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Enzyme activities of the marine-derived fungus *Alternaria alternata* cultivated on selected agricultural wastes

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Abstract

Studies were carried out on the enzyme activities of the marine- derived fungus *Alternaria alternata* cultivated in various agro-wastes. The fungus produced cellulase, xylanase, pectinase and laccase by both solid-stat fermentation (SSF) and submerged fermentation (SMF) techniques on different agricultural wastes. The level of cellulase, xylanase, pectinase and laccase production by SSF (9.68, 24.84, 52.5 and 1126.62 U/g solid substrate, respectively) on wheat bran after 10 days of incubation, which considered higher than that obtained by SMF(2.61, 16.31, 35.28 and 531.94 U/g solid substrate, respectively). The highest extraction yield of cellulase and xylanase was achieved with distilled water while the highest pectinase and laccase extraction was obtained with acetate buffer (0.05 M, pH 5). Therefore, the high enzyme production along with the very low cost of the substrate, showed the suitability of the system *A. alternata*-SSF for industrial purposes, especially for the use of these enzymes in biotechnological processes in high salt concentration conditions.

Keywords: Marine fungi, lignocellulolytic enzymes, cellulase, xylanase, pectinase, laccase, agricultural wastes, *A. alternata*.

INTRODUCTION

Historical Background

Marine-derived fungi are able to produce biologically active secondary metabolites different from those produced by their terrestrial counterparts because they are adapted to the salinity found in marine environments [1] and [2]. However, only recently this group of microorganisms has attracted attention as potential source of new generation of natural products and in biodegradation process [3]. In this context, the study of extracellular enzymes production by these microorganisms is very important in applied biotechnology. Among the extracellular enzymes produced by filamentous fungi the lignocellulytic system is of great significance in environmental remediation.

Plant cell walls are the most abundant renewable source of fermentable sugars on earth [4] and [5] and are the major reservoir of fixed carbon in nature [6]. The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component [7]. Plant biomass comprises on average 23% lignin, 40% cellulose and 33% hemicellulose dry weight [8].

Lignocellulosic wastes are the largest group of wastes present on this plant causing environmental pollution [9]. Various agro-industrial wastes such as wheat bran [10], sugarcane bagasse [11] and [12], coffee pulp [13], lemon peel [14] and apple pomace [15] have been explored for the microbial production of pectinase.

SSF is a technique that has been well know for centuries [16].The technique is defined as any fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients (Minerals, Vitamins, ect.) in absence of free flowing liquid [17]. SSF offers numerous advantages for the production of bulk chemicals and enzymes [18], [19] and [20]. In the field of production, several natural solids have been successfully employed: e.g., wheat, corn, rice, sugar cane and beet, banana waste, potato, tea, coccus, apple and citrus fruits, wheat flours and corn [19]. The advantages of (SSF) in comparison to traditional submerged fermentation (SMF) techniques are better yields, easier recovery of products, the absence of foam formation and smaller reactor volumes. Moreover, contamination risks are significantly reduced due to the low water contents and, consequently, the volume of effluents decreases [21]. It has been shown that for some specific processes, particularly enzyme production, the costs of these techniques are lower and the production higher than SMF [22].

Marine-derived fungi are a potential for the search of new compounds with relevant features. Among these, the lignocellulytic enzymes have potential applications in a large number of fields, including the environmental and industrial sectors. This work aimed to evaluate the enzymatic activities of the marine-derived fungus *A. alternata* under different fermentation conditions**.**

MATERIALS AND METHODS

Chemicals

2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diamm- onium salt (ABTS) was obtained from MP Biomedicals,LLC.29525 Fountain PKwy, Solon, OH 44139 (USA).

Organism and culture conditions

The filamentous marine-derived fungus *A. alternata* used in this study was isolated from decayed wood pieces of old fishing boats from Ismalia, Egypt. Identified in the National Research Centre, Chemistry of Natural and Microbial Products Dept. [Microbial Culture Collection Unit (MCCU)] according to [23]. Stock cultures of the fungus was maintained on malt extract agar slants (biomalt 20g/l, agar15g/l, 800 ml sterile sea water and 200 ml distilled water) [24] at 4°C and periodically subcultured.

