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Isolation and Screening of Pectinolytic *Streptomyces Sp.* From Soil Samples of Egypt.

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ABSTRACT

Pectin is a major part of all plants. Pectinases hydrolyze pectic substances. The purpose of the present study was to isolate and screen pectinolytic *Streptomyces sp*. from different soil samples of Egypt. Total 526 *Streptomyces sp*. were isolated. Primary screening of pectinolytic *Streptomyces sp*. was made by pectin agar plates. Two hundred thirty three isolates were had pectinolytic activity, providing clear zones, represented (44%). These positive isolates divided into two group's fair and good one. Fair group that contain clear zone diameter <10 mm, represented 42.9% and good one that contain clear zone diameter ≥10 mm represented 57.1%. By secondary screening of the good isolates using pectin broth medium, five isolates (2, 5, 8, 72 and 103) give the best results (≥15 U/mg). By screening the five isolates using different media (M1, M2, M3 and M4), isolates (2, 5 and 103) provide the best results on M2 pectin nitrate broth medium, while 8 and 72 give the best results with M1 pectin broth medium. By screening using different agro-industrial waste products as lemon peel, orange peel, rice husk and wheat bran, isolate 5 give maximum activity (21.5 U/mg) with lemon peel nitrate medium.

Keywords: Pectinases, Pectinolytic *Streptomyces sp*., Different media, Different agro-industrial wastes.

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INTRODUCTION

Biotechnological products like enzymes are most significant products for human needs in large scale part of environmental, industrial and food technology especially the one that obtained from microbial origin. Pectinases are cluster of enzymes that help in breakdown of pectic parts which are mostly within plant tissues. The pectin, an indispensable component present in between middle lamella and cell wall of plant cells composed of as many as seventeen various monosaccharides and at least seven various polysaccharides forming a most complicated bio-macromolecule in nature [1]. Pectinases are naturally produced by different organisms such as fungi, bacteria, yeasts, nematodes, other harmful microrganisms, plants and insects. A quarter sales of a global food enzymes rely upon microbial pectinases. Even though fungal pectinases are being industrially exploited, pectinase creation from actinomycetes has also been reported earlier [1, 2, 3, 4, 5, 6, 7 and 8].

Actinomycetes plays a major role in the vegetable residue degradation. Actinomycetes produce extracellular enzymes like cellulase, xylanase and pectinase as they are effective degraders of plant debris. It is a specific class of gram-positive bacteria (Actinomycetales) of prokaryote. In 16SrDNA tree, actinomycetes form a definite phylogenetic series and have been a major technological importance in the past years, with the discovery of numerous range of metabolites produced by its various genera [9]. The diversity in actinobacterial populace in sediment and samples of water from the submarine environment of Tamil Nadu and diverse genera viz. *Streptomyces, Actinopolyspora, Actinomadura, Nocardiopsis, Micromonospora* and Actinomycete have been observed in earlier studies [10]. They have ability to synthesize chemically diverse and commercially important bioactive substances like pigments, antibiotics and enzymes, etc [11].

Streptomyces sp. is significant one in soil ecology and present worldwide in soil. They provide the characteristic earthy smell to soils [12]. They are considered as one of all significant bacteria, due to their capacity to develop the soil properties as well as producing several extracellular enzymes as secondary products [13, 14]. Pectin degrading enzymes reported from actinomycetes are mainly pectate lyases [15].

Pectate lyase from the actinomycete *Thermomonospora fusca* was purified and characterized [16]. Pectate lyase has lately been detected from *Streptomyces viridochromogenes* [17]. Pectinolytic enzymes from actinomycetes have been used for the degumming of ramie pan fibers [18]. Pectinase was also produced and purified from *Streptomyces sp. QG-11-3* [19]. Also pectinase was produced and purified from *Streptomyces lydicus* by [1].

The use of wastes as low cost residues, inexpensive, available and safe substrates in enzyme production is especially interesting for countries where agro-industrial residues are abundant. The Disposal of fruit peels is becoming the threatened problem over fruit industries. So, fruit peels will be used as a potential substrate for the production of pectinase. Among them, citrus, apple and orange are most widely used because it contains large amount of pectin. For using in industry, pectinases can be made out of pectin-containing wastes [20], such as apple pomace and grape skin area [21, 22], tamarind kernel [23] and citrus peel [24]. All of these materials contain variable levels of pectin: lemon peel (around 35%, [25] and apple pomace (up to15%; [22], whereas grape skin and tamarind kernel have minimum pectin content 4%; [21] and <2%; [26], respectively.

