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A Thermostable and Alkalitolerant Arabinofuranosidase by *Streptomyces lividus*

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Authors' contributions

This work was carried out in collaboration among all authors. Author AAO designed the study. Author OA performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author FCA managed the analyses, proofread and effected necessary corrections and managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: The study aimed at producing and purifying thermostable and alkalitolerant microbial arabinofuranosidase using local Palm Kernel Cake (PKC) as substrate.
Study Design: This is an experimental design in which samples were collected thrice and subjected to laboratory analyses from which quantitative data were obtained and analysed.
Place and Duration of Study: Ibadan, Nigeria, Five months.
Methodology: Bacterial strains were isolated from degrading PKC by serial dilution and pour plate technique on formulated Modified Basal Salt Agar Medium and incubated at 50°C for enzyme activity screening. Plates were afterwards flooded with 1% congo red solution for visualization of hydrolysis zone. Its arabinofuranosidase activity was optimized in solid state fermentation in PKC. Production temperature, pH, moisture content, inoculum size and agitation were studied for optimization test. Optimal production temperature and pH for arabinofuranosidase by isolate was 45°C and pH 9. Produced arabinofuranosidase was purified to apparent homogeneity with ammonium sulphate precipitation, dialysis and column chromatography techniques. Stability of arabinofuranofuranosidase obtained to temperature, pH, substrate concentration and some ions was determined as well as its molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

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Results: Isolate with highest arabinofuranosidase activity was selected and identified as *Streptomyces lividus*. Purity level attained was 16.36 fold. Enzyme had a specific activity of 25.4 U/mg, and total enzyme activity of 13.2 U. Molecular weight of enzyme appeared as a band of 30 kDa. Purified arabinofuranosidase enzyme revealed optimum temperature and pH as 60^{o}C and 9 respectively. Enzyme was stable over a broad pH range of 3-11, and temperature of 30-80^{o}C. Residual activity after incubating for 1 hour at 70^{o}C was 64%. Enzyme kinetics studies showed Km and Vmax values for P-nitrophenyl arabinofuranoside were 2.3mM and 0.7U/min respectively.
Conclusion: Apart from Solid State Fermentation (SSF) of PKC being a potential fermentation technique for production of arabinofuranosidase by *Streptomyces lividus*, the enzyme was highly stable.

Keywords: Arabinofuranosidase; Streptomyces lividus; thermostable; palm kernel cake.

1. INTRODUCTION

Agricultural residues like Palm Kernel Cake (PKC) are sources of cheap renewable energy from which useful industrial enzymes may be produced using solid state fermentation (SSF) systems. α -L- arabinofuranosidases (α -L-AFases) is one of such enzymes. They are enzymes that act together with other hemicellulases and pectic enzymes for the complete hydrolysis of hemicelluloses and pectin found in most agricultural substrates [1]. They are promising tools in various agro-industrial processes including production of important medicinal compounds, improvement of wine flavors, pulp treatment, production of bioethanol, and synthesis of oligosaccharides [2]. Thermostable arabinofuranosidases have been isolated from a number of bacterial and fungal sources and their production have been optimized in fermentation mediums by various researchers to improve their yield [3,4,5]. None of such study had been done on thermostable arabinofuranosidase from Actinomycetes such as *Streptomyces lividus*. Purification of the arabinofuranosidase is necessary for its characterisation which will determine its potential for industrial production. The aim of this work was to isolate and identify hemicellulose degrading bacteria from PKC, to optimize process conditions for producing arabinofuranosidase enzyme, and to purify and characterise the arabinofuranosidase obtained from identified *Streptomyces lividus*.

2. MATERIALS AND METHODS

P-nitrophenyl arabinofuranoside was purchased from Sigma-Aldrich (USA). The Palm Kernel Cake (PKC) used was obtained locally from Palm Oil refineries. All other chemicals used were of analytical grade.

2.1 Sample Collection and Preparation

Palm Kernel Cake (PKC) was purchased from four local Palm Kernel Oil Refineries in Ibadan, Nigeria. The substrate was prepared as described by Khandeparkar and Bhosle [6]. PKC was washed twice in distilled water and then boiled in distilled water for 10 to 15 min. The water was decanted and substrate dried in an oven at 50°C. It was grinded using a home mixer (GASA, QBL-18l40). The grinded substrate was sieved using a 40µm mesh and stored at 4°C until used.

2.2 Enrichment and Isolation of Bacteria from PKC

Enrichment was carried out in Erlenmeyer flasks containing modified basal salt medium (MBSM) and 0.5% w/v of carbon source (PKC) according to the modified method of Khandeparkar and Bhosle [6] After obtaining consistent microbial growth, 1mL of the culture broth was appropriately diluted and pour plated on Modified Basal Salt Medium (MBSM) plates in the composition: NaCl 25.00 g, KCl 0.75 g, $MgSO_4$7.00 g, NH_4Cl 0.50 g, K_2HPO_4 (10%) 7.00 mL KH_2PO_4 (10%) 3.00 mL, Yeast extract 0.60 g, Peptone 0.20 g, Trace metals solution, 1.00 mL, Distilled water 1000 mL, and appropriate amount of agar. The medium was adjusted to pH 9.0 using 1M NaOH. Nutrient agar plates enriched with PKC were also plated out. These plates were incubated for 48 hours at 50°C. The individual colonies were collected, re-streaked on MBSM and Nutrient agar plates. The purity of the bacterial isolates was further confirmed by repeated plating on Nutrient agar plates enriched with PKC. The isolates were then subjected to Gram-staining tests to determine their Gram-reaction. The pure cultures obtained were maintained on Nutrient agar slants at 4°C.

