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AN ECONOMICAL AND ENERGY SAVING APPROACH FOR AMYLASE PRODUCTION FROM *BACILLUS* SP. MTZ-1 BY SUBMERGED FERMENTATION UNDER STATIC CONDITIONS: APPLICATION AND ANTIMICROBIAL POTENTIAL OF CRUDE PREPARATIONS

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ABSTRACT

A *Bacillus* sp. MTZ-1 was examined for amylase production along with its substrate specificity, applications, pH, carbon source and NaCl concentration at different time intervals. Maximum enzyme activity was recorded at pH 7 after96 h of incubation while addition of 0.5% NaCl and 1% maltose increased the production of enzyme. Substrate specificity was maximum against malt starch followed by wheat starch, rice starch, corn starch and soluble starch, respectively. Crude enzyme showed fruit juice clarification when introduced into fresh apple juice to remove haze. Enzyme preparations showed antibacterial activity against Vancomycin-Resistant *Enterococci* (VRE), *Bacilus subtilis, Pseudomonas* sp., *Acinetobacter sp.,* Enteropathogenic *Escherichia coli* (EPEC), Methicillin-Resistant *S. aureus* (MRSA), *Klebsiella sp., Shigella sp., Staphylococcus epidermiditis, E. coli* and *S. aureus*. Antifungal activity of the crude enzyme also showed inhibition of many fungal pathogens including *Aspergillus niger, A. flavus, A. tereus, Penicillium sp., Rhizopus sp., Trichophyton tonsurans* and *Mucor* sp. by Agar well diffusion method.

Keywords: Amylase production; *Bacillus* sp. MTZ-1;Antimicrobial activity, crude enzyme preparations.

INTRODUCTION

Amylases (α-amylase, β-amylase, γ-amylase and glucoamylase) are cellular enzymes which are extraneous in nature and hydrolyze starch residues to form a variety of glucose polymers like dextrin and small macromolecules successively (Windish and Mhatre, 1995). Enzymes for industrial use have been synthesized from animal, plant and microbial sources, however, the use of enzymes from plants and animals is confined to small scale applications due to certain limitations. Plant and animal sources provide less enzyme yield which is not sufficient for various industrial procedures such as starch treatment due to scarcity or limitations in availability of plants and animal proteins (Saroja *et al*., 2000;Teodoro and Martin, 2000; Shiau and Hung, 2003; Sajedi *et al*., 2005; Sodhi *et al*., 2005; Ten *et al*., 2005; Sajitha *et al*., 2011; Mohammadabadi and Chaji, 2012; Ahmadi, 2012).Microbial enzymes have got significant place and value in various industries due to their ability to maintain pressure induced shape, chemical stability, cost effectiveness, wide availability, environmental security and easy production on large scale(Sajitha *et al*., 2011; Bakri *et al*., 2009).Amylases comprise approximately 30% of the world's enzyme yield with a broad range of amylase applications in industries like food (for baking),breweries (for fruit juice clarification), textile, paper, (for starch liquefaction) as well as in pharmaceutical and clinical grounds (Pandey, 2000).*Bacillus* species are considered as the most trusted and potent producers of different exo-acting cellular enzymes and amylases. Several *Bacillus* species which include *B. licheniformis, B. stearothermophilus, B. amyloliquefaciens,* and *B. subtilis* have been recorded to produce 60% commercial enzymes (Burhan *et al*., 2003). There are numerous advantages of amylase production by genus *Bacillus* such as, modifications can be done simply to obtain the enzyme of desirable properties, ability to secrete variety of proteins in the surrounding medium, safe to deal with, environment friendly, rapid fermentation process, easy and less expensive production, maximum enzyme production at high pH, osmolarity, temperature and pressure, etc. Due to these reasons, Bacillus has been used as potent bacterial machineries for producing wide range of enzymes and biological chemicals over a longer period of time (Demirkan, 2005).The purpose of this study was to carryout amylase production under static conditions in order to combat the problems faced by industries in developing countries like electricity failure, limited financial resources, malfunction in experimental devices and less availability of equipment etc. Studies were conducted to optimize the production of amylase through submerged fermentation by *Bacillus* sp. in terms of pH, temperature and incubation period (Vidyalakshmi *et al*., 2009; Singh *et al*., 2012). Solid state fermentation procedure was used along with submerged fermentation to optimize the production of amylase(Raj and Hemashenpagam, 2012)while amylase

production was also carried out in one of the studies using agro based industrial residues through shake flask fermentation (Abd-Elhalem *et al*., 2015).