Corn cobs, wheat bran, wheat straw, rice straw and saw dust were obtained locally and used as fermentation substrates. 3g. of the solid substrates were moistened with 15 ml of sea water in SSF, But with 50 ml of sea water under shaking conditions 150 rpm in SMF. Then autoclaved for 20 min at 121 ºC and inoculated aseptically with 1ml mycelial suspension (inoculum). Incubation was carried out at 25-28 ºC in 250 ml Erlenmeyer flasks for up to 21 days.

Enzyme extraction

The culture broth from submerged cultivation was centrifuged at 5,000 rpm for 15 min and the supernatants were used for enzyme assays. In the case of solid state fermentation, the solid cultures were suspended in 50 ml of distilled water and placed on a shaker for 1h. The suspension was filtered through a nylon cloth, and then centrifuges at 5,000 rpm for 15 min. The filtrates obtained were used for determination of enzyme activities [25].

Effect of extraction method on the activity of the selected enzymes.

Distilled water, acetate buffer (0.5 M) and non-ionic surfactant were used for separation and recovery of the enzymes from the heterogeneous solid-liquid fermented slurries.

Assay for enzyme activities

CMC ase activity

This was done according to the method of Mandels and Weber [26]. 0.5 ml of diluted enzyme solution was added to 0.5 ml of 1% carboxymethylcellulose (CMC) in 0.05 M acetae buffer (pH 5.00). Incubation of the reaction mixture was performed for 30 min at 50°C. The released reducing sugars were determined by the method of [27].

Xylanase activity

This was done according to the method of Warzywoda [28]. 0.5 ml of diluted enzyme solution was added to 0.5 ml of 1% xylan in 0.05 M acetae buffer (pH 5.00). Incubation of the reaction mixture was performed for 30 min at 50°C. The released reducing sugars were determined by the method of [27] as xylose.

Pectinase activity

This was performed according to the method of Silva [29]. Enzyme activity was evaluated by mixing 0.2 ml enzymatic extract and 0.8 ml of citrus pectin (0.5 % pectin in 0.05 M acetate buffer, pH 5.0). Samples were incubated at 50°C for 10 min and the reducing sugars were determined by Somogyi method [30]. One unit of exo - PG activity was defined as the amount of enzyme releasing 1 µ mol of reducing sugars (as galacturonic acid) per min per ml of enzyme solution.

Laccase (Lac)

Laccase activity was measured by using the method described by [31] based on the oxidation of the substrate 2,2'-azino–bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS). The rate of ABTS oxidation was determined spectrophotometrically at 420 nm.

The reaction mixture contained 600 µL sodium acetate buffer (0.1 M, pH 5.0 at 27 ºC), 300 µL ABTS (5 mM), 300 µL mycelial liquid fraction and 1400 µL distilled water. The mixture was then incubated for 2 min at 30 ºC. The absorbance was measured immediately in one-minute intervals. One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 mole of ABTS (ε_{420}= 36,000 M^{-1} cm^{-1}) per minute.

The blank contained all the assay constituents except the active enzyme; heated inactivated enzyme was used in its place.

RESULTS AND DISCUSSION

Effect of substrate type on enzyme activity in SSF and SMF

The results obtained from these studies indicated that the marine-derived fungus *A. alternata* isolated from decayed wood samples able to produce the following enzymes CMCase, Xylanase, Pectinase and Laccase, these results agree with that reported by [32] who indicated the presence of laccase, cellulase and xylanase activities in several facultative and obligate marine fungi isolated from mangrove and sea grass leaves and sediments from mangrove stands.

The activity of extracellular hydrolytic and oxidative enzymes greatly depended on the fermentation technique, vegetal substrate type and time of incubation. However, the CMCase, Xylanase and Pectinase productions by SSF were better when wheat bran was used as substrate after 10 days of incubation Figure 2, 6 and 11 and this concedes with that found by [25] for the CMCase, Xylanase and Pectinase productions by *Trichoderma reesei* and *Trichoderma viridae* by SSF and SMF fermentation.

