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Agro wastes residues as strategy to produce cellulase.

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Abstract: Agro wastes such as carrot peel, onion peel, potato peel and sugar beet peel were utilized for the production of cellulase. The agro wastes produced cellulase enzyme and activity was obtained on 6th day for potato peel and 7th day for carrot peel, onion peel and sugar beet peel. The maximum cellulase activity was observed with sugar beet for all parameters; at the pH of 6.5 (1.68 U/ml) and at temperature 40ºC (1.76U/ml). With different carbon and nitrogen sources, the maximum enzyme activity was observed with manitol and sugar beet peel as substrate (2.32U/ml) and with potassium nitrate, sugar beet peel also as substrate (1.69 U/ml). The kinetics enzyme has optimal temperature of 40°C, optimal pH of 8, the ions of $Mg^{2+,}$ Mn $^{2+}$and cu^{2+} and EDTA in low concentrations of 2mM. EDTA was found to inhibit enzyme ctivity of cellulase, while the Zn^{2+} and Fe^{2+} activated the cellulase. Different metal ions increased ordecreased inhibition of cellulase activity at 2mM. Michaelis-Menten equation constant (Km and Vmax) values of purified cellulase were 0.22 µg /mL and 2.34 U/ml.
Key words: Fungi, agro wastes, cellulase, carbon and nitrogen sources.

Introduction

The biological wastes are organic in nature and easily assimilated by the microorganisms mainly fungi which make such wastes very appropriate for enzyme production under submerged fermentation. Therefore, submerged fermentation is finding increasing application in the production of value added products from wastes mostly from lignocelluloses agro wastes. The carrot peel, onion peel, potato peel, and sugar beet peel are mainly composed of cellulose, hemi-cellulose and lignin which are used as bioresources of raw materials for industries considering that cellulose has been produced in large quantities. Wastes and their disposal have become an environmental concern worldwide especially when these wastes are biodegradable into useful goods and services [1]. Cellulolytic wastes can be used to produce important compounds such as alcohol, thereby assisting in controlling environmental pollution [2]. Cellulases (EC 3.2.1.21) refer to a class of enzymes produced chiefly by fungi, bacteria and protozoan that catalyze the hydrolysis of cellulose [3] . Cellulose is the major component of plant biomass and the major biopolymer found in abundance on earth, and much of the cellulose exists as wastes. *Penicillium crustosum* is a food borne ubiquitous fungal species, frequently isolated from agro wastes, vegetables, decayed food and fruits. It is also common in the soil rhizosphere of vegetables [4]**.** Proper biotechnological utilization of agro wastes in the environment will eliminate pollution and convert them into useful by products [5]. *Penicillium crustosum* has been reported under the name of *Penicillium cyclopium* and it has rarely been found in extremely cold environments [6]. Approximately 90% of all industrial enzymes are produced by submerged fermentation, often using genetically modified microorganisms[7]. The present study describes the isolation of *Penicillium crustosum* from agro wastes and analyzes the enzymatic activity produced by *Penicillium crustosum* cultivated using different agro wastes residues as substrates in submerged fermentation (SmF). The kinetic properties and effect of metal ion on the enzyme activity were investigated.

Materials and Method.

Substrates pretreatment and optimization

Agro wastes sample such as carrot peel, onion peel, potato peel, and sugar beet peel were collected from different parts of rural Bangalore. It was subjected to profound water washes to free from dust. Thereafter, it was chopped into pieces of 10 mm length; the samples were dried under sun for 3-5 days depending of on the moisture content. The dried material was shredded and then sieved to obtain uniform particle size of 150 µm. The powdered 3.5g of the substrates was mixed with 60 ml of distilled water containing 0.8% of ammonium nitrate in 250 ml of Erlenmeyer flask and sterilized [8].

Fungi isolation and identification

The fungus used in this study was isolated by screening the agro waste samples collected from Bangalore (India). Fungi were isolated and identified by serial dilution and wet mount technique [9].

Effect of incubation period, pH, temperature, carbon and nitrogen source on cellulase production.