MATERIALS AND METHODS

Screening of amylase producing bacteria

A *Bacillus* sp. MTZ-1 from the culture collection of Microbiology Research Laboratory, Department of Microbiology, Federal Urdu University of Arts, Science and Technology, Karachi, Pakistan was screened for the production of amylase through streaking on starch agar plates. The plates were then incubated at 37^0C for 24 h in incubator. After 24 h, 1% Iodine solution was flooded over the colonies to examine the appearance of clear zone of hydrolysis indicating a positive result (Parka and Son, 2007). The colonies of the strain were small, smooth, regular, convex and pinkish orange in color while bacterial cells were observed scattered in arrangement, rod-shaped and spore forming in microscopic examination.

Production of amylase by submerged fermentation in static condition

To cultivate *Bacillus* sp. MTZ-1, growth medium was prepared with the following composition: (g/L)Starch:10; peptone: 10; yeast extract: 20; KH_2PO_4:0.05; $MnCl_2.4H_2O$: 0.015; $MgSO_4.7H_2O$: 0.25; $CaCl_2.2H_2O$: 0.05; $FeSO_4.7H_2O$: 0.01(Rao *et al*., 2011) and 250 mL Erlenmeyer flask containing 150 mL growth medium was inoculated with a loop full of 24 h bacterial culture and placed in incubator under static conditions at 37^0C for 24 h. After incubation 10% of growth medium with *Bacillus* sp. MTZ-1 was aseptically transferred into 150ml of the production medium with same composition. The flask was kept in static condition at 37^0C in incubator followed by vigorous manual shaking at 24 h intervals. After incubation, fermented broth was centrifuged at 2000 rpm for 20 minutes. Broth was then filtered and pellet was discarded while cell free culture supernatant was used as source of crude enzyme. Amylase production was carried out in static conditions and effects of NaCl concentrations, pH, sugars and starch substrates on amylase production were demonstrated.

Enzyme assay

Modified spectrophotometric method of Fisher and Stein (1961) was used to detect amylase. Control tubes (C) containing 0.5 mL sample (crude enzyme filtrate); 0.5 mL starch solution (1%); 0.5 mL Tris buffer (0.2M) and 1 mL DNS (3,5-Dinitrosalicylic acid**)** were placed in water bath (Lab Companion BW-20H) at 35^0C for 1h along with sample test tubes (T) which contained similar contents as in control tubes except DNS. 1mL DNS was added in the sample test tubes other than control tubes after incubation. All the tubes were kept in boiling water bath for 5 minutes then cooled at room temperature. Optical density of each sample was recorded through spectrophotometer (JENWAY 6310 Spectrophotometer) at 540 nm wavelength. One enzyme unit was defined as the amount of enzyme required to hydrolyze 1µmol of maltose per ml (U/mL).

Effect of concentration of NaCl on amylase production

Effect of NaCl concentration on enzyme activity and production was observed by varying the concentrations of NaCl i.e. 0.2%, 0.5% and 1%in 150 mL of production medium.

Effect of initial pH on amylase production

Enzyme activity was checked at different initial pH i.e. pH: 6, 7, 8 in 150 mL of production medium by adjusting the pH through 0.2 M Tris buffer.

Effect of different sugars on amylase production

Different sugars (1%)i.e., glucose, maltose and fructose were added in 150 mL production medium to investigate their effect on amylase activity.