2.3 Screening for Arabinofuranosidase Producing Bacteria

Screening of bacterial isolates for arabinofuranosidase production was done according to the method described by Sumardi et al. [7] with slight modifications. 0.5 g Palm Kernel Cake was enriched in 100 ml basal medium containing 2.5 g NaCl, 0.075 g KCl, 0.7 g $MgSO_4$, 0.05 g NH_4Cl, 0.06 g Yeast extract, 0.02g Peptone, K_2HPO_4 solution (1.4 mL), KH_2PO_4 solution (0.6 mL) and Trace elements at 50°C for two days, with agitation (100 rpm). 0.5 mL of each enrichment culture was spread aerobically on the same medium onto agar plates containing 0.5 g Birchwood Xylan instead of PKC. It was incubated for 48 hours at 50°C. The isolates that degraded xylan showed a clear zone around the colony after staining with a solution of 0.1% congo red for 15 minutes, and destaining through repeated washing with 1 M NaCl.

2.4 Optimization of Arabinofuranosidase Production in Solid State Fermentation

The best isolate was evaluated for its ability to produce a-L-AFase when grown under solid state fermentation (SSF). SSF was carried out in 100 mL Erlenmeyer flasks containing 10 g of PKC as carbon source and moisturized with 30 mL of MBSM (substrate to moisture ratio 1:3 w/v). The flasks were sterilized at 121°C for 15 minutes, allowed to cool to ambient temperature and each was inoculated with 1 mL of culture broth and incubated under static condition at room temperature (28 ± 2°C) for 72 h. Optimization of arabinofuranosidase production, took into consideration the effect of: Moisture, Inoculum sizes, Incubation Temperature (25-55°C), Agitation rate, pH of substrate (pH 5.0-9.0) and various Mineral salts [8]. The moisture content of the substrates was adjusted at different ratios between the substrate and the basal medium added. This was achieved by varying the ratio of PKC to the liquid basal medium used (w/v) thus; 1:0.5(50%v/w), 1:1(100%v/w), 1:1.50 (150%v/w). The effect of inoculum size was determined by adding bacterial suspension in dilutions 10^{-2}, 10^{-5}, and 10^{-8} to the substrate. Agitation rates of 50, 100, and 150rpm were used to optimize enzyme production by *Streptomyces lividus*. The mineral salts tested on arabinofuranosidase production were: $FeSO_4.7H_2O$; $MnSO_4.4H_2O$; $ZnSO_4.7H_2O$, and $CoCl_2.6H_2O$. The sulphate salts were each used to substitute Magnesium sulphate while Cobalt chloride salt was used in place of potassium chloride in the minimum basal salt medium used in this work and in the same concentration i.e. 7 g/l and 0.75 g/l respectively in the MBSM.

2.5 Enzyme Assay

The α-L-AFase activity in the supernatants was estimated following the method described by Gilead and Shoham [9]. The assay was based on the hydrolysis of p-nitrophenyl-a-L-arabinofuranoside (p-NPAF) (Sigma). The reaction mixture contained 80 µl of appropriately diluted enzyme sample, 80 µl of 50 mM sodium phosphate buffer (pH 8.0), and 40 µl of p-NPAF (4 mg/mL) in the same buffer. Tubes were incubated at 80°C for 10 min, and the reaction was then terminated by the addition of ice-cold 1 M Na_2CO_3. The absorbance of the yellow color produced by the release of p-nitrophenol (pNP) was measured at 420 nm using UV-Vis spectrophotometer [10].

α-L-AFase unit: One international unit (U) of a-L-AFase activity was defined as the amount of enzyme which produces 1 µmol of p-nitrophenol (pNP) per min under the standard assay conditions described above.

2.6 Enzyme Purification

Fresh crude arabinofuranosidase was precipitated out in salting out process using ammonium sulphate at different saturation levels (20% - 80%) in an overnight reaction. Each fraction was tested for protein content and α-L-arabinofuranosidase activity. Fractions with considerable α-L-arabinofuranosidase activity were pooled together being centrifuged in a cold centrifuge (Model C-24BL) at 5,000 rpm for 1 hour at 4°C and dialysed against 100mM sodium acetate buffer (pH 5). During dialysis, buffer was replaced every 4 hours with fresh one. The dialysate was applied to a Sephadex gel G-100 Column (2 cm by 30 cm) was equilibrated with 10 mM Tris buffer (pH 5). The absorbed proteins were eluted using 0.5M NaCl in the same buffer at a flow rate of 0.1 mL/min and collected in fractions. Collected fractions were analysed for protein content [11] and α-L-arabinofuranosidase activity. CM-cellulose column (3.5 by 50 cm) was equilibrated with 10 mM. The purity of the α-L-AFase in the purification process was evaluated by SDS-PAGE [12]. Molecular weight marker, middle range (Wako Pure Chemical Industries) was used as a molecular mass standard. The electrophoresed gel was stained with 0.2% coomassie brilliant blue R-250 to enhance the

visualisation of the protein band in a Benchtop Transilluminator (No: 95 – 0180 - 01).