MATERIALS AND METHODS

Isolation of *Streptomyces sp.* from soil samples

Twenty five cultivated agriculture soil samples were collected from different areas of Egypt. Pectinolytic *Streptomyces sp*. were isolated from collected soil samples by serial dilution method and spread on starch nitrate agar plates. Serial dilution was done by taking one gram of soils were transferred into 100 ml of sterilized distilled water, shaking for two hours. The resulting soil suspensions were serially diluted and plated onto starch nitrate agar [27]. Following pouring plate technique, the inoculated plates were incubated at 28 ^{o}C for 7-14 days. Firm cartilaginous rough chalky colonies of *Streptomyces sp.* were selected and purified. Starch nitrate agar medium contains Starch, 20g; KNO_3 2.0g; K_2HPO_4 (anhydrous), 1.0g; $MgSO_4.7H_2O$, 0.5g; NaCl, 0.5g; $CaCO_3$ 3.0g; $FeSO_4.7H_2O$, 0.01g; Distilled water 1000 ml.

Preliminary screening of pectinase activity on solid plates

The cultures were screened for their ability to grow and utilize polygalacturonase using pectin as a sole carbon source in a defined synthetic basal medium. Fresh cultures of the *Sterptomyces* isolates were screened for utilization of pectin using a solid medium containing: KH_2PO4 (anhydrous), 4.0g, Na_2HPO4 (anhydrous), 6.0g, Pectin (from apples), 5.0g, $(NH_4)_2SO_4$, 2.0g, Yeast extract, 1.0 g, $MgSO_4$. $7H_2O$, 0.0001g, $CaCl_2$ 0.001g, agar, 10.0g, distilled water, 1000 ml. Hydrolysis zones were detected after 4 and 6 days by flooding plates with an aqueous solution of cetrimide solution (hexadecyl-trimethyl-ammonium-bromide) (1% w/v) [28].

Secondary screening of Polygalactouranase by active isolates by submerged fermentation medium

Heavy spore suspensions (1ml) of the active *Streptomyces* strains used as inoculum for 50 ml broth medium in 250 ml conical flasks, which were incubated on a rotary shaker (NEW BRUNSWICK SCIENTIFIC, EDISON, N., J., USA) (180 r.p.m) at 28 ^{o}C for five days. After incubation the supernatants were separated by filtration, the filtrates were used as crude enzymes sources. 250 ml conical flasks contains 50 ml of the following medium: KH_2PO_4 (anhydrous), 4.0 g, Na_2HPO_4 (anhydrous), 6.0g, Pectin (from apples), 5.0 g, $(NH_4)_2SO_4$, 2.0 g, Yeast extract, 1.0g, $MgSO_4$. $7H_2O$, 0.0001g, $CaCl_2$ 0.001g, distilled water, 1000 ml using pectin as a sole carbon source [28].

Polygalacturonase assay:

polygalacturonase was determined according to [29] using 1.0 % (w/v) polygalactourinic acid (Sigma) in 0.2 M phosphate buffer pH 7.0 as substrate for enzyme reaction. The assay reaction mixture contain 0.5 ml substrate + 0.5 ml diluted enzyme solution in the buffer was incubated at 40 ^{o}C for 10 min, the reaction was stopped by addition of 1ml of dinitrosalisilic acid (DNS) reagent solution followed by keeping in boiling water for 3 mints, then put in cold ice. The amount of reducing sugars liberated was determined by the DNS method [30] using D-galactourinic acid (Sigma) as a standard.

One unit (1U) of enzyme activity is equal to the 1 µ Mol of reducing sugars released per ml per min, measured in terms of D-galacturonic acid, produced as a result of enzyme substrate reaction.

Protein determination

The protein concentration was determined in the enzyme solution according to [31]. Bovine serum albumin was used as a standard.

Screening of the most active *Streptomyces isolates* for polygalacturonase using different media

The most active isolates that showed clear zone (equal or more than ten mm) were screened for polygalacturonase production in submerged medium using the following different media.

