# Bioprospecting *Rhizopus oryzae* MT4489 for production of polygalacturonase and cellulase enzymes using solid state fermentation

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## Abstract

The secretion of cell wall-degrading enzymes (CWDEs) is a vital virulence factor determining the infectivity of phytopathogenic fungi. In this study, we investigated the potential of *Rhizopus oryzae* MT4489 to secrete polygalacturonase (PGase) and cellulase enzymes using agro-industrial residues for solid state fermentation (SSF). Enzymes produced by *R. oryzae* in different substrates were evaluated at six concentrations: 0.25, 0.50, 0.75, 1.0, 1.25 and 1.50 g $L^{-1}$. Optimization of various substrate parameters including temperature, pH, fermentation time, moisture content and inoculum concentration was also evaluated. This experiment consisted of thirteen treatments, twelve were inoculated with *R. oryzae* strain and the control (without application). The laboratory assay was laid out in a completely randomized design and replicated three times. The highest PGase enzyme production (442.03 µmole $mg^{-1}$) was recorded at 1.25 g $L^{-1}$ in wheat bran inoculated with *R. oryzae*, while corn cobs yielded the greatest cellulase (488.44 µmole $mg^{-1}$) output at same concentration. Optimal levels of enzyme production were obtained at temperature and pH range values of 40 to 50 °C and 5 to 6, respectively. Maximum enzyme yield was recorded at inoculum concentration of $1\times10^5$ and 72 to 96 h incubation time. Wheat bran and corn cobs were the best substrates for producing PGase and cellulase enzymes, respectively. The ability *R. oryzae* to produce CWDEs in this study suggests that it may exhibit virulence factors required to initiate disease in crops. This knowledge will be helpful to breeders in developing inhibitors of the enzymes in plants to stem the ravages of *R. oyzae* rot disease.
**Keywords:** Enzyme, Inoculum, Optimization, Substrate, Virulence.

## Introduction

*Rhizopus* species have been associated with rot disease in many fruits and tuber crops. The infection often results in maceration of the infected tissue and eventual decay of lesions. It is a filamentous fungus which has the ability to thrive on different substrates under suitable environmental conditions where it produces various cell wall-degrading enzymes (CWDEs). *Rhizopus oyzae* Went & Prinsen Geer. has been implicated in fruit rot of stone fruits (Mari *et al*., 2002) and tomato (Hahn, 2004). Dania *et al*. (2021) reported *R. oryzae* as the causal organism of soft rot disease in sweet potato tubers. Similarly, Nishijima *et al*. (1990) attributed papaya soft to the pathogen.

The infective propagules produced by plant pathogens need to pass through the host cell wall which acts as a barrier to ensure successful pathogenesis. The production of CWDEs is an important virulent factor determining the ability of fungi to cause plant diseases (Quoc and Chau, 2017). Degradation of polysaccharide constituents of the complex structure in plant cell wall including cellulose, hemicellulose, pectin and aromatic polymers by CWDEs enables fungi to absorb smaller structural polysaccharide units of the host tissue as nutrients (Zhang *et al*., 2013; Fernandez *et al*., 2014). Although crops differ in the relative structural composition of their cell walls, they contain cellulose submerged within a pectin web, structural proteins, lignin and hemicellulose (Kubicek *et al*., 2014).

Solid state fermentation (SSF) involves the use of agricultural residues and by-products in the production of enzymes from microorganisms. They usually contain a significant reservoir vital for the growth of several plant pathogens particularly fungi notable for converting these substrates to micromolecules. The enzyme secretion ability of fungi is largely influenced by indices such as temperature, moisture content, and fermentation duration, occurrence of nitrogenous substances and pH of substrate (Ferriera *et al.,* 2014).The adoption of SSF method for identification of the secretion of enzymes by plant pathogenic fungi enhances the process of rapid screening of fungal isolates for the production or otherwise of specific enzymes of interest (Peeran *et al*., 2018; Evstatieva *et al*., 2020).