2.7 Enzymatic Properties

pH optimum of the arabinofuranosidase enzyme was determined by measuring its activity at pH values ranging from 3.0-11.0. The reaction mixture included 0.5 mL of 50 mM Sodium citrate buffer at different pH levels (pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0), and 0.5mL of the partially purified enzyme. The reaction was initiated by addition of 0.5mL solution of P-nitrophenyl arabinofuranoside in 4mg/ml concentration. These were held in test tubes and maintained at 50°C for 10 minutes. The reaction was terminated by the addition of 2 mL of 1M Na_2CO_3. The absorbance of *p*-Nitrophenol released was measured using a spectrophotometer at a wavelength of 405 nm [13]. The pH stability of the enzyme was determined by incubating the enzyme in Sodium citrate buffer with pH ranges of 3 to 11 (with difference of 1 unit in between each pH) for 1 hour at 50°C after which the residual enzyme activity was measured according to standard assay procedure.

The effect of temperature on α -L-AFase activity was carried out at different temperature values (30-80°C) in 50mM sodium citrate buffer at pH 9.0 using a water bath. The reaction mixture included 0.5mL of 50mM sodium citrate buffer pH 9.0 and 0.5 mL solution of of the partially purified enzyme. The reaction was initiated by the addition of 0.5 mL of the substrate P-nitrophenyl arabinofuranoside in 4 mg/ml concentration. These were held in test tubes and maintained at different temperatures (30°C, 40°C, 50°C, 60°C, 70°C and 80°C for 10 minutes). The reactions were terminated by the addition of 2 mL of 1M Na_2CO_3. Thermo stability of the enzyme was determined by assaying for residual enzyme activity after incubating the reaction mixture at various temperatures (30-80°C) for 1 hour in sodium citrate buffer (pH 9.0) before termination by the addition of 2 mL of 1M Na_2CO_3. The absorbance of *p*-nitrophenol released was measured using the spectrophotometer at a wavelength of 405 nm.

2.8 Kinetic Parameters

Kinetic parameters (Vmax and *Km*) of purified arabinofuranosidase in Sodium phosphate buffer (50mM, pH 7.0) was determined by studying its reaction rate at varied concentration of *p*-nitrophenyl-α,L-arabinofuranoside (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM). The reaction was initiated by the addition of 0.5mL of the different substrate concentration in solution to the purified enzyme held in different test tubes and maintained at 50°C. After which reactions were terminated by addition of 2 mL of 1M Na_2CO_3. Absorbance of *p*-nitrophenol released was measured using spectrophotometer at 405 nm. Substrate concentration was plotted against the reaction rate to determine whether the enzyme obeys Michaelis–Menten kinetics, and Km and Vmax values were determined from the Lineweaver–Burk double reciprocal plot [14].

3. RESULTS

3.1 Production of α-L-AFase

Temperature and pH for arabinofuranosidase production was optimal at 45°C and 9

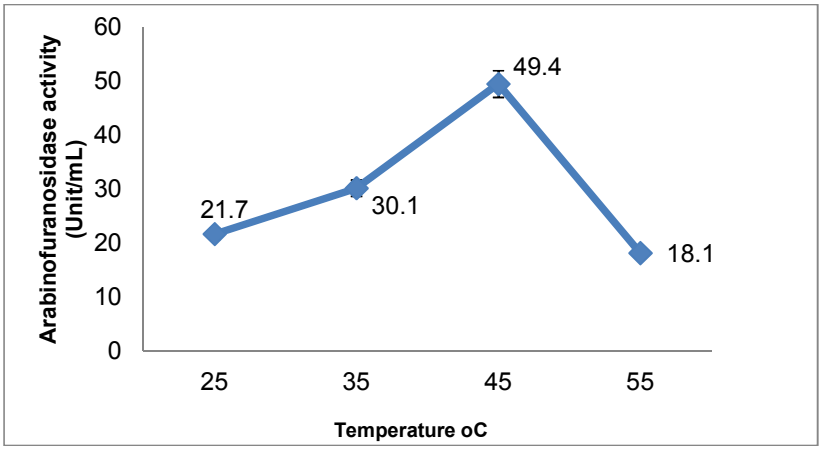

Fig. 1. Effect of temperature on arabinofuranosidase production by *Streptomyces lividus* in SSF of palm kernel cake

respectively (Figs. 1 and 2). The effect of moisture content on arabinofuranosidase production is shown on Fig. 3. Arabinofuranosidase production was optimal at 150% (v/w) at a value of 40.7±2.68 Units/mL. It was found that the higher the inoculum concentration, the higher the enzyme produced, as indicated by the enzyme activities shown in Fig. 4. The optimum agitation speed was generally at 150rpm and it favoured the synthesis of arabinofuranosidase enzyme. Activity increased with agitation speed in direct proportion as indicated Fig. 5. Enzyme activity was generally high with varying mineral salts used. $MnSO_4$ however gave the highest activity (60.5±1.47 Units/mL) when it was added as the only mineral salt to the basal medium used for the fermentation process (Fig. 6).