1- Hankin medium, represented M1, [28] containing (g/l): KH_2PO_4 (anhydrous), 4.0 g, Na_2HPO_4 (anhydrous), 6.0g, Pectin (from apples), 5g, $(NH4)_2SO_4$, 2.0g, Yeast extract, 1.0g, $MgSO_4$. $7H_2O$, 0.0001g, $CaCl_2$ 0.001g, distilled water, 1000 ml. using pectin as a sole carbon source.
2- Pectin nitrate medium, represented M2, [27], containing (g/l): Pectin, 20g; KNO_3 2.0g; K_2HPO_4 (anhydrous), 1.0g; $MgSO_4$. $7H_2O$, 0.5g; NaCl, 0.5g; $CaCO_3$ 3.0g; $FeSO_4$. $7H_2O$, 0.01g; Distilled water 1000 ml.
3- Horikoshi medium, represented M3, [32], containing (g/l): glucose 5.0, peptone 5.0, yeast extract 5.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.1, pH 8.0 was used as a basal medium.
4- Modified Horikoshi medium, represented M4, containing (g/l): pectin 5.0, glucose 5.0, peptone 5.0, yeast extract 5.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.1, pH 8.0 was used as a basal medium.

Screening by using different industrial and agricultural wastes

Screening the most active *Streptomyces* isolates by using the best medium for polygalacturonase production for each isolate by using different industrial and agriculture wastes like lemon peel, orange peel,

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rice husk and wheat bran. Orange bagasse (Ob), lemon peels (Lp), Wheat bran (Wb) and rice husk (Rs) were obtained from local market in Egypt. Orange bagasse (Ob) and lemon peels (Lp) cut into small pieces (~2 mm), washed with tap water several times in order to remove all water soluble compound then dried and grinded.

RESULTS AND DISCUSSION

Isolation of *Streptomyces sp.*

The current study, isolation and screening process of pectinase producing *Streptomyces* was carried out from the agricultural soil gathered from different places of Egypt. Streptomycetes are usually round, convex shaped colonies, moreover their rooting growth into the medium. Based on these characteristics, 526 streptomycetes colonies were randomly chosen and isolated from different soil sources of Egypt on the basal starch nitrate medium. Starch nitrate agar medium was found to be very effective for the isolation of the actinomycetes in confirmation with the reports [1, 3, 4, 5, 33 and 34]. Results showed that El Sharkia showed the largest number of the isolates, (200 isolates) which represented 38%, followed by El Qalioubia, (120 isolates), which represented 23%, El Esmailia, (41 isolates), which represented 8%, Aswan, (50 isolates), which represented 9%, Luxor, 45 isolates, represented 9% and Assuit, (70 isolates), which represented 13% respectively, as shown in figure 1.

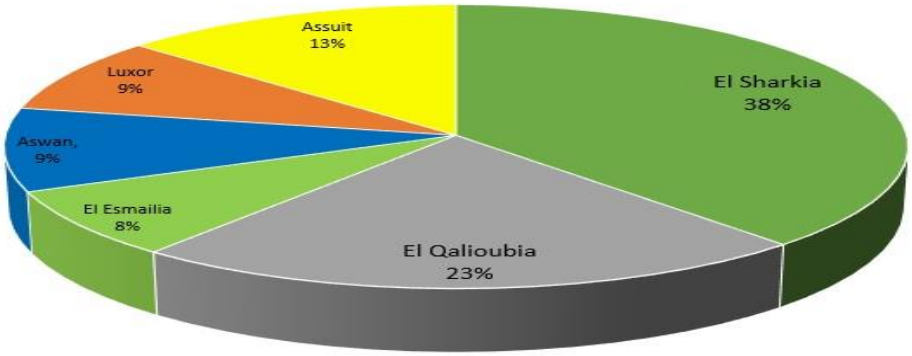

Figure 1: Total number of streptomycetes isolated from different soil sources of Egypt.

Preliminary screening of pectinase activity on solid plates

The *Streptomyces* isolates were screened for their ability to grow and produce pectinase using solid petri dishes. The results showed, that, the number of isolates which non produced pectinase were 293 isolates represented 56%. Two hundred thirty three isolates produced pectinase activity represented 44% as shown in figure 2 and 3. The selected isolates were checked out for their ability to hydrolyze pectin in the main screening step. The strains were spotted on to pectin-agar plates and four days after inoculation, when the plates were flooded with cetrimide solution (1%), clear zones appeared around effective colonies, indicating the usage of pectin. White coloring appeared in the left over area of the dish, this is because of reaction of cetrimide with the remained pectin present. The efficiency of cetrimide in finding pectinolytic activity is well established [1, 5 and 8].