A. alternata gave the highest production of CMCase 9.68 U/g of wheat bran by SSF Figure 2, compared to 2.61 U/g of corn cobs Figure 1 and with lesser efficiency on wheat straw and rice straw Figure 3 and 4, respectively, after 10 days of incubation which was nearly similar to that obtained by *Asperigllus awamori* (9.6 U/g) on grape pomace [33]. A previous study described in the literature shows that different agricultural wastes containing lignin inhibit cellulase activity on cellulose [34]. This observation could also explain the lower values for the enzyme studied here. Also [35] reported the CMCase production by *Penicillium decumbens* on a mixture of corn straw and wheat bran (9.05 U/g).

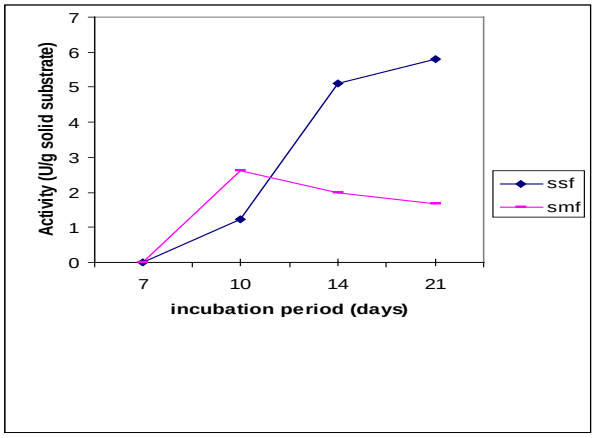

Fig 1. Production of cellulase by *Alternaria alternata* on corn cobs by ssf and smf

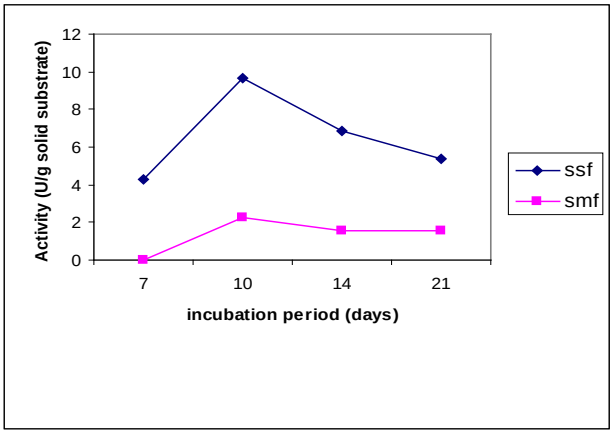

Fig 2. Production of cellulase by *Alternaria alternata* on wheat bran by ssf and smf.

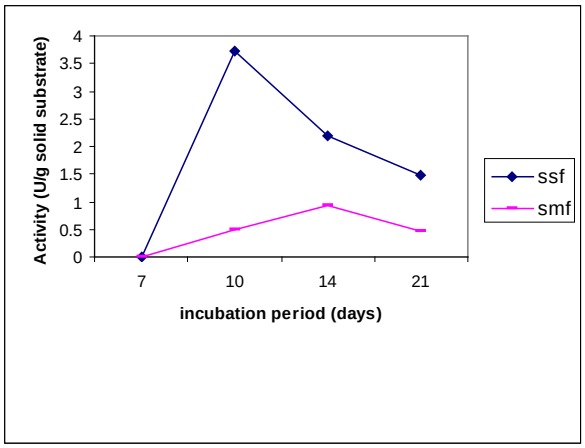

Fig 3. Production of cellulase by *Alternaria alternata* on wheat straw by ssf and smf.