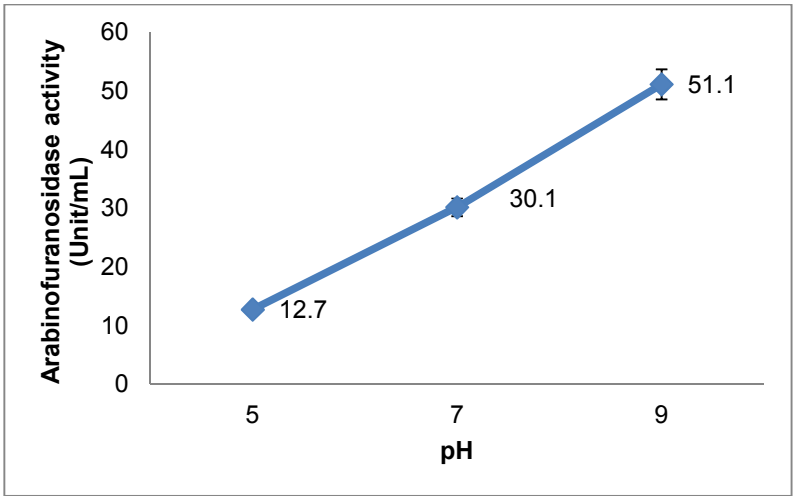

Fig. 2. Effect of pH concentration on arabinofuranosidase production by *Streptomyces lividus* in SSF of palm kernel cake

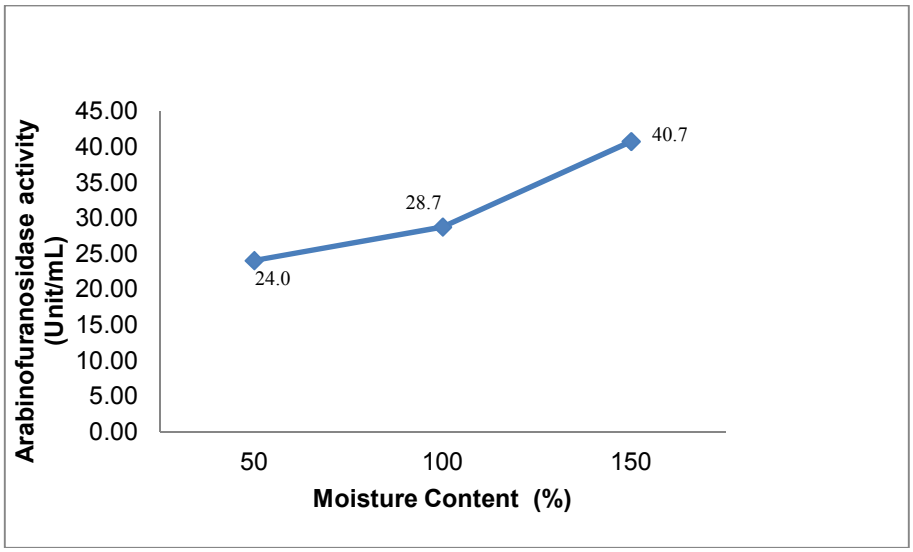

Fig. 3. Effect of moisture content on arabinofuranosidase production by *Streptomyces lividus* in SSF of palm kernel cake

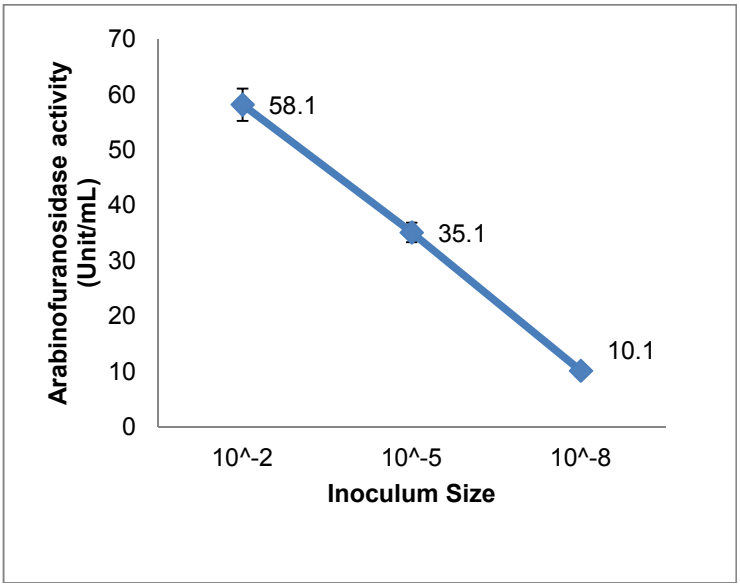

Fig. 4. Effect of inoculum concentration on arabinofuranosidase production by *Streptomyces lividus* in SSF of palm kernel cake