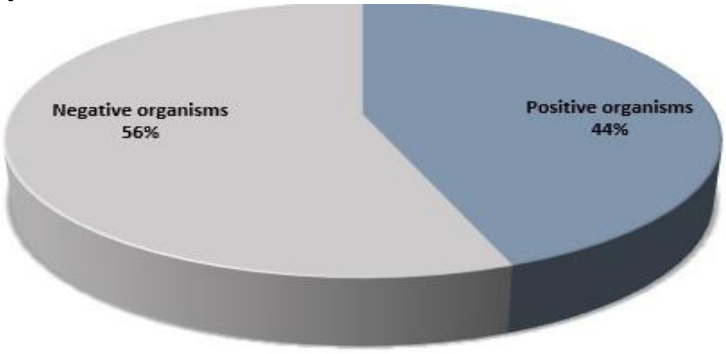

Figure 2: Survey of the isolated streptomycetes for pectinase production.

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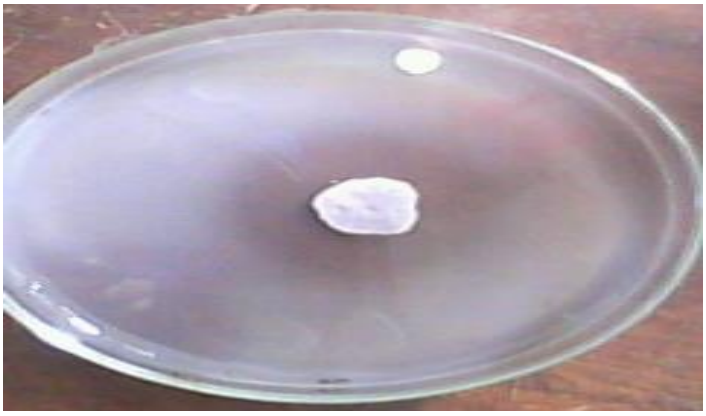

Figure 3: Photograph showing pectin clear zones of *Streptomyces sp.* pectin clear zone, in millimeter diameter.

In primary screening, 293 isolates did not give zone of clearance, represented negative group (56%) and 233 *Streptomyces* isolates (44%) showed zone of clearance as shown in figure 2. The positive isolates were classified as good and fair producers, selected on the basis of pectin clear zone in millimeter diameter for their pectinase activity. One hundred thirty three isolates were found to be good producers that have clear zone more than 10 mm in diameter represents 57.1%, other fair one contains 100 isolates that have clear zone diameter of less than 10 mm in diameter represents 42.9% as shown in figure 4.

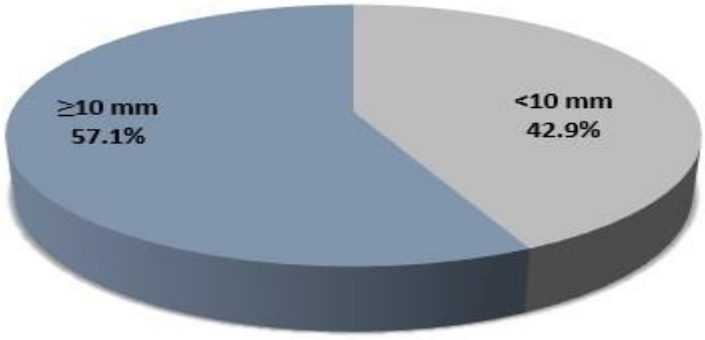

Figure 4: The pectinolytic *Streptomyces sp.* divided into two clusters, good and fair producers

Survey of the good group of streptomycetes isolates for polygalactouranase production in submerged medium

Since the present investigation was focused on good polygalacturonase producers, 133 isolates which showed dish clearance more than ten millimeter represented (57. 1%), were exposed to secondary screening process by submerged fermentation by using pectin synthetic medium [28]. The results showed that, among the 133 isolates, five isolates were more than 15U/mg specific activity, represents (4%), 27 isolates range from 10 to 15 U/mg, represents (20%), and 72 isolates range from 5 to 10 U/mg, represents (54%) and 29 isolates were up to 5 U/mg, represents (22%), as shown in figure 5.