Substrate specificity

Ability of enzyme to hydrolyze different starch substrates like corn, rice, purified starch, wheat, malt and potato was also examined by the above mentioned procedure (Fisher and Stein, 1961)in which 1% of all above mentioned starch substrates were used.

Applications of crude enzyme filtrate

Antibacterial and antifungal activity of crude enzyme preparations

Antibacterial potential of crude enzyme preparations was checked against different bacterial pathogens like Vancomycin-Resistant *Enterococci* (VRE), *Bacillus subtilis, Pseudomonas sp., Acinetobacter sp.*, Enteropathogenic *E.coli* (EPEC), Methicillin-Resistant *S.aureus* (MRSA), *Klebsiella* sp., *Shigella* sp., *Staphylococcus epidermidis*, *Escherichia coli* and *Staphylococcus aureus*. Antifungal activity of the crude enzyme filtrate was also inspected against many fungal pathogens including *Aspergillus niger, Aspergillus flavus, Aspergillus tereus, Penicillium* sp., *Trichophyton tonsurans* and *Mucor* sp. by agar well diffusion assays (Jahangirian *et al*., 2013). All the above mentioned organisms were inoculated on their respective agar plates and after 24h incubation, 100 µL cell free supernatant was dispensed in agar wells (diameter 6mm) under aseptic conditions. For antibacterial activity, bacterial suspensions with turbidity of 0.5 McFarland (1.5×10^8 colony-forming units (CFU/mL) were used for making growth lawn on agar plates. Plates were then incubated at 37°C for 24 h after which zones of growth inhibition were measured.

Apple juice clarification

Clarification of apple juice by crude amylase has been reported in several studies (Dey and Banerjee, 2014; Sorrivas, 2006).Clarification effect of enzyme was determined on apple juice by mixing 0.5 ml crude enzyme filtrate with 5 mL of fresh apple juice in test tube marked as 'T' while 0.5 mL distilled water was used in place of crude enzyme filtrate in control test tube marked as 'C' followed by vigorous shaking. Both the tubes were left for 30 min in water bath at 37^{O}C and changes in the tubes were observed after every 5 min. Tubes were then left for overnight incubation at room temperature (Madden, 2000).

RESULTS AND DISCUSSION

Amylases are the centre of focus in industrial sector as they can be acquired from microorganisms instead of other expensive sources and its productivity can easily be increased by optimizing temperature, pH, incubation period and carbon sources, etc. Usually amylase production has been carried out by Shake flask fermentation (Joseph and Charles, 2013; Deb *et al*., 2013)or solid state fermentation experiments(Akcan *et al*., 2012; Raul *et al*., 2014) while in the present study, it is conducted by Submerged fermentation under static condition to combat energy crises like short fall of electricity, shortage or lack of advanced equipment, etc.

Screening of amylase producing bacteria

Bacillus sp. MTZ-1 showed prominent zone of starch hydrolysis after flooding 1% Iodine solution (Fig. 1).

Effect of concentration of NaCl on amylase production

Among all three NaCl concentrations (0.2, 0.5 and 1%), maximum enzyme activity was detected in 0.5% NaCl at 24 h (1720 U/mL / min) while minimum activity was observed at 1% after 168 h (350 U/mL), moreover, considerable enzyme activities were also recorded with 0.25 and 1% NaCl (Fig. 2). NaCl tolerance by the strain up to 1% makes it suitable to be used in detergent formulations which are of high salt concentrations. Moreover, amylase production with 1% NaCl would also decrease the risk of contamination. Studies have been conducted on greater amylase production under high NaCl concentrations by different halo-tolerant Bacillus species (Rao*et al*., 2011; Sarethy *et al*., 2012; Chovatiya *et al*., 2014).