The influence of all parameters on enzyme activity was determined by measuring cellulase activity at incubation period varying from 6-7days, pH (4.5-7.5) and temperature varying from (20-50°C). Different agro wastes, like carrot peel, onion peel, potato peel and sugar beet peel were powdered in a laboratory and were used as substrates for cellulase production. 3.5 grams of each finely powered agro wastes was taken in an Erlenmeyer flask of 250 mL capacity. The medium was supplemented with additives 0.5g equal of different carbon sources including manitol, sucrose, fructose and nitrogen sources such as $NaNO_3$, KNO_3, and NH_4Cl. All factors influencing on enzyme activity were determined by measuring cellulase activity[10] in 0.05M citrate buffer of pH 4.8.

Enzyme molecular weights and zymogram

The molecular weights of cellulase were estimated using the technique of sodium dodecy sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [11]. The zymogram, the SDS-PAGE was carried out for the samples with 12% separating gel with a addition of 0.1% CMC. 70µl of the crude enzyme (supernatant) was mixed with 30µl of gel loading dye and mixed, they were not boiled. 70 µl of the mixture was loaded in the gel and the elcectrophoresis was carried out at 25mA current and 100V. After the dye front reached the end of the gel, electrophoresis was stopped and the gel was gently shaken and removed using 20% Isoproponal and was placed immersed in 20% isoproponal at 40C for an hour followed by rinsing with water to remove the excess of isoproponal. The gel was then immersed in 100mM acetate buffer pH 6.0 and incubated at 60^0C for an hour to digest the substrate by the enzyme. The gel was then stained with congo red 0.1 g (w/v) in water for 30mins and washed with 1M Nacl for 15mins [12].

Determination of kinetic parameters

The kinetic parameter of the cellulase enzyme was determined at the optimum pH 3.0 - 9.0. The pH was adjusted using two the following buffers; 0.1M citrate buffer (pH 3- 6) and 0.1M phosphate buffer (pH 7– 9.0), temperatures between 20 to 60° C. Metal ions which include $MgSO_4.7H_2O$, $MnSO_4.5H_2O$, $FeSO_4.7H_2O$, $CuSO_4.7H_2O$ and $ZnSO_4.7H_2O$ at 2 mM and EDTA at 5mM were tested for their effects of enzyme activity and the substrate on the enzyme activity was also essayed. Cellulase activities were measured at different concentrations of substrate, using CMC at concentration range of 1.0 to 10 mg/ml. The Michaelis-Menten constant, Km for each substrate was determined from the Lineweaver-Burk plot.

Results and discussion

Incubation time

Penicillium crustosum was inoculated in ammonium nitrate 150 mL conical flask and incubated at 28◦C for a period of 7 days. The production of cellulase increased with increase in incubation time. The highest amount of cellulase was recorded on 6th day for potato peel with maximum cellulase activity (8.21 U/ml) (Fig. 2); whereas onion peel, sugar beet peel and carrot beet peel showed maximum cellulose production at 7th day with maximum cellulase activity of (24.57U/ml); (23.38 U/ml) and (26.56 U/ml) respectively (Fig. 1). There was increase in the cellulase enzyme production when carrot peel used as substrate on 7th day of incubation

whereas there was decrease in enzyme production after 6th day of incubation when potato peel was used as a substrate. Incubation time affects the enzyme production. Sonjoy et al., (1995) [13] reported that in short incubation period of enzyme production offers the potential for inexpensive production and it varies from enzyme to enzyme from single substrate. It was found that incubation period needed for enzyme production is shorter in solid state fermentation than in submerged fermentation process [14,15]. Gomes [16] also reported the maximum time of celllulase production on 7th day of fermentation period for *T.viride* which was similar to our findings. Zhang et al., (1999)[17] reported the maximum production of cellulase after 6 days of fermentation period. Patil et al., (2006)[18] reported that the period of fermentation depends upon the nature of medium, fermenting organisms, concentration of nutrients and the process physiological conditions.

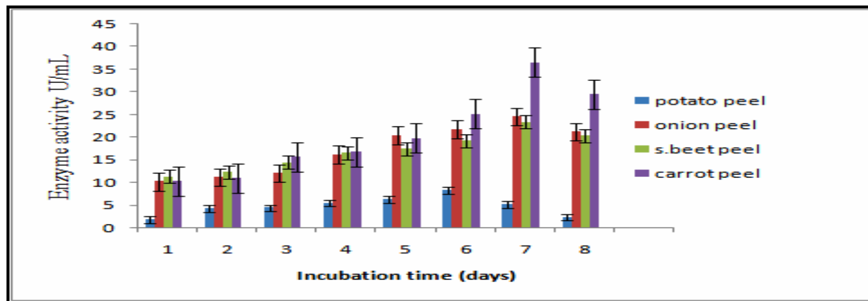

Figure 1- Effect of incubation time on enzyme activity by using different substrates.