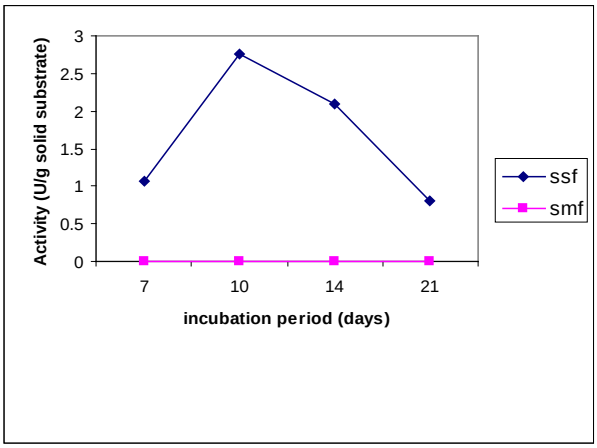

Fig 4. Production of cellulase by *Alternaria alternata* on rice straw by ssf and smf.

Table 1. Effect of extraction method on the activity of the selected enzymes from solid state fermentation of wheat bran.

Methods of extraction	Enzyme activities (U/gm solid substrate)			
	CMCase	xylanase	Pectinase	Laccase
Distilled Water	9.86	24.84	52.5	1126.62
Acetate buffer (0.5M-pH5)	6.04	23.66	97.08	1721.43
non-ionic surfactant Tween 80 (1%)	8.81	19.86	95.83	824.82

Figures 5,6,7,8 and 9 showed, the highest activity of xylanase obtained by SSF using wheat bran as solid substrate (24.84 U/g) after 10 days of incubation, which was higher than that reported by [35] when used a mixture of corn straw and wheat bran (13.59 U/g) of xylanase. Also [36] studied the production of xylanase by *Aspergillus niger* using wheat bran as the solid substrate. In the same line, the production of xylan – degrading enzymes by a Koji mold. *Aspergillus oryzae* RIB 128, has been tested on dried wheat bran, rice bran and orange beel [37]. *A. alternata* produced xylanase by SMF but with lesser degree (16.31 U/g) on wheat straw after 21 days of incubation Figure 7.

From Figures 10, 11, 12 and 13 the highest production of Pectinase (exo-poly galacturonase) 52.5 U/g was obtained on wheat bran by SSF, which was higher than that obtained by *Aspergillus awamori* (25 U/g) on grape pomace [33] and on wheat (20 – 25 U/g) reported by [38]. *A. alternata* produced pectinase by SMF but with lesser degree (35.28 U/g) of wheat bran.

This fungus was found to be able to produce laccase by SSF (1126.62 U/g) and SMF (531.83 U/g) especially on wheat bran Figure 15, but with lesser degree on other agricultural wastes used Figures 14, 16 and 17.

A. alternata not able to produce neither laccase nor cellulase on saw dust by both SSF and SMF even after 21 days of incubation while, [39] reported the production of laccase, xylanase and cellulase by *Ceriporiopsis subvermispora* on wood chips under SSF. And [40] recorded significant delignification of spruce wood shavings after 3 days treatment with *Ceriporiopsis subvermispora* and *lloPhlebia tremesa*.

It was also observed that our fungus did not produce laccase on rice straw by SMF. In contrast, Belal and El-Mahrouk [41] reported the production of laccase by *Phanerochaete chrysosporium* on rice straw.

A. alternata a marine fungus was able to produce lignocellulose-degrading enzymes (cellulase, xylanase, pectinase and laccase) as that produced by marine fungi isolated from salt marsh grass [42]. Also Rohrmann and Molitoris [43] have reported the presence of laccase in addition to the above enzymes in marine fungi isolated from algae.

A. alternata produced higher levels of the lignocellulose-degrading enzymes (cellulase, xylanase, pectinase and laccase) on wheat bran by SSF than by SMF, Widespread suitability of wheat bran may be due to the presence of sufficient nutrients and its ability to remain loose even in moist conditions thus providing a large surface area [44],and this concedes with that reported by Patil and Dayanand [45] who recorded the maximum production of exo-Pectinase (45.9 U/g) by *Aspergillus niger* DMF 45 in SSF when compared to 30.3 U/g by DMF 27 in SMF. This also agreed with that reported by [46] for laccase production by *Aspergillus niger*. And [25] found that *Trichoderma reesei* and *Trichoderma viride* produced higher cellulolytic, xylanolytic and pectinolytic activities when grown in SSF than in SMF.