Although submerged fermentation is commonly used in production of industrial enzymes, there is the need to minimize its high cost of production using alternative techniques such as SSF method which is cheap, readily available and reliable (Demir and Tari, 2014). Fungi are most suited to SSF because of their mycelial growth pattern and inherent

physiological processes (Barrios-Gonz´alez, 2012; Soccol *et al*., 2017). This study aimed to evaluate the potentials of *R. oryzae* to produce cellulase and polygalacturonase enzymes and optimization using selected ago-industrial residues as substrates.

## Materials and Methods

### Source of *Rhizopus* isolate

The fungal strain used in this study was *Rhizopus oryzae* MT4489 which was previously isolated from sweet potato tubers and characterized using both morphological and molecular methods (Dania *et al*., 2021). The fungus was initially cultured on a selective medium, potato sucrose agar with the following composition: potato (200 g), sucrose (20 g), agar powder (15 g) and 1 L of sterile distilled water (SDW). The isolate was later purified and maintained on potato dextrose agar in slants and kept in the refrigerator at 4 °C prior to further laboratory analyses.

### Solid State Fermentation for enzyme production

Different agricultural residues (wheat bran, corn cobs, rice bran, banana peels, groundnut shells, coconut residues, yam flour, cassava peels, potato peels, cowpea haulms, plantain peel and sorghum bran) were obtained from local markets and farmers' fields at Ibadan, Oyo State, Nigeria. The substrates were screened for the ability of *R. oryzae* to secrete PGase and cellulase enzymes. The residues were washed three times with SDW in order to eliminate water-soluble compounds, and sun-dried at ambient temperature (29-31°C) for two weeks to ensure almost complete dehydration. The dried agro-residues were ground to a fine powder using a Waring blender 57BL33 Commercial Power, USA prior to cellulase and PGase enzymes assay. Substrate powder was sterilized in 1000 mL conical flask at 121 °C for 20 min and allowed to cool to 45 °C. They were mixed thoroughly with SDW to 75% moisture content. A 100 g powder sample of each test substrate was uniformly spread in sterilized plastic trays measuring 25 × 10 × 8 cm under aseptic conditions. Each substrate was homogenized using a sterile spatula inoculated with spore suspension of *Rhizopus oryzae* MT4489 at $10^5$ spores $g^{-1}$ of inoculum which was quantified using hemocytometer. Enzyme produced by *R. oryzae* in different substrates was evaluated at six concentrations: 0.25, 0.50, 0.75, 1.0, 1.25 and 1.50 g $L^{-1}$.

Control treatments comprised substrates without inoculum application. The various weights were suspended in 100 mL of SDW at 28–30 °C and vortexed in a rotary shaker (200 rpm) for 30 min. The slurry was decanted through a sterile muslin cloth and the content centrifuged for 20 min to eliminate insoluble substances. The transparent supernatant was obtained and used for enzyme analyses. The influence of incubation temperature and pH on enzyme production was assessed at a range of 10–90 °C. The substrates weighing 10 g each were incubated in conical flasks and within a pH range of 3.0 to 11.0 for 48 h (Rao *et al*., 2014). Optimum fermentation time was determined over a period 12–120 h for cellulase and PGase enzymes production.

### Bioassay of *Rhizopus oryzae* for cellulase and PGase enzymes production

*Rhizopus* isolate was evaluated for cellulase enzyme production using the carboxyl methyl cellulose medium with the following composition (g $L^{-1}$): $K_2HPO_4$ (1.0); $MgSO_4$ (0.05), $NaNO_3$ (2.0), $FeSO_4$ (0.01), KCl (0.1), glucose (20) and agar (15). The pH of the medium was adjusted to 4.5 using aqueous HCl solution and sterilized at 121 °C for 15 min and 1.05 k Pas pressure. Petri dishes containing 15 mL of the medium were inoculated with the pathogen using single spore and incubated at 28–30 °C for four days. The Petri dishes were flooded with 15 mL of 5% Congo Red staining solution for 20 min and decanted. The plates were thereafter flooded with 15 mL of 2 M NaCl solution for 10 min and filtrated to expose the clear areas of cellulose production. Pure cultures of the cellulase-producing *Rhizopus* were further purified on PDA plates and sub-cultured into McCartney bottles. The fully grown cultures were refrigerated at 4 °C until when required for further analysis. The secretion of PGase enzyme was evaluated according to the modified method of Yoshida *et al.* (2003). An aliquot of 5 M HCl was added onto 0.5% polygalacturonic acid (Sigma-Aldrich, Burlington MA, USA) and 2.0 mm EDTA in 0.7 mm sodium acetate buffer with pH 5.5. Standard PGase solution EC 3.2.1.15 was added and thereafter adjusted to between 5 and 0.01 units and diluted with SDW up to 10%. The clear supernatant was used as a source of PGase enzyme.