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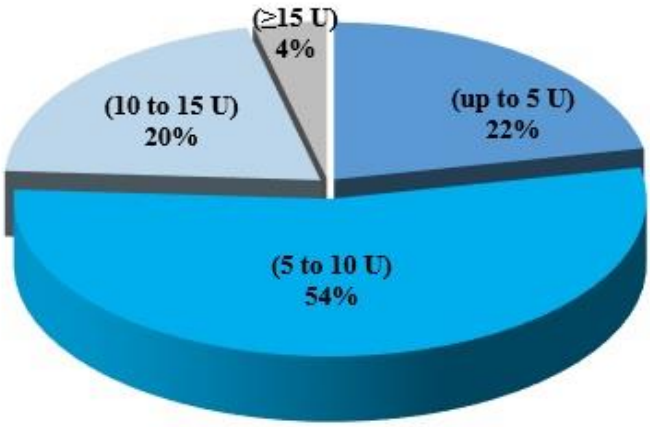

Figure 5: Survey of the good group of *Streptomyces* isolates for polygalacturonase production.

Screening of the most active *Streptomyces sp.* for polygalacturonase using different media

Screening of the most active five *Streptomyces* isolates (no. 2, 5, 8, 72 and 103) that have maximum polygalacturonase activity (≥15 U/mg) by using different media (M1, M2, M3 and M4).

The results showed that isolate no. 2, 5 and 103 provide the best results on pectin nitrate medium (M2) with pectin as only carbon source, while isolate no. 8 and 72 provide the best results on Hankin medium (M1) with pectin as only carbon source as shown in figure 6. Out of the four growth media processed, two have been reported for actinomycetes (Horikoshi medium and pectin nitrate). Decrease or increase of enzyme level production by using other media may be due to amount of carbon and nitrogen not well suited for production [1]. This was comparatively greater than pectinase from *Streptomyces sp. J9* [4], 1122 U/L and 923 U/L by using apple and orange pomace respectively, polygalacturonase from *Streptomyces lydicus* was18. 3 U/mL [1] and enzyme activity and enzyme activity from *Streptococcus sp. SB5* was 12.75 ± 0.12I U/ mg protein [35].

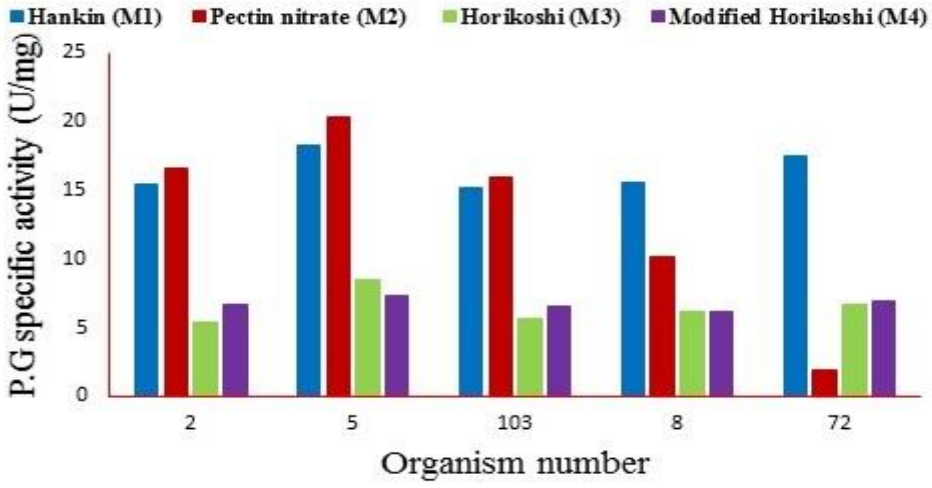

Figure 6: Screening of the most active five *Streptomyces* Isolates for polygalacturonase using different media.

Screening of the most active five isolates by using different industrial and agricultural wastes by using best medium for each isolate

The use of wastes as low cost residues, inexpensive, available very safe substrates in chemical production is especially interesting for countries where agro-industrial residues are abundant. This type of in turn helps to alleviate global warm in and environmental pollution. In this study we record, successful usage of agro-industrial waste products for polygalacturonase production.