In contrast to that reported by [47] who detected all these enzymes (CMC, xylanase, pectinase and laccase) production by *Coniochaeta ligniaria* NRRL 30616 but at higher levels in SMF than in SSF.

Effect of different leaching agents on extraction of extracellular hydrolases and laccase from fermented slurry in solid – state cultivations using wheat bran as substrate.

One of the major difficulties in SSF is the extraction or leaching of enzyme from fermented materials, which is critical in determining the economic feasibility of enzyme production [48]. In this study, distilled water, acetate buffer (0.5 M) and non-ionic surfactant were used for separation and recovery of enzymes from the heterogeneous solid-liquid fermented slurries. As shown in Table 1 that the recovery of CMCase and xylanase were better with distilled water than with other extraction agents. While the pectinase and laccase extraction was higher with acetate buffer where the activity increased about 1.85 and 1.53 fold, respectively compared to the control. This finding is in contrast with other studies [25].

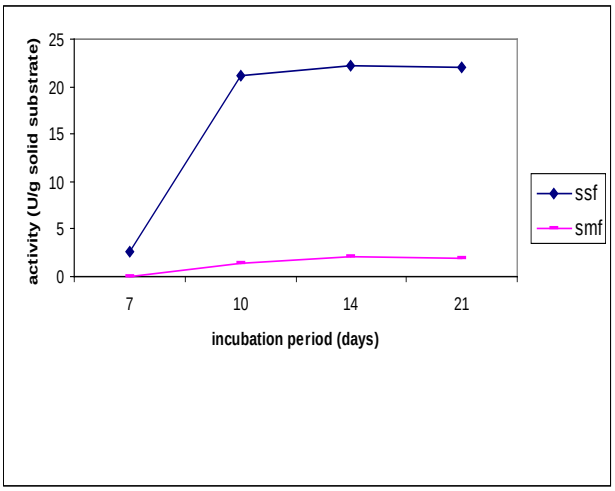

Fig 5. Production of xylanase by *Alternaria alternata* on corn cobs by ssf and smf.

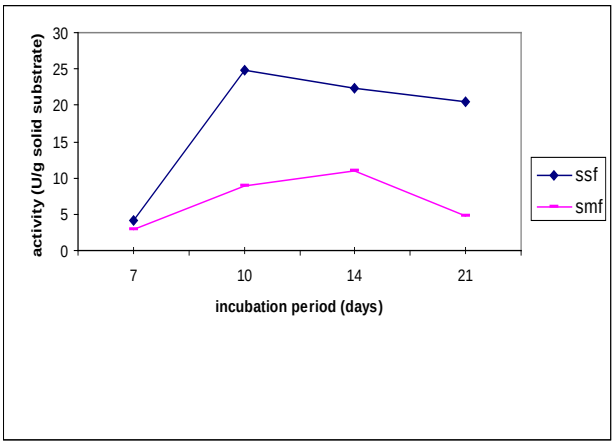

Fig 6. Production of xylanase by *Alternaria alternataon* wheat bran by ssf and smf.

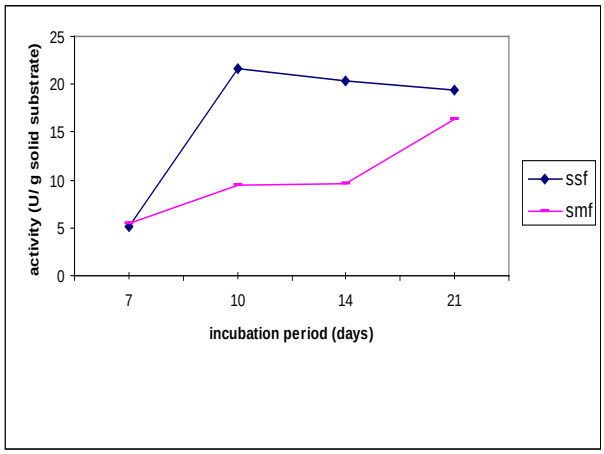

Fig 7. Production of xylanase by *Alternaria alternata* on wheat straw by ssf and smf.