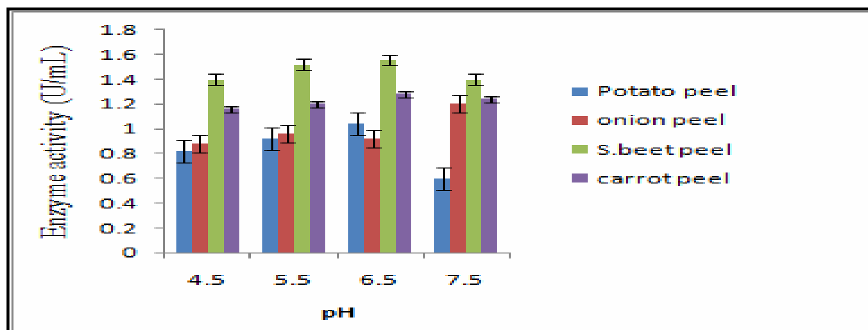

Figure 2- Effect of pH on enzyme activity by using different substrates.

Effect of pH on enzyme production

Cellulase enzyme from *Penicillium crustosum* was found active over a pH range of 5.5-7.5 with maximum activity at pH 7 and onion peel as substrate. The enzyme activity varied with the pH (Fig. 2). For the substrate potato peel the activity of the enzyme varies from pH 4.5 to pH 5.5, where maximum activity (0.8U/ml) was observed and decreased gradually from pH 6.5 to the pH of 7.5. Similarly the enzyme activity for the sugar beet peel substrate increased up to the pH 6.5 with the maximum activity (1.68U/ml) and decreased at the pH 4.5. Onion peel as the substrate the enzyme activity increased up to pH7.5 (1.72U/ml) and decreased at pH 4.5. Similarly the carrot peel as substrate the enzyme activity was highest at pH of 5.5 (1.28U/ml) and the lowest activity was found in pH 4.5. In comparison with the other substrates the onion peel at pH 7.5 maximum activity of the enzyme cellulose was observed. Peciulyte [19] isolated cellulolytic fungi from waste paper gradual recycling materials and stated the optimum pH of 4.5, 5.5, 6.5 and 6.0 for *Aspergillus niger* DPK-cl-12, *Gliomastix rorum, Stachybotrys chartarum* DPK-cl-111 and *Penicillium funiculosum* DPK-cl-19 respecitvely at 30ºC.

Soni [20] observed a wide variations pH of cellulase produced by different fungi such as *Aspergillus sp* showing optimum pH of 6.0; *A. terreus* pH 6.0 and *M. fergusii* T41 showing the optimum pH of 4.0. Lee et al., (2008) [21] purified and characterized the cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull and reported the optimum pH of 7.0 which was near to our findings. Optimum pH of 6.5 for cellulose was also reported by [22] isolated from marine bacterium *Bacillus subtilis* subsp. *subtilis* A-53 were in accordance to our results. The wide variation in pH might be due to the different substrates and different microbial origin. Our finding are similar with [23] who reported that maximum CMCase activity was recorded at pH 7.5 by *Aspergillus niger* (*Z10* wild type strain) when among the tested pH range between 4-9.

Effect of temperature

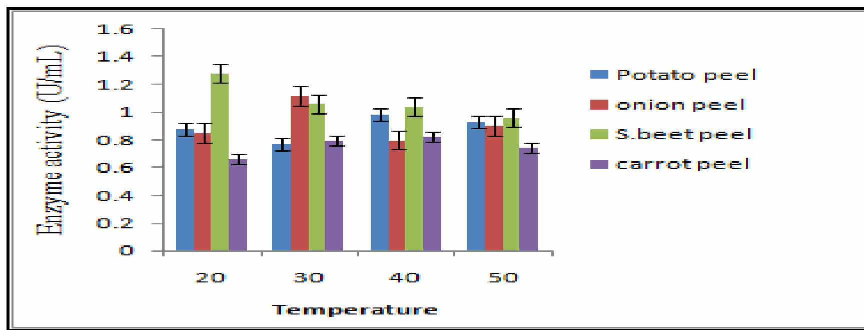

Figure 3- Effect of temperature on enzyme activity using different substrates.