### Optimization of factors influencing enzyme secretion

PGase and cellulase enzymes were secreted using SSF as basal substrate with combination of wheat bran and corn cobs in a ratio of 3:1 (which were the best of the screened substrates for enzymatic activity) as described in the screening experiment above. Optimization of the different factors influencing enzyme secretion was carried out by varying each single factor at once while other parameters remained fixed with three replications for all treatments.

Thermo stability of crude extract was assessed in a water bath between a range of 40 and 90 °C for 30 min. The crude extract was diluted in acetate buffer 200 mM, pH 3.5–6.5 and 0.1 M glycine-NaOH, pH 9.5 to determine the effect of pH variability on PGase enzyme activity (Bari *et al*., 2010), while 0.1 M tris-HCl, pH 7.5-8.5; 0.1 M glycine-HCl, pH 2.5 buffers were used for cellulase.

All samples were incubated at 4 °C for 24 h. Measurement of moisture content was done according to Santos *et al.* (2004). To determine the optimum fermentation time, enzyme secretion was assessed over a 10–120 min incubation period using the modified method of Kaur and Gupta (2017). Moisture content was determined by weighing 100 g at 24 h interval, until a constant weight was attained. Thereafter, the samples were oven-dried at 70 °C for 72 h to obtain dry weight values. Percent moisture content was determined as follows:

$$Moisture = mi - msx100 / ms$$

Where: mi = initial weight of sample, ms = dry weight of sample

**Statistical analysis**

All the treatments in the experiments were replicated three times. Data were subjected to one-way analysis of variance and means were separated using the Duncan Multiple Range Test at 5% level of significance.

## Results

The production of PGase enzyme was generally reduced at lower substrate concentrations. At the lowest level of 0.25 g $L^{-1}$, the quantity of the enzyme produced varied from 58.72 to 251.3 µmole $mg^{-1}$ (Table 1). Yam peels yielded the least amount of enzyme (58.72 µmole $mg^{-1}$), while wheat bran produced the highest quantity (251.13 µmole $mg^{-1}$). The ability of *R. oryzae* to produce PGase varied and increased with higher substrate concentrations. It was observed that the peak of enzyme production in the various substrates was between 1.25 and 1.50 g $L^{-1}$. Overall enzyme secretion by *R. oryzae* was highest (442.03 µmole $mg^{-1}$) at 1.25 g $L^{-1}$ which was significantly higher ($P \leq 0.05$) than other treatments, while yam peels (153.06 µmole/mg) was least suitable substrate. The production pattern of cellulase enzyme by *R. oryzae* also increased with higher substrate concentrations. The highest amount of cellulase produced was recorded in corn cob substrate (488.44 µmole $mg^{-1}$) at 1.50 g $L^{-1}$ which was closely followed by cassava peels with 484.33 µmole/mg (Table 2). Although both substrates were not significantly different in cellulase yield from the fungus, they were, however, significantly higher ($P \leq 0.05$) than other treatments. The quantity of cellulase produced by *R. oryzae* was significantly lower in cowpea haulms and wheat bran with values of 215.20 and 219.09 33 µmole $mg^{-1}$, respectively than the other substrates.