Effect of pH on amylase production

When the effect of initial pH on amylase production was checked, the highest enzyme activity was observed at pH 7(after 96 h i.e. 1250 U/mL/min) which was in accordance with the results of some previous studies (Rao *et al*., 2011; Nusrat and Rahman, 2007; Sankaralingam *et al*., 2012; Alariya *et al*., 2013) while at 24 h, the activity was minimum (320 U/mL) at the same pH. This might be due to the increase in bacterial cells during exponential phase at 24 h or any metabolite production interfering with enzyme activity, while this reduction was overcome at 96 h, followed by a slight decrease at 168 h which could be due to the accumulation of some waste products or limitation of nutrients in production medium. The same suggests the neutral nature of the enzyme as its synthesis was maximum at pH 7. Moreover, the enzyme activity was also cogent at pH 6 and 8 (Fig. 3).

Effect of different sugars on amylase production

Analysis of amylase production with different sugars showed maximum enzyme activity in the presence of maltose (320 U/mL) while it was minimum in case of fructose i.e. 70 U/mL (Fig. 4). Contrary to this, maximum enzyme activity was also observed in previously conducted study in the presence of maltose and fructose by plant growth promoting Rhizobacteria (Karnwal, 2011). Sugars like glucose and maltose stimulate the production of α-

amylase as it is an inductive enzyme (Morkeberg, 1995). Enzyme production was not detected during the presence of glucose which may be due to catabolic repression because in the presence of glucose, the strain might be utilizing it as a carbon source instead of enzyme production for utilization of starch which was in accordance with some other antecedent studies (Nusrat and Rahman, 2007; Afunasyeva *et al*., 1978).Catabolic repression occurs in *Bacillus sp.* due to the presence of high glucose concentration and different readily processed substrates (Nihashi and Fujita, 1984).

Fig. 1. Zone of starch hydrolysis by the amylase producing strain *Bacillus* sp. MTZ-1.

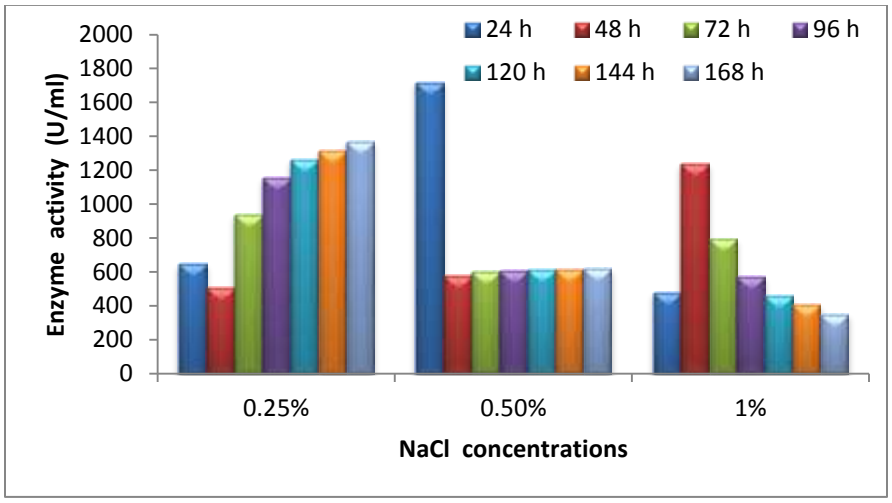

Fig. 2. Effect of NaCl concentrations on enzyme activity.