The effect of environmental factor such as temperature was found to be an important parameter that influenced enzyme activity and production. The optimal temperature for cellulase enzyme production by *Penicillium crustosum* was found to be around 20 to 40^{0}C and the highest production obtained at 40 º C (Fig.3), there was increase in enzyme activity in all substrates such as potato peel (0.66U/ml), onion peel(1.76U/ml), sugar beet peel (1.3U/ml) and carrot peel (0.37 U/ml) the enzyme activity increased at 40º C. Whereas the enzyme activity decreased at 50ºC; for substrates onion peel and sugar beet peel substrate meanwhile potato peel and carrot peel enzyme activity decreased at 20ºC. In comparison with other substrates, sugar beet peel exhibited highest cellulase activity at 40 ºC. In many reports different temperatures for maximum cellulase production either in flask or in fermentor studies using *Penicillium genus* suggesting that the optimal temperature for cellulase production depends on the strain variation of the microorganism was reported. Rahna et al., (2011) [24] reported that an optimal temperature for cellulase activity in the range of 20 - 50°C for *Streptomyces* sp using fruit waste as substrate which was similar to ours findings. Furthermore, [25] reported an optimal temperature for cellulase activity in the range of 40 -55°C for several *Streptomyces* species including *Streptomyces lividans, Streptomyces flavogrisus*, and *Streptomyces nitrosporus.* Coral [23] reported, the optimum temperature for CMCase activity was 40 °C in *A. niger Z10* strain.

Effect of Nitrogen sources on cellulase production

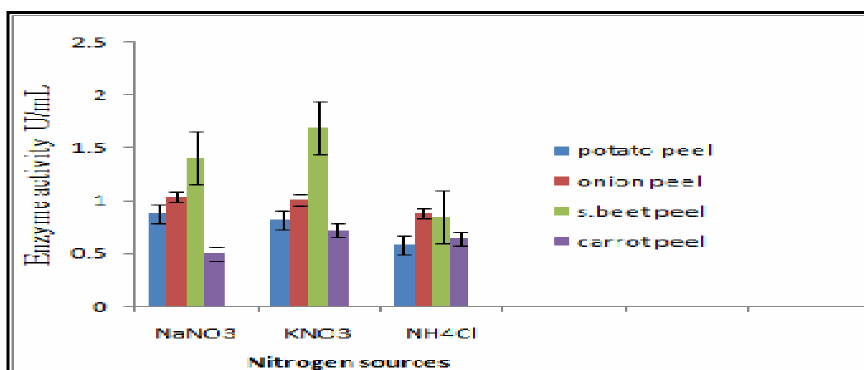

Figure 4- Effect of nitrogen sources on enzyme productivity by using different substrates.

The source of nitrogen in the growth medium has a very important role in microbial growth and enzyme production [26]. The highest cellulase activity was sugar beet peel with potassium nitrate and sodium nitrate (1.69 U/ml), (1.49U/ml) and ammonium chloride (0.88 U/ml). The lowest enzyme activity with potato peel and onion peel (0.58 U/ml), (0.88 U/ml) with ammonium chloride respectively. In comparison with others substrates sugar beet peel had the highest enzyme activity with potassium nitrate (Fig. 4). Among the nitrogen sources, potassium nitrate was found as a best source of nitrogen. Potassium nitrate (KNO_3) showed the highest yield among the other inorganic nitrogen sources which are $NaNO_3$ and NH_4Cl. According to (Kubisi et al., 1999)[27] potassium nitrate is a naturally occurring mineral source of nitrogen. Rosma et al., (2007) [28] have found that for the fermentation medium with 0.01% (w/v) nitrogen content, potassium nitrate (KNO_3) showed the highest yield among the other inorganic nitrogen sources which are $NH_4H_2PO_4$ and $(NH_4)_2SO_4$.