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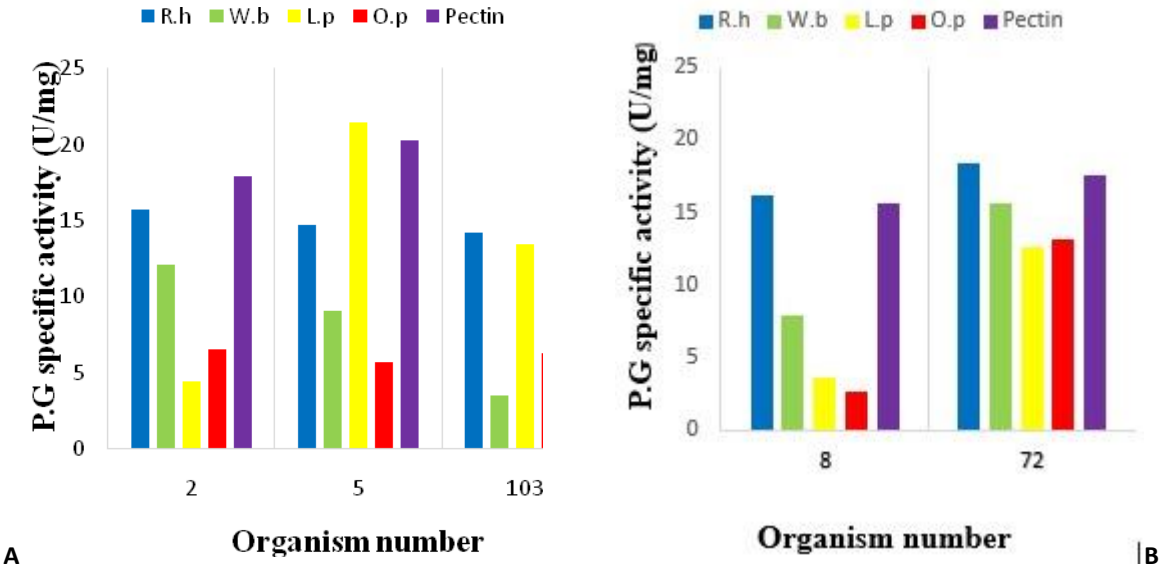

Figure 7: Screening for polygalacturonase using different agro-industrial wastes, (A) screening of isolates no. 2, 5 and 103 using M2 medium and (B) screening of isolates no. 8 and 72 using M1 medium.

From the results we find that M2 is the best medium for polygalacturonase by isolate no. 2, 5 and 103 using pectin as the sole carbon source as shown in figure 6. By screening using agro-industrial wastes like orange peel, lemon peel, rice husk, wheat bran instead of pectin in M2 medium for isolate (no. 2, 5 and 103), the isolate no. 5 give maximum enzyme activity by using lemon peel as the sole carbon source (21.5 U/mg) as compared to pectin (20.29 U/mg), while using rice husk, wheat bran and orange peel give lower enzyme activity (14.7, 9.1 and 5.7 U/mg respectively as compared to pectin (20.29 U/mg). In case of isolates no. 2 and 103 by using different agro-industrial wastes instead of pectin in M2 medium, enzyme activity lower by using all used agro-industrial wastes (lemon peel, orange peel rice husk and wheat bran) as compared to pectin as shown in figure 7(A).

In case of isolates (no. 8 and 72), we found that, M1 is the best medium for enzyme activity by using pectin as the sole carbon source as shown in figure 6. By screening of these isolates using agro-industrial wastes like orange peel, lemon peel, rice husk, wheat bran instead of pectin by using M1 medium, both isolates no. 8 and 72 give higher enzyme activity using rice husk 16.2 U/mg and 18.4 U/mg respectively as compared to pectin in medium 15.57 U/mg and 17.55 U/mg respectively, while using lemon peel, orange peel and wheat bran give lower enzyme activity as compared to pectin in medium as shown in figure 7(B).

Complex carbon sources such as agro-industrial wastes and pectin in fermentation medium have been reported as substrates that induce the pectinase activity in *Streptomyces sp.* This really reported by [19 and 36]. *Lentinus edodes*, also called Chinese mushroom, was successfully grown on apple pomace and other lignocellulosic wastes. Saadoun, *et al.,* 2013 also use agriculture waste for pectinase production [4]. Hours *et al.,* 1988 reported that, production of pectinase by *Aspergillus foetidus* from apple pomace fermentation [37]. Many researchers reported pectinase production using fruit processing industrial waste materials [38, 39 and 40].

CONCLUSION

The agriculture soil was seemed to be good source for isolation of pectinase producing *Streptomyces sp.* The results obtained in our study strongly support the suitability of *Streptomyces sp.* as good prospect for polygalacturonase production. We can produce enough amount of pectinase at a cheaper rate using agriculture wastes which will become a boon for our industries that are utilizing these enzymes for several processes. Further studies were made for taxonomical, identification of pectinase producing *Streptomyces sp.* and maximization for pectinase production.

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