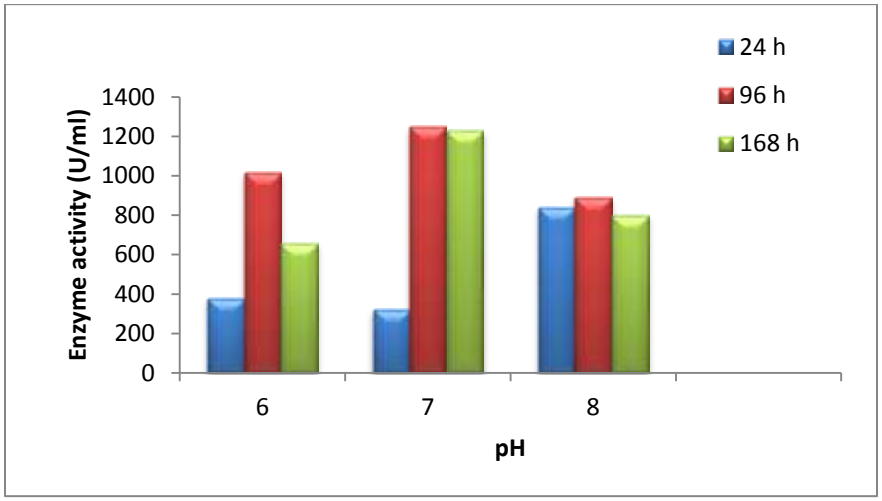

Fig. 3. Effect of pH on enzyme activity.

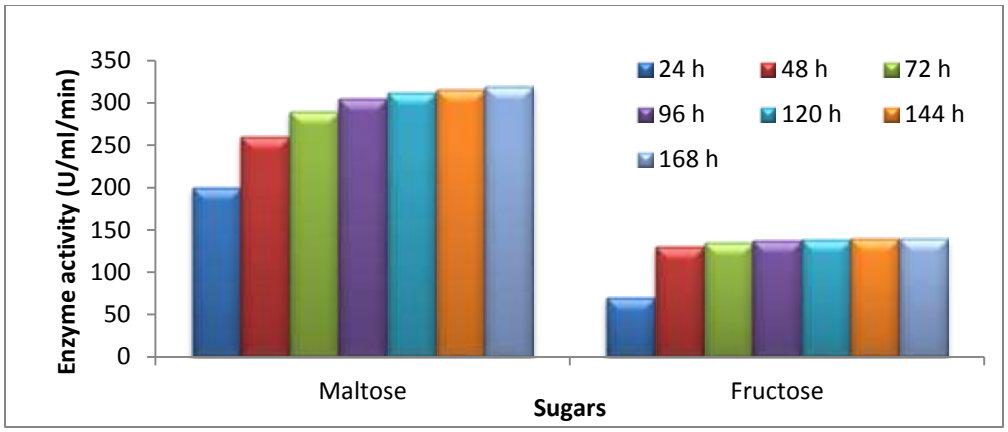

Fig. 4. Effect of carbon source on enzyme activity.

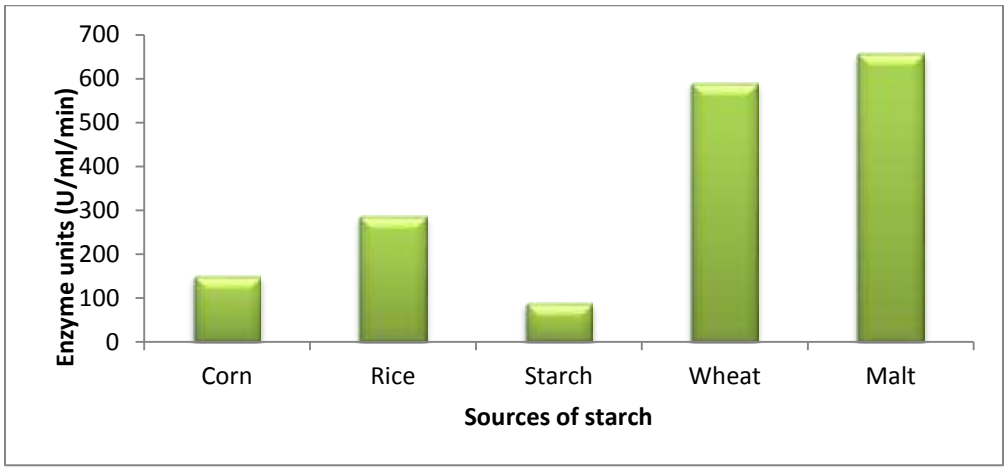

Fig. 5. Effect of different starch sources on enzyme activity.