Effect of Carbon sources on cellulase production

Carbon source is very essential component for microbial growth and product formation. Sometimes it enhances the product formation as well as growth of the microorganism. Enzyme activity was found to be maximum with potato peel as substrate (0.45 U/ml) with fructose, and the lowest enzyme activity found with manitol at (0.32U/ml). Carrot peel, onion peel and sugar beet peel showed highest enzyme activity with manitol (0.4U/ml), (0.98U/ml) and (2.32U/ml) respectively. The lowest enzyme activity was observed with carrot peel and sugar beet peel (0.33 U/ml), and (0.82 U/ml with fructose and (1.25U/ml) with sucrose for sugar beet peel. The highest in enzyme activity with sugar beet peel supplemented with manitol for all substrates (Fig 5). Some previous studies reported that the agricultural wastes of lignocellulosics are used as a carbohydrate source to produce commercially important products such as ethanol, glucose and single cell protein [29]. Priscila da Silva Delabona et al., (2012)[30] observed that the nature of the carbon source in the culture medium had a significant influence on endoglucanase production. Gautam et al.,(2010)[31] optimized the medium constituents for cellulose production by *Trichoderma viride* in submerged fermentation.

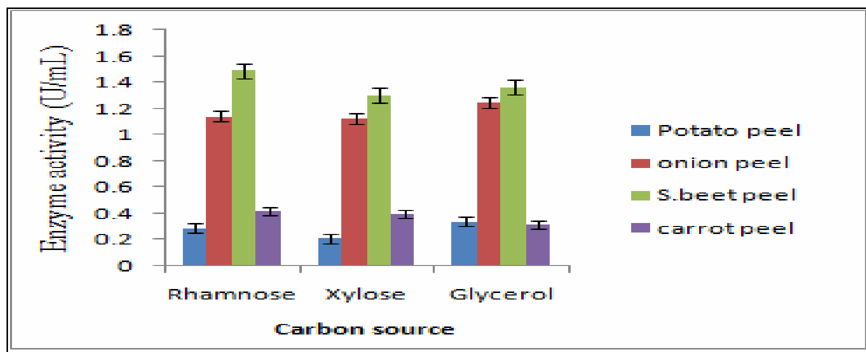

Figure 5- Effect of carbon sources on enzyme activity by using different substrates.

Effect of pH (3-9) on kinetics of cellulase activity

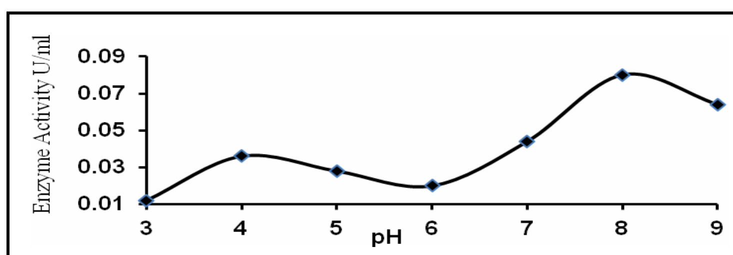

Figure 6- Effect of pH on kinetics on cellulase activity of *Penicillium crustosum.*

Different buffers of varying pH 3 to 9 were used in order to check the effect of pH on cellulase from *Penicillium crustosum.* Maximum activity of cellulase enzyme was found at pH 4 and 8 (Fig. 6). Our results are in agreement with findings of [32] who obtained maximum cellulase activity from *A. niger* ANL301 at pH between pH 3 to 9 with three major activity 3.5; 5.5 and 7. Coral [23] reported pH between 3 to 9 for a wild strain of *A. niger* Z10.

Effect of temperature (20-50°C) on kinetics of enzymes activity

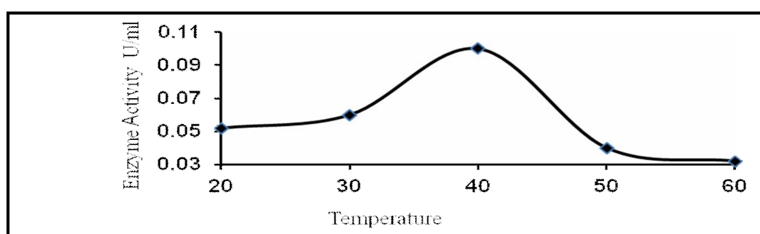

Figure 7- Effect of temperature (20- 50°C) on cellulase activity of *Penicillium crustosum*.