The activity of PGase enzyme increased progressively with rise in temperature and attained a maximum at 40 °C. Thereafter, it showed decline in activity as the temperature was further increased to 50 °C and beyond (Fig. 1). Comparatively, cellulase activity peaked at 50 °C with a steady decrease at subsequent increase in temperature. The relative activity of PGase enzyme increased with pH and peaked at 6.2 with a 100% performance (Fig. 2). Thereafter, the enzyme activity decreased with further increase in pH. Thus, the optimum pH for PGase activity was 5.0. The effect of substrate pH on cellulase enzyme also followed the same pattern. The enzyme attained maximum activity (100%) at pH 6.0. A further increase in the pH beyond this threshold resulted in diminished output of the enzyme. Therefore, the optimal pH of the substrate for the production of cellulase enzyme by *R. oryzae* was 6.0. There was a steady increase in the activity of PGase enzyme secretion beginning from 12 to 120 h of fermentation time (Fig. 3). Enzymatic activity peaked at 96 h with enzyme yield of 600 µmole $mg^{-1}$. Comparatively, the production of cellulase was maximum at 72 h of incubation and declined thereafter reaching the lowest level at 120 h after inoculation. The quantity of enzyme produced increased with rise in substrate moisture content reaching a maximum at 70% (Fig. 4). Higher amount of cellulase was produced (800 µmole $mg^{-1}$) at 70% moisture content than PG (900 µmole $mg^{-1}$) at the optimum moisture content of 80%.

Maximum enzymatic activity of 900 and 800 µmole $mg^{-1}$ was attained by PGase and cellulase enzymes respectively at 100000 spores $g^{-1}$ (Fig. 5). The enzymatic yield progressed steadily with increased inoculum concentration and peaked at 100000 spores/g-1, thereafter there was a decline of activity for both enzymes.

## Discussion

*Rhizopus oryzae* MT334 produced significant amount of PGase and cellulase enzymes among all the twelve substrates evaluated in this study. Several authors have reported that agro-industrial residues are good substrates for the secretion of pectinolytic enzymes (Demir *et al*., 2014; Rao *et al*., 2014; Kaur and Gupta, 2017). The secretion of PGase enzyme by *Bacillus* species in *Solanum tuberosum* (Dharmik and Gomashe, 2013) and wheat bran (Chiliveri *et al*., 2016) has also been reported. Although treatments that were inoculated with *R. oryzae* produced significantly higher amounts of both PGase and cellulase, the uninoculated control substrates still yielded some amount of the enzymes though in lower proportions. It must be stated that although plants have the inherent ability to produce cellulase enzymes, they however lack the potency to initiate bulk breakdown of cellulose polymers. Instead, plant-produced cellulase is mainly involved in the arrangement of cellulose microfibrils during its biosynthesis (Carrasco *et al*., 2016). Similarly, Rhadha *et al*. (2019) submitted that although polygalacturonasese enzymes are produced mainly by microorganisms, most plants are also able to secrete these biological substances at the ripening stage in fruits.

Enzyme production increased progressively and reached a maximum of 40 °C and 50 °C for

PGase and cellulase, respectively in this study. Abbasi *et al*. (2011) reported that PGase produced by fungi is more active at a temperature of 40 to 55 °C. These enzymes interfere with the lamella tissue which reduces viscosity of the pectin medium (Gomes *et al*., 2011). Patil *et al*. (2012) reported that the optimum temperature range of PGase enzyme was between 10 and 40 °C, while thermal stability was halved at 50 °C after 4 h incubation period. Incubation temperature is a very essential factor determining the effectiveness of fermentation stage in the production of enzymes by microbes because of its ability to aid their growth and metabolic processes. Most times, the quality of enzymes relating to the influence of temperature is dependent on production indices such as incubation temperature, type of microorganism and nutrient availability in the substrate. Temperature enhances enzyme secretion rate by microorganisms because of the increase in heat generated during substrate fermentation, which spontaneously affects its sporulation potential and product yield. Although the heat produced in such circumstance is desired in composting of substrate for agricultural production, it is detrimental in biological processes involving enzyme secretion since they are usually denatured at higher temperatures (Maller *et al*., 2011). Kaur and Gupta (2017) suggested that the decrease in enzyme secretion by *Bacillus* species at high temperatures above optimal levels may be attributed to a reduction in the substrate moisture content.