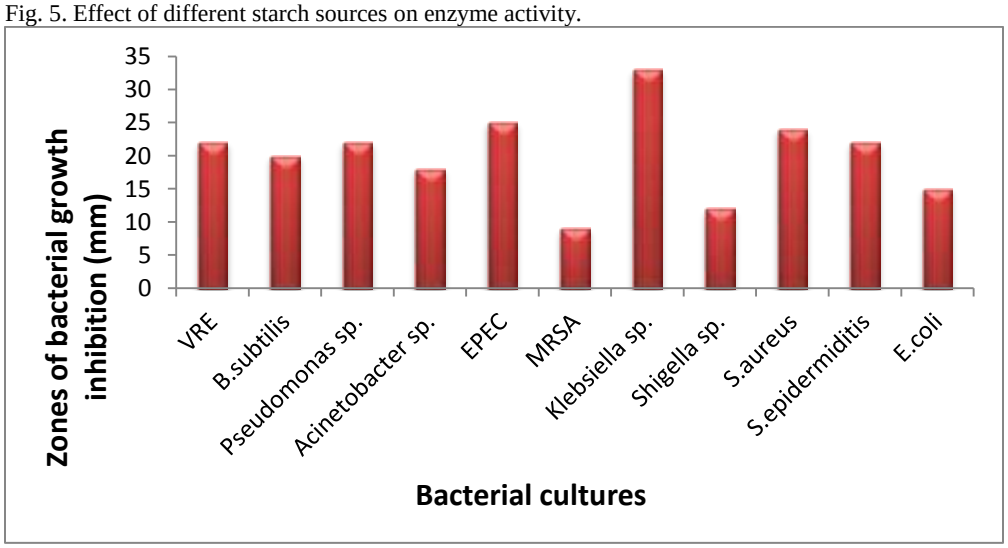

Fig. 6. Bacterialgrowth inhibition zones (mm).

Substrate specificity

Effect of different starch substrates was examined to study the substrate specificity of crude enzyme filtrate. The highest hydrolytic activity was recorded in the presence of malt starch (658 U/mL) while the enzyme was also capable to carry out the hydrolysis of corn, rice, wheat and purified starch except potato starch. According to these results, enzyme showed greater specificity with malt while it did not show specificity with potato starch (Fig. 5).

Hydrolysis of these starch substrates make this enzyme suitable to be used in industries (food, textile and paper industries) where starch hydrolysis is required during different treatment processes.

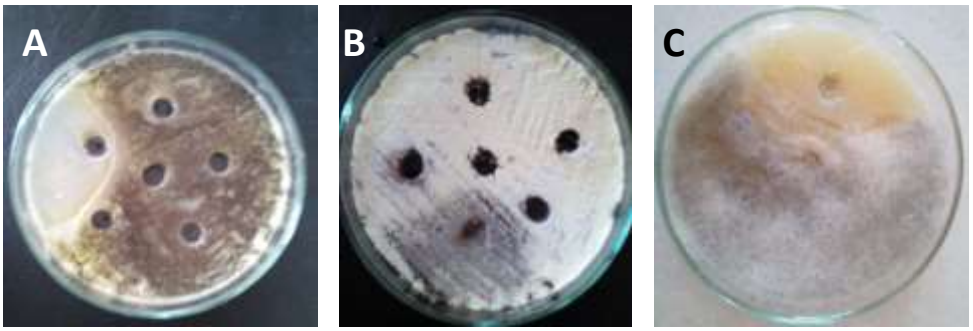

Fig. 7. **(**A) (B) and (C) zones of antifungal activity of crude enzyme preparations against *Aspergillus niger*, *Trichophyton tonsurans* and *Mucor* sp., respectively.