In order to determine the effect of different temperature on enzyme activity, cellulase activities at different temperatures ranging from 20 to 60°C were measured. The results of these measurements indicated

that the enzyme showed highest activity at 40°C. Therefore at temperatures above 40°C decrease in cellulase activity was observed (Fig. 7). Our findings are similar with [23] who reported optimal temperature of 40°C for the carboxymethyl cellulase enzyme of wild type strain of *A. niger* Z10. Tao [33] reported that the optimum temperature was 50°C for endoglucunase obtained from *A. glucus* XC9. Some commercial cellulase enzyme was stable at 60 °C reported by [34]

Enzyme molecular weights and zymogram

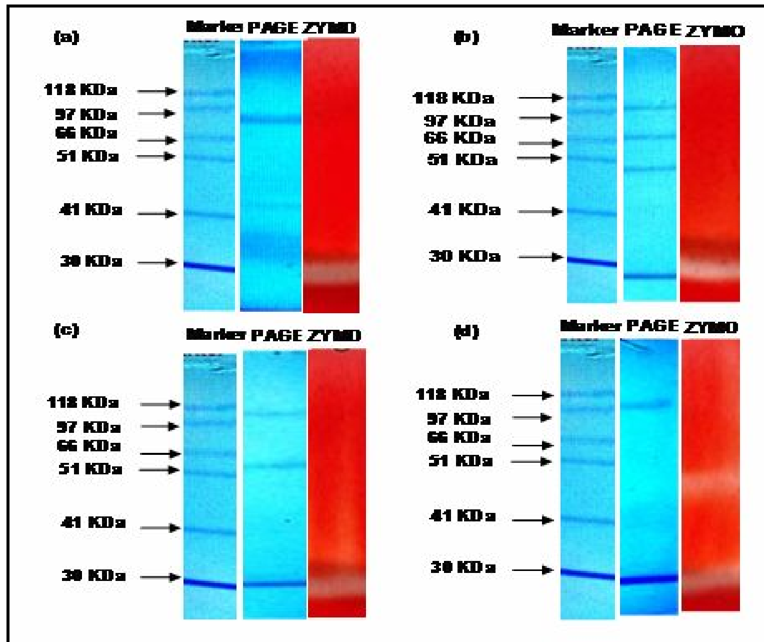

Figure 8- SDS-PAGE electrophoreses cellulase and zymogram stain with Congo red. (a) Potato Peel (b) Carrot Peel (c) Onion peel (d) Sugar beet peel.

The crude enzyme obtained from carrot peel; onion peel; sugar beet peel and potato peel, were differentiated on the basis of their molecular weight. The molecular weight of the protein bands ranged from 51kDa and 118 kDa for potato peel, 96 and 118 kDa for carrot peel, 96 and 118 kDa for onion peel and 118kDa for sugar beet peel. Zymogram analysis for cellulase activity as measured by degradation of carboxymethyl cellulose showed that at least one enzyme is responsible for the measured cellulase activity in potato peel, onion peel and carrot peel, thereafter at least two bands of cellulase appear in sugar beet peel. One major zones of clearing were observed on the zymogram gel, with sizes of approximately of 29 kDa on potato peel, carrot peel, onion peel and 2 bands 29 kDa, 90 kDa on sugar beet peel (Fig 8). Our results are close to the findings of [35] who isolated a CMCase with 54 kDa and Kumar et al., (2012)[36] reported the molecular weight of cellulase obtained from *Bacillus* sp, were 29 kDa alkaline cellulose, from *Bacillus pumilus,* 30-65 kDa cellulose, from *Paenibacillus polymyxa*, 72 kDa, from *Sinorhizobium fredii*, 94 kDa and from *Aspergillus niger* 83 and 50 kDa CMCase. Immanuel [34] reported molecular weight of cellulase obtained from A. niger were 23, 36 kDa.

Effect of metal ions and EDTA on the cellulase activity

Effects of metals ions and EDTA on cellulase activity are tabulated in Table 1. Manganese (Mn^{+2}) and (Zinc^{2+}) increased the enzyme activity by 51 and 24 % respectively. EDTA and some metal ions inhibited the enzyme activity. The percentage inhibition on the enzyme activity were 71.3, 68, 70, 52 % for Mg^{2+}, Mn^{2+}, Cu^{2+} and EDTA respectively. Although a number of studies have been focused on the role of metal ions and anions on cellulase, few studies were related to the relationship between ionic concentrations and enzymatic kinetics. In fact, many metal ions and anions with different concentrations are used in the industry [37]. EDTA was inhibitory to the activities of the cellulase from *Penicillium crustosum.* It is a metal chelating agent and inhibition of the enzymes and suggests that the enzyme activities depend on chemical activities and may contain inorganic groups, which is inactive with EDTA (ethylene diamino tetra acetic acid) [38]. Our results are in agreement with the findings of [39, 40]. EDTA (ethylene diamino tetra acetic acid) was found to be inhibitory to the activity of cellulase used in this study. The control (without metal ions, surfactants, chelating agents and inhibitors) was considered to be having 100% activity [41].