Temperature variation is important in the analysis of enzyme activity because it has the ability to either activate or deactivate the activity of enzymes produced by microorganisms depending on the prevailing circumstances. An arbitrary increase in substrate temperatures below or above optimum threshold level will degrade the constituent protein involving catabolism of large units of polypeptides (Gomes *et al*., 2011). In contrast, inactivation of enzymes at cooling occurs at lower temperatures which decrease the interactive potential and dissociation of small subunits of polypeptides thereby reducing the activity of enzymes (Lara-Márquez *et al*., 2011).

Maximum PGase activity was attained at pH 6.0 while the optimal pH for cellulase enzyme was 5.0. The results agree with previous findings of Zheng *et al.* (2003) who reported that PGase secreted by most fungi are more potent at pH values between 3.5 and 6.5. Microbial growth and the synthesis of CWDEs are regulated by substrate pH. The acidity or alkalinity of the growth medium also plays a prominent role in determining cell wall permeability and enzyme stability. The optimum pH for the production of PGase and cellulase enzymes by *R. oryzae* was 5.0 and 6.0, respectively. Rao *et al*. (2014) reported a pH optimal range of 4.0 to 5.0 for the secretion of pectinase enzyme in substrate inoculated with *Aspergillus* species. Enzymes as proteins, are influenced by variation in substrate pH. Higher or lower pH values will activate ionization of amino acid molecules which alters the structural composition and functionality of proteins. Extremes of pH will cause absolute deactivation of several enzymes. The secretion of enzyme by microbes is often affected by the composition of growth medium and physical factors including thermal stability, fermentation time, pH and inoculum concentration (Muthulakshm *et al*., 2011).

Optimum amounts of cellulase (800 µmole $mg^{-1}$) was produced at 70% moisture content while PG production reached a maximum (900 µmole $mg^{-1}$) when the moisture content was elevated to 80%. The results are consistent with the findings of previous authors who reported optimum moisture content for cellulase secretion at 72% (Chaurasia *et al*., 2013) and 80% for PGase activity (El-Shishtawy *et al*., 2014). The optimum moisture content facilitates the permeability of nutrients across cell wall and this encourages maximum production of enzymes in microorganisms. Joko *et al*. (2014) attributed reduced enzyme secretion at lower moisture levels by microbes to decreased growth because of poor solubility of nutrients contained in substrate medium, while depreciation in enzyme activity at higher moisture levels may be due to inadequate oxygen circulation within the substrate leading to plasticity. Moisture is a vital determinant of enzymatic activity which affects substrate texture and nutrient utilization by the pathogen and the ultimate by-product. A higher moisture content will lead to a proportionate reduction in permeability of substrate and decreased oxygen circulation in the substrate. The implication is that there will be decline in the activities of aerobic microorganisms, especially bacteria. Also, moisture stress at low levels will result in poor mobilization and circulation of essential nutrient elements required for the pathogen growth and replication. This reduces the secretion of required enzymes (Gaur and Gupta, 2017).

Variation in the duration of fermentation of substrate evaluated between 12 to 120 h showed that the production of PGase and cellulase enzymes reached a maximum at 72 and 96 h, respectively after inoculation with *R. oryzae*. This implies that the production of cellulase by the pathogen took a shorter time relative to PGase enzyme. It was observed that there was a decrease in enzyme activity with a further increase in fermentation time which may be attributed to depletion of requisite nutrients. Jayani *et al*. (2010) reported an optimum incubation time of 72 h for the production of PGase by *Bacillus sphaericus*. Also, Bensal *et al*. (2012) found that the production of cellulase enzyme in *Aspergillus* decreased with incubation time.

The activity of both enzymes increased until they attained a peak at $10^5$ spores $g^{-1}$ inoculum concentration and then began a decline. Various

authors have reported that the optimal range of inoculum concentration for initiation of most fungal infection is between $10^5$ and $10^6$. (Dania *et al*., 2021). There is usually competition of nutrients in the substrate medium at higher concentrations with a proportionate decrease in the quantity of enzymes produced. Inoculum concentration is a vital biological factor, determining biomass yield during microbial fermentation. Optimal yield tends to occur when an equilibrium is reached between biomass and nutrients in the substrate (Abdullah *et al*., 2016).