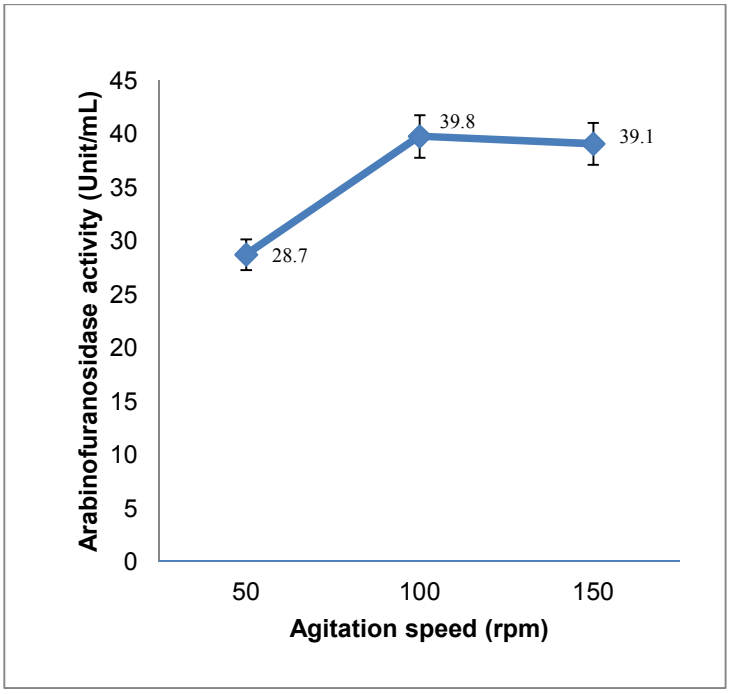

Fig. 5. Effect of agitation on arabinofuranosidase production by *Streptomyces lividus* in SSF of palm kernel cake

3.2 Purification of α-L-AFase

The purity level attained was 16.4 fold. This resulted in an increase in specific activity from 1.6 to 25.4 U/mg protein, and a total enzyme activity of 13.2 U. The recovery after gel filtration with sephadex G-100 was 5.92% (Table 1). A single prominent band of 30 kDA was obtained as the molecular weight of enzyme after subjection to SDS-PAGE (Fig. 7).

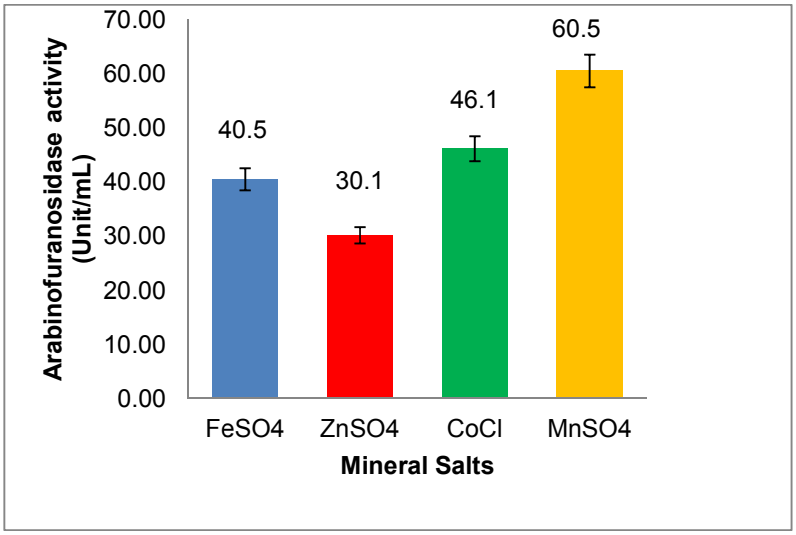

Fig. 6. Effect of different mineral salts on arabinofuranosidase production by *Streptomyces lividus* in SSF of palm kernel cake

Table 1. Purification of arabinofuranosidase from *Streptomyces lividus*

Purification steps	Volume (mL)	Total enzyme activity (U)	Total protein (mg)	Specifc activity (U/mgprotein)	Purification fold	Recovery (%)
Crude enzyme	100.0	222.6	143.5	1.6	1.0	100.0
$(NH4)_2SO4$ precipitation	100.0	131.8	12.3	10.7	6.9	52.2
Dialysis	100.0	47.5	3.1	15.8	9.8	21.3
Sephadex G-100	10.0	13.2	0.5	25.4	16.4	5.9