Table 1- Effects of metal ions and EDTA (ethylene diamino tetra acetic acid) on the cellulase enzyme of *Penicillium crustosum*.

Salts	Metals ions	Concentrations (mM)	% Activity	% Inhibition
Control (none)	-	-	100	-
$MgSO_4.7H_2O$	Mg^{2+}	2.0	28.3	71.3
$MnSO_4.5H_2O$	Mn^{2+}	2.0	32	68
$FeSO_4.7H_2O$	Fe^{2+}	2.0	151	-
$CuSO_4.7H_2O$	Cu^{2+}	2.0	30	70
$ZnSO_4.7H_2O$	Zn^{2+}	2.0	124	-

Linewear Burk plot

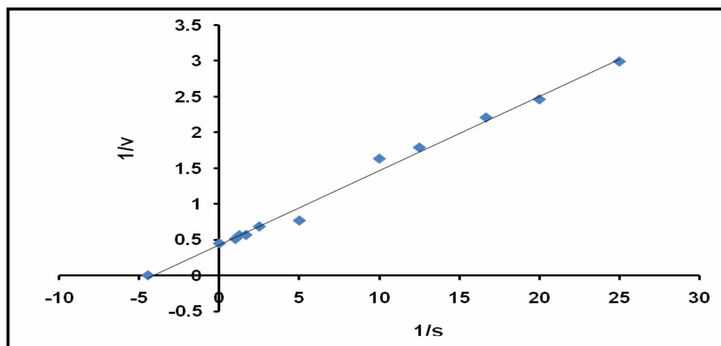

Figure 9- Linewearver- burk plot 1/V versus 1/S of the cellulase of the *Penicillium crustosum*

The enzymatic extracts produced by submerge fermentation (SmF) were used to estimate the cellulase kinetic parameters Km and Vmax. These values were apparent values, since the extracts used were not purified. The Michaelis constant, Km, is a parameter related to the affinity of an enzyme to the substrate, with the value of the substrate concentration at which the enzyme acts at a rate corresponding to half the maximum reaction rate, Vmax. Therefore, the higher the value of the constant Km, the lower the affinity of the enzyme to the substrate. The kinetic parameters K_m and V_{max} of cellulase were determined by typical Michaelis–Menten hyperbolic and Lineweaver–Burk double reciprocal plots $\frac{1}{V} = 0.094 \frac{1}{[S]} + 0.43$. Different concentrations of substrate *viz.* 1 to 10% were used to essay carboxymethyl cellulase produced by *Penicillium crustosum.* The Line weaver-Burk plot (1/V viz 1/[S]) (Fig.9), the V_{max} and K_m values obtained were 2.34 U/ml and 0.22 µg/ml respectively. Our results indicate small Km value of cellulase which demonstrates high affinity of enzyme with the substrate [42]. Our study is in agreement with [43] who observed the effect of substrate level on the cellulase activity of *Aspergillus niger* NRRL 567 with optimum substrate level at 4% CMC. Duenas et al., (1995) [44] also studied maximum substrate level to be 5% with CMCase produced by mixed culture of *Trichoderma reesei* and *Aspergillus phoenicis*. Brimer [45] reported that the difference in substrates levels used by many researchers for different cellulolytic microorganisms depend upon the composition of inducer substrate and optimum media in various studies.

Conclusion

Several microorganisms capable of converting cellulose into simple carbohydrates had been discovered for decades. However, needs for newly isolated cellulolytic microbes still remained unexplored. In this study we have isolated and identified efficient cellulase producing fungi from cellulose rich agro wastes. The isolate *Penicillium crustosum* showed a potential to produce cellulase using agro wastes as a substrate and its enzyme production efficiency increased by optimization of cultural conditions and media components.

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