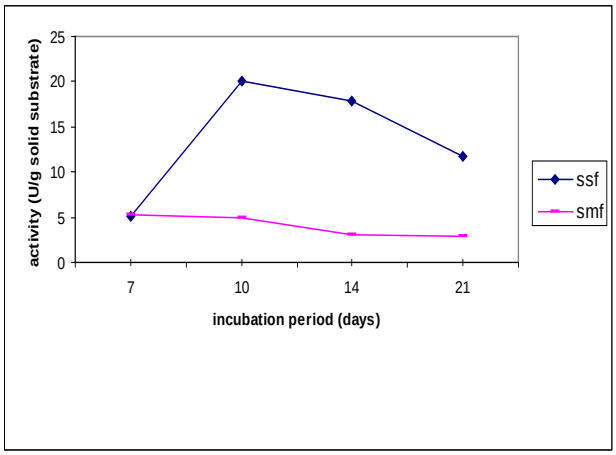

Fig 8. Production of xylanase by *Alternaria alternataon* rice straw by ssf and smf.

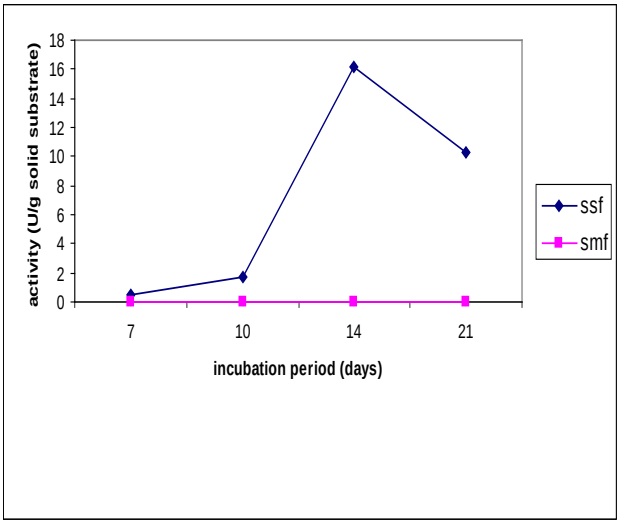

Fig 9. Production of xylanase by *Alternaria alternata* on saw dust by ssf and smf.

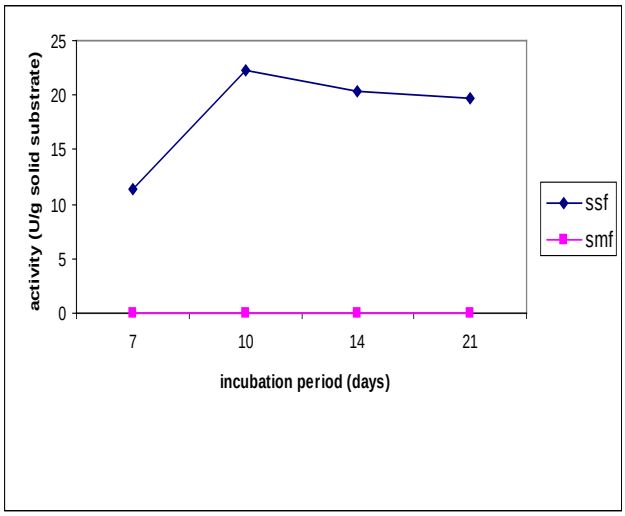

Fig 10. Production of pectinase by *Alternaria alternata* on corn cobs by ssf and smf.