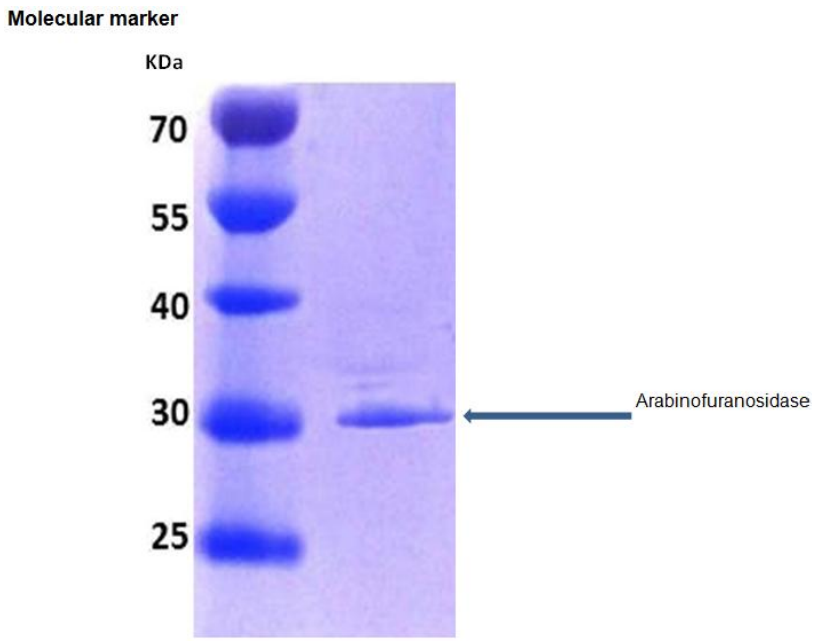

Fig. 7. Electrophoretogram of purified arabinofuranosidase enzyme using SDS-Polyacrylamide gel electrophoresis

3.3 The Effects of pH and Temperature on the Activity and Stability of α-L-AFase

The arabinofuranosidase enzyme produced by *Streptomyces lividus* was optimal at pH 9 (43.6 Units/mL), and was lowest at pH 3 (12.0 Units/mL). The enzyme was however found to be stable over a wide range of pH, although there was a sharp decrease in enzyme activity at pH 11 (Fig. 8). The arabinofuranosidase enzyme retained 66% of its activity after incubation for 60 minutes at pH 9, the activity reduced to about 44 % after incubation for 90 minutes at the same pH (Fig. 9). The stability of the enzyme was determined at different temperature levels, ranging from 30-80°C. The optimal temperature for stability was found to be at 60°C at an enzyme activity of 54.4 Units/mL, although the enzyme was relatively active across the range of temperature investigated. The activity began to decline after 60°C (Fig. 10), although it still retained about 64% of its activity at 70°C after 1 hour of incubation. At 80°C, only 46% of the enzyme activity was retained (Fig. 11).

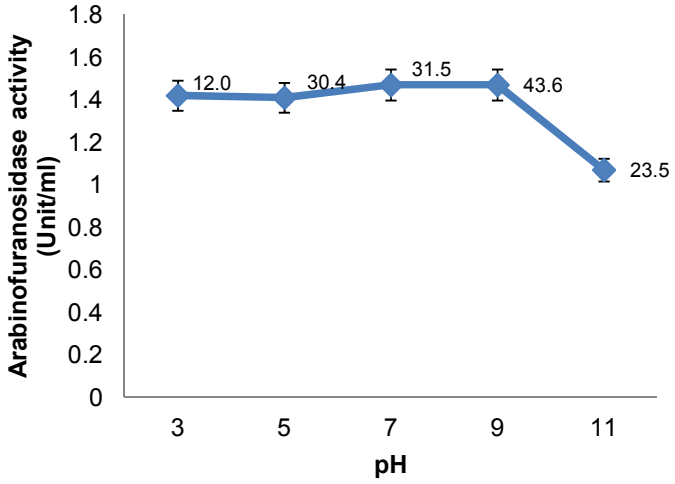

Fig. 8. Effect of different pH on the activity of arabinofuranosidase produced by *Streptomyces lividus* in Solid state fermentation

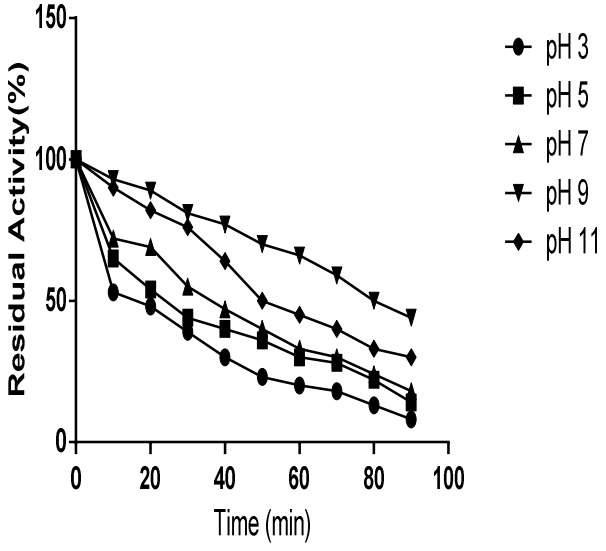

Fig. 9. pH stability of arabinofuranosidase enzyme after 1h incubation at different pH levels