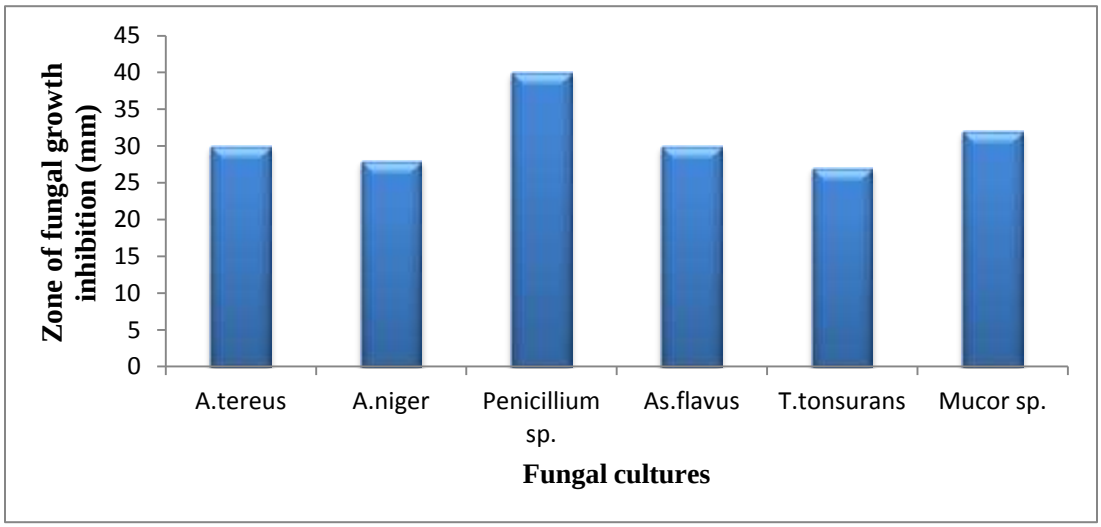

Fig. 8. Fungal growth inhibition zone sizes with respect to crude enzyme preparations.

Fig. 9.Apple juice clarification after 24 h.
Test tubes "**C**" and "**T**" representing **"control"** and **"test"** samples.

Applications of crude enzyme

Antibacterial and antifungal activity of crude enzyme filtrate

Inhibitory effect of the crude enzyme filtrate was observed against various pathogenic bacteria such as Vancomycin-Resistant *Enterococci* (VRE), *B. subtilis, Pseudomonas* sp., *Acinetobacter* sp., Enteropathogenic *E. coli* (EPEC), Methicillin-Resistant *S. aureus* (MRSA), *Klebsiella* sp., *Shigella* sp., *S. epidermiditis, E. coli* and *S. aureus*. Maximum inhibitory effect was recorded against *Klebsiella* sp. (33 mm) while minimum against Methicillin-Resistant *S. aureus* (MRSA) i.e. 9 mm. Growth inhibition zones (mm) against all the above mentioned bacterial strains have been displayed in the graph (Fig. 6). Cell free supernatant also showed antifungal activity against some fungal strains like *A. niger, A. flavus, Penicillium* sp., *A. tereus, T. tonsurans,* and *Mucor* sp. (Fig. 7). Among all fungal strains, inhibitory effect against *Penicillium* sp. was the maximum (40 mm) while it was the minimum against *T. tonsurans* (27 mm) (Fig. 8).

Apple juice clarification

Apple juice was clarified after 24 h (Fig. 9) which showed that hazy fruit juices can be clarified by the enzyme which is less harmful as compared to other fruit juice clarifying agents and chemicals. Starch hydrolyzing enzymes (e.g. amylases and amyloglucosidase) remove the pectin residues and break starch into minuscule residues to promote post bottling haziness by accumulating with each other or by making starch-protein complexes (Stocke, 1998). Moreover, by using enzyme formulations taste of fruit juice is not or least affected. Furthermore, due to its antibacterial and antifungal potential it can also be used as preservative or stabilizer of food stuff in food industry where biological food stabilizers are in great demand to prolong the shelf life.

CONCLUSION

Hydrolytic enzymes can be acquired in bulk with less expenses and convenient production processes. Production of amylase in current research has been conducted by submerged fermentation under static conditions to infer the possibility of amylase production without mechanical shaking or agitation minimizing the cost of process. The *Bacillus sp.* MTZ-1 proved to be a potent candidate for amylase production, and amylase produced by this strain can be further purified and characterized for its effective utilization in various industries.

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