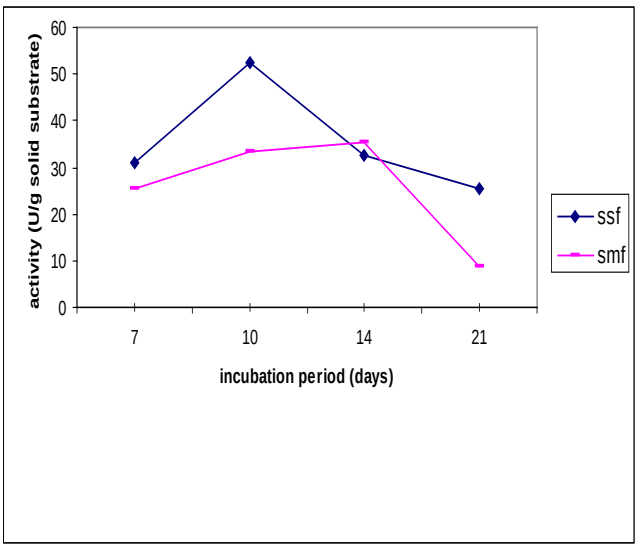

Fig 11. Production of pectinase by *Alternaria alternata* on wheat bran by ssf and smf

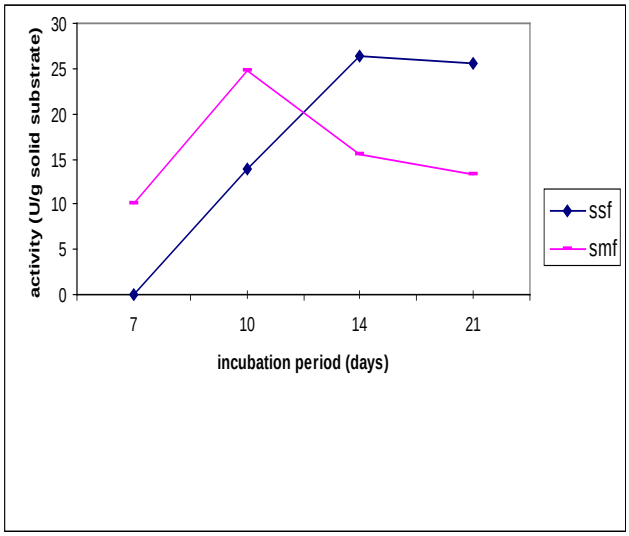

Fig 12. Production of pectinase by *Alternaria alternata* on wheat straw by ssf and smf.

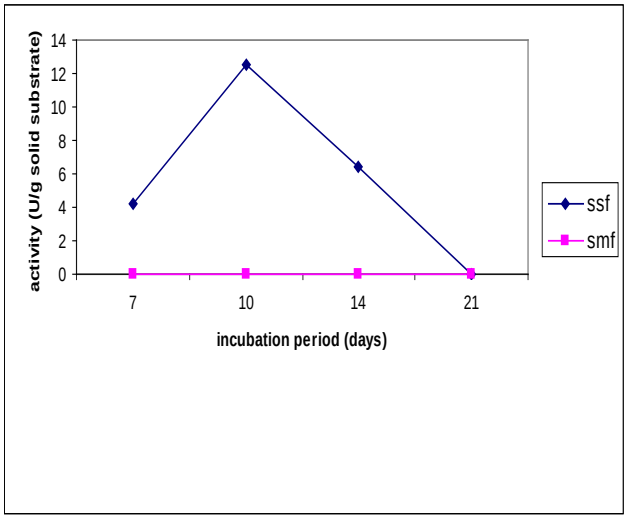

Fig 13. Production of pectinase by *Alternaria alternata* on rice straw by ssf and smf

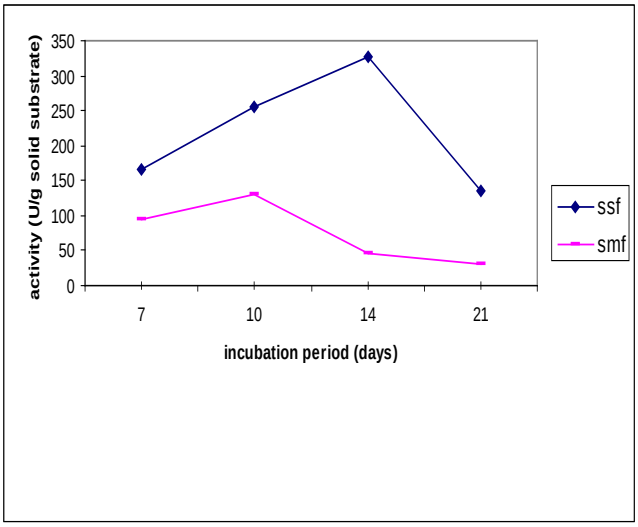

Fig 14. Production of laccase by *Alternaria alternata* on corn cobs by ssf and smf.

