17 demonstrated that Tween 80, β-mercaptoethanol, EDTA and SDS showed pectinase residual activity of 20.8%, 20.1%, 15.5% and 6.2% respectively.

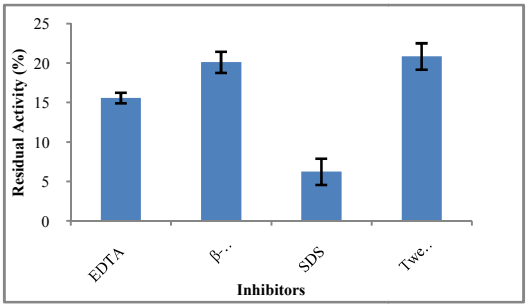

Fig 17 Effect of different inhibitors on activity of pectinase produced by *Bacillus subtilis* strain arium1115

It was seen that the activity of pectinase enzyme from *Bacillus subtilis arium* 1115 got inhibited by these compounds, where the maximum inhibition was exhibited by SDS and the least inhibition was exhibited by Tween 80. Similar results were reported for pectinase enzyme activity from *Bacillus halodurans* M29 (Mei *et al*., 2013) However, the pectinase enzyme from Bacillus sp. strain BR1390 was inhibited by 5 mM EDTA while SDS had no effect and Tween 80 stimulated the activity of it (Rastegari and Karbalaei-Heidari, 2014).

Purification of pectinase enzyme

The enzyme was precipitated at 80% saturation by ammonium sulphate and purified by dialysis (Table 1). The partial purification of pectinase was carried out in our study by using ammonium sulphate precipitation followed by dialysis. The enzyme was precipitated at 80 % saturation by ammonium sulphate. In case of *Bacillus subtilis* BKDS1, the enzyme activity was found in the 40 - 100% salt saturation fraction (Kavuthodi *et al.,* 2015). In case of *Saccharomyces cerevisiae*, crude enzyme samples were partially purified by fractionation using ammonium sulphate (Merck) with 20–80% saturation (Poondla *et al*., 2015).

Table 1 Purification of pectinase enzyme produced by *Bacillus subtilis* strain arium 1115

Enzyme	Enzyme activity (U/ml)
Crude enzyme	31.234
Undialyzed precipitate	34.265
Dialyzed enzyme	37.190

The pectinase enzyme produced by *Bacillus subtilis* strain arium1115 showed 33% of clarity (Table 2 and Fig.18). Pectinases are now prerequisite in juice clarification because they not only bring down bitterness and cloudiness of fruit juices but also reduce viscosity of fruits, improve pressability of pulp and disintegrate jelly like pectin (Soares *et al*., 2001:Sandri *et al*., 2011; Kohli and Gupta, 2015; Poondla *et al*., 2015; Tapre and Jain, 2014; Singh and Gupta, 2004). The application of crude pectinase enzyme as a clarifying agent in fruit juice was evaluated in our present study. The results showed that pectinases have juice turbidity clarifying action and thus. can be used in iuice and wine industries.

Table 2 Application of pectinase enzyme

Absorbance of control	Absorbance of test	% Clarity
1.27	0.85	33%

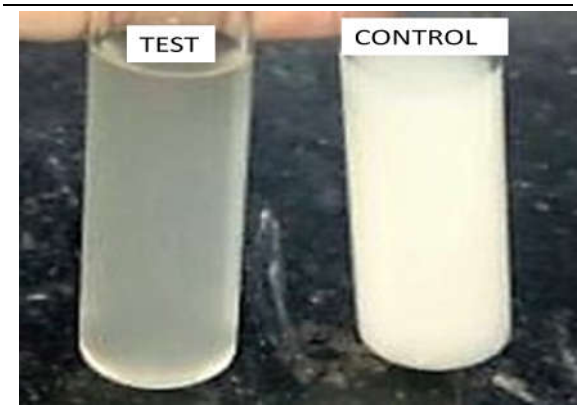

Fig 18 Application of pectinase enzyme produced by *Bacillus subtilis* strain arium1115 as a clarifying agent in banana juice.

Similar result was reported for pectinase enzyme from *Bacillus* sp. MBRL576 which was used to clarify apple pulp (Bhardwaj and Garg, 2014).

CONCLUSION

In conclusion, it can be said that *Bacillus subtilis* strain arium 1115 isolate is a good source of pectinase which is active at neutral pH and at 45°C and showed clarification of juice which is an important application. Attempts should be made to adapt the enzyme to conditions that makes it more useful in terms of commercial applicability in industries such as juice industry, paper, textiles and tea industries.

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