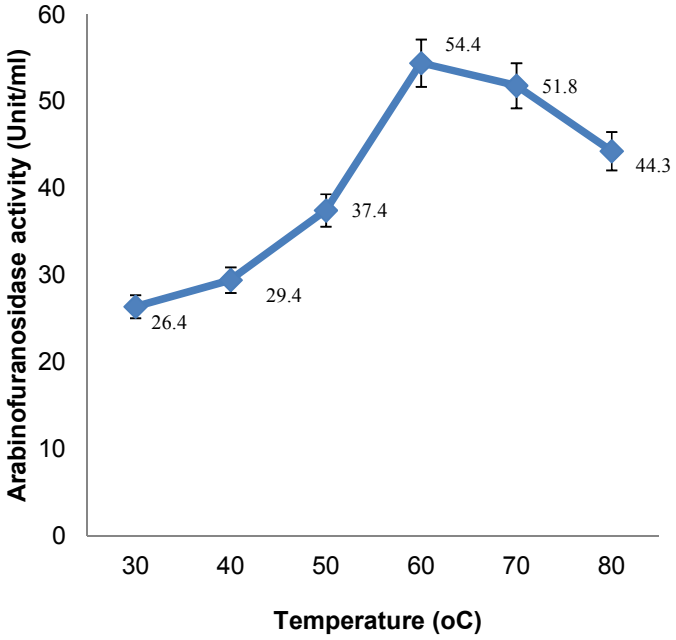

Fig. 10. Effect of varying temperature on the activity of arabinofuranosidase produced by *Streptomyces lividus* in Solid state fermentation

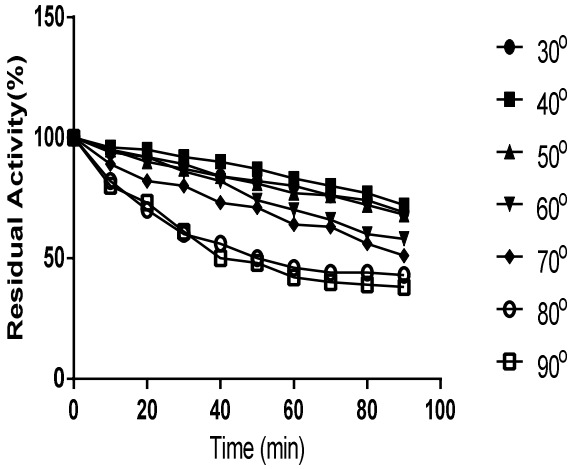

Fig. 11. Thermal stability of arabinofuranosidase enzyme produced by *Streptomyces lividus* after 1 h of incubation at different temperature

3.4 Kinetic Parameters

The *Km* and Vmax values of the partially purified arabinofuranosidase enzyme were determined to be 2.33mM and 0.71U/min respectively (Fig. 12). The low *Km* value obtained for the enzyme is indicative of a relatively high affinity for its substrate.

4. DISCUSSION

This study reports the production, purification and characterization of α-L-Afase enzyme from *Streptomyces lividus*. The ability of this organism to produce the enzyme may have been influenced by the PKC used as substrate. Numan and Bhosle [2], noted that efficient

production may generally be influenced by arabinose-containing substrates of which PKC is one [15]. PKC has been used in solid state fermentation experiments for the production of various enzymes including Mannanase, Xylanase, Amylase and Cellulase [16,17,18,7]. This is the first report to the best of our knowledge on the production of α-L-AFase from PKC using a bacterial culture. The optimal temperature and pH for enzyme production in this work is different from that which was observed in *Pseudomonas cellulosa* and *Bacillus pumilus* PS213 [19,20]. It may be indicated therefore that the optimum temperature for enzyme production by any microorganism has to do with the optimum growth temperature of the producing organism in solid state fermentation experiments [21]. Other factors including moisture content, air flow and oxygen level may also influence the optimum temperature for enzyme production in solid state fermentation experiments [22]. Adequate moisture for growth during fermentation of substrate in solid state is important for improved aeration as well as to increase penetrability between particles. Moisture demand could be as low as 50% (v/w) and as high as 150% (v/w). Optimum enzyme activity was at 150%(v/w) moisture content probably because PKC requires a lot of water to be moistened, so absorbs water faster and because the microorganism is not a fungus which requires more moisture content for solid state fermentation being more of a bacterium than a fungus [22,23]. This is in line with what was observed in this study. There is a direct correlation between the inoculum size and enzyme production. In this study, it was observed that enzyme production increased with increase in inoculum concentration, the optimal being at 10^{-2} concentration of the bacterial suspension. A similar trend was noted in the work of Battan et al. [24] during production of xylanase by *Bacillus pumilus*. In solid and liquid state fermentation systems, optimum aeration and agitation are mandatory to ensure availability of oxygen, and other important materials to the microorganisms [25]. The optimum agitation speed for *Streptomyces lividus* was at 100rpm and it favoured the synthesis of arabinofuranosidase enzyme. The composition of mineral salts for enzyme production is an important factor in solid state fermentation systems. Several authors have reported that the composition of mineral salts could either enhance or inhibit enzyme production. This may be dependent also on the type of mineral and salt, its concentration, and the microorganism producing the enzyme [26,1]. It was noted that $MnSO_4$ gave the highest activity for *Streptomyces lividus* when it was added as the only mineral salt to the basal medium used for the fermentation process.