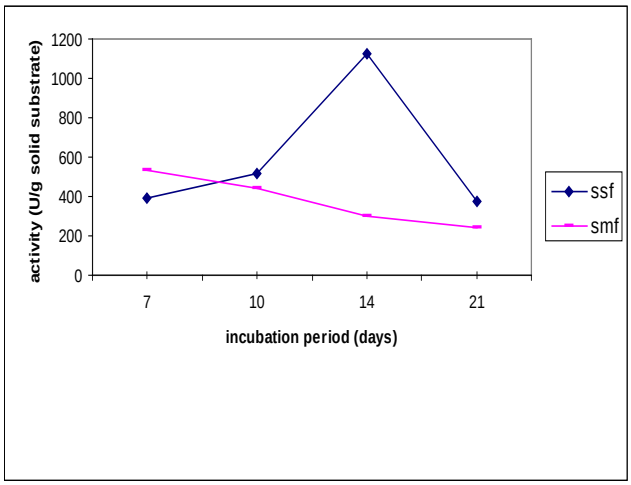

Fig 15. Production of laccase by *Alternaria alternata* on wheat bran by ssf and smf.

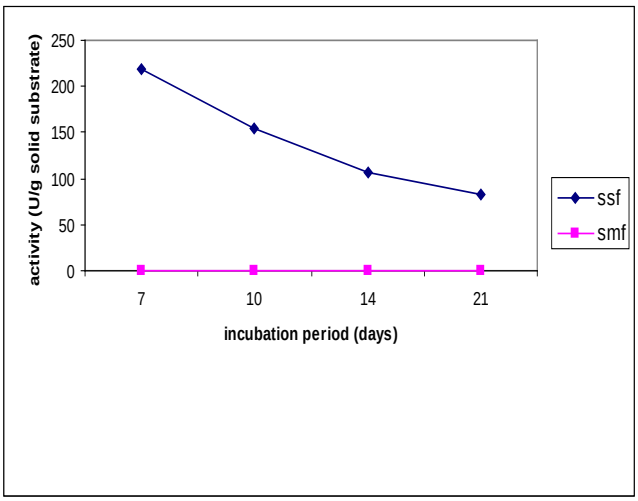

Fig 16. Production of laccase by *Alternaria alternata* on wheat straw by ssf and smf.

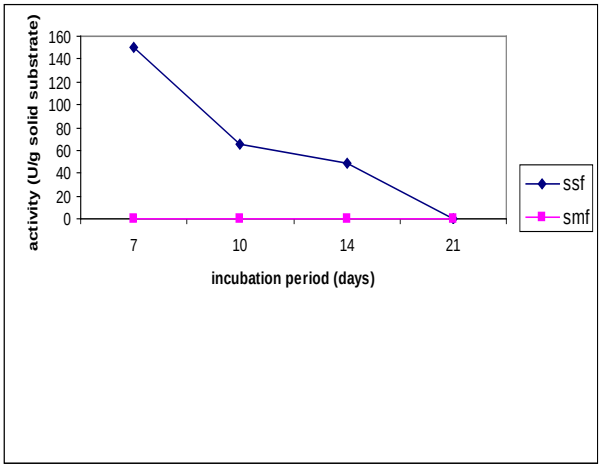

Fig 17. Production of laccase by *Alternaria alternata* on rice straw by ssf and smf.

CONCLUSION

The marine-derived fungus *A. alternata* isolated from decayed wood samples able to produce the following enzymes CMCase, Xylanase, Pectinase and Laccase by solid-stat fermentation (SSF) of the agriculture waste wheat bran higher than the other tested wastes. The highest extraction yield of cellulase and xylanase was achieved with distilled water while the highest pectinase and laccase extraction was obtained with acetate buffer (0.05 M, pH 5).

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