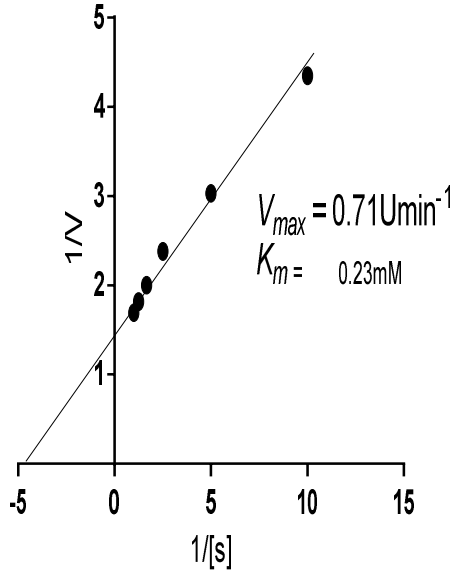

Fig. 12. Double reciprocal plot for the reaction kinetics of arabinofuranosidase from *Streptomyces lividus* against *p*-nitrophenyl-α,L-arabinofuranoside at pH 7 and temperature of 50°C

Enzymes exhibit different catalytic activities within ranges of physical conditions such as pH, temperature or presence of metal ions [27]. The activity of the purified arabinofuranosidase enzyme from *Streptomyces lividus* was optimal at 60°C, although the enzyme was relatively active across the range of temperature investigated. Inacio et al. [28] also noted the the same with the arabinofuranosidase enzyme Abf2 obtained from *Bacillus subtilis*. The residual activity of the purified arabinofuranosidase enzyme obtained from this study retained 70% activity at 60°C after incubation for 1 hour. The residual activity declined to 46% at 80°C after 1 hour. Researchers like Miyazaki [29], Yan et al. [4], Ahmed et al. [1], Lee and Lee [5], Ratna et al. [30] have reported optimal activity of arabinofuranosidase enzyme ranging from 50-90°C. The source from which the enzyme is produced and characterized from may account for the variation in its optimum temperature be an indication of its activity at different temperature [2]. The enzyme showed stability over a wide range of pH 3-11, and it retained 50% of its activity after incubation for 80 minutes at pH 9.

The reaction kinetics of arabinofuranosidase enzyme from *Streptomyces lividus* revealed its *Km* and *Vmax* values to be 2.3mM and 0.7U/min respectively. Inacio et al. [28] who performed kinetic experiments at 37°C reported *Km* and *Vmax* values for two arabinofuranosidase enzymes AbfA and Abf2 from *Bacillus subtilis* to be 0.498 mM, 0.421 mM, and of 317 U mg^{-1} and 311 U mg^{-1} for AbfA and Abf2, respectively.

The arabinofuranosidase enzyme was apparently purified to near homogeneity. The overall recovery level was 5.9%. This value corresponded to its purification fold of 16.4. Degrassi et al. [20] however reported a higher recovery level (26.6%) of arabinofuranosidase enzyme from *Bacillus pumilus* PS213. Tuncer and Ball [31] reported a mean percentage (%) yield of 16.0. The purification fold in this study is similar to the result of Ratna et al. [30] who reported a purification fold of 17.6 fold. As expected, the specific activity of the enzyme increased with each successive purification step from 1.6U/mg protein the crude enzyme to 25.4U/mg protein after chromatographic purification. Similar trend was observed in the works of Lim et al. [3], Yan et al. [4] and Lee and Lee [5].

The molecular mass of the purified enzyme after subjection to analysis using SDS PAGE was obtained as a prominent band of 30kDa. A similar molecular weight of 34kDa was obtained from purified arabinofuranosidase enzyme CtGH43 gotten from *Clostridium thermocellum* [1]. Microbial α -L-AFases however vary in their molecular masses depending on the source of the enzyme [2].

The results obtained from this study shows clearly that Palm Kernel Cake (PKC) was able to induce arabinofuranosidase enzyme production. Since PKC is a cheap agricultural residue obtained from the Palm Oil Industry, Solid State Fermentation (SSF) system using PKC as substrate is therefore a cost-effective technique for the production of arabinofuranosidase. The cultivation system may be modified optimally to enhance the production, and facilitate scale up processes for mass production of this very important industrial enzyme.

5. CONCLUSION

Solid State Fermentation (SSF) of PKC is a potential fermentation technique for production of this arabinofuranosidase by *Streptomyces lividus*. Also the enzyme was highly stable at relatively high temperature and alkali pH.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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