## Conclusion

The study has demonstrated that various agricultural residues are good substrates for the production of PGase and cellulase enzymes by *R. oryzae*. Wheat bran and corn cobs were the best substrates for the production of both enzymes. The ability to produce CWDEs in all the test substrates suggests that the pathogen possess virulence factors necessary for disease infection in plants. Additionally, it will be helpful to breeders in developing inhibitors of the enzymes in plants to stem the ravages of *R. oyzae* rot disease.

**Table 1:** Effect of substrate concentration on activity of polygalacturonase enzyme (µmole $mg^{-1}$) at varying concentrations.

| Substrate | Concentration (g $L^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | **0.25** | **0.5** | **0.75** | **1.0** | **1.25** | **1.5** |
| Wheat bran | 251.13a | 301.08a | 366.21a | 400.33a | 442.03a | 455.44a |
| Corn cobs | 87.55f | 103.22h | 131.22g | 150.62g | 155.33g | 170.73h |
| Rice bran | 177.08b | 223.8c | 287.02b | 333.45c | 342.77d | 361.11d |
| Banana peels | 178.29b | 203.05d | 256.44c | 308.22d | 401.01b | 431.3b |
| Groundnut shells | 77.31g | 120.89f | 143.17f | 153.19g | 180.17f | 188.55g |
| Coconut fibre | 102.38e | 111.54g | 163.33d | 177.03f | 199.09e | 249.52e |
| Yam peels | 58.72h | 83.41i | 101.1h | 113.66h | 138.1h | 174.06h |
| Cassava peels | 78.33g | 90.04i | 105.37h | 123.66h | 141.09h | 173.77h |
| Potato peels | 101.22e | 112.08g | 130.98g | 148.21g | 155.06g | 185.62g |
| Cowpea haulms | 155.88c | 246.18b | 288.01b | 365.32b | 388.08c | 396.99c |
| Plantain peels | 122.01d | 134.88e | 153.45e | 187.72e | 201.33e | 247.33e |
| Sorghum bran | 100.87e | 111.11g | 134.55g | 177.77f | 208.32e | 222.44f |
| Control | 10.73i | 17..08j | 22.28i | 27.70i | 31i | 39.66i |

Within a column, means followed by same letter are not significantly different using Duncan Multiple Range Test at $P \leq 0.05$.

**Table 2:** Effect of substrate concentration on activity of cellulase enzyme (µmole $mg^{-1}$) at varying concentrations.

| Substrate | Concentration (g $L^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | **0.25** | **0.5** | **0.75** | **1.0** | **1.25** | **1.50** |
| Wheat bran | 101.66h | 124.09i | 155.33g | 196.01h | 233.42g | 219.06h |
| Corn cob | 277.65a | 344.77a | 397.4a | 422.71a | 447.33b | 488.44a |
| Rice bran | 133.60f | 151.76f | 208.4e | 278.65e | 338.33e | 383.08d |
| Banana peels | 88.82i | 122.47i | 143.05h | 154.77i | 208.90i | 291.88f |
| Groundnut shells | 144.22e | 180.31e | 231.77d | 282.11e | 300.88f | 321.02e |
| Coconut fibre | 117.33g | 125.20i | 169.23f | 202.41g | 217.67h | 250.82g |
| Yam peels | 203.33c | 267.34c | 312.65b | 352.66d | 461.32a | 474.11b |
| Cassava peels | 233.06b | 277.81b | 320.14b | 396.42b | 460.22a | 484.33a |
| Potato peels | 113.01g | 144.67g | 171.90f | 231.02f | 402.63c | 450.40c |
| Cowpea haulms | 87.31i | 122.41i | 154.07g | 198.03h | 237.22g | 215.20h |
| Plantain peels | 177.01d | 207.77d | 282.64c | 301.24d | 378.01d | 389.72d |
| Sorghum bran | 104.51h | 140.03h | 171.88f | 227.33f | 301.42f | 318.70e |
| Control | 4.14j | 11.42j | 16.91±i | 20.88±j | 28.03i | 33.99i |

Within a column, means followed by same letter are not significantly different using Duncan Multiple Range Test (DMRT) at $P \leq 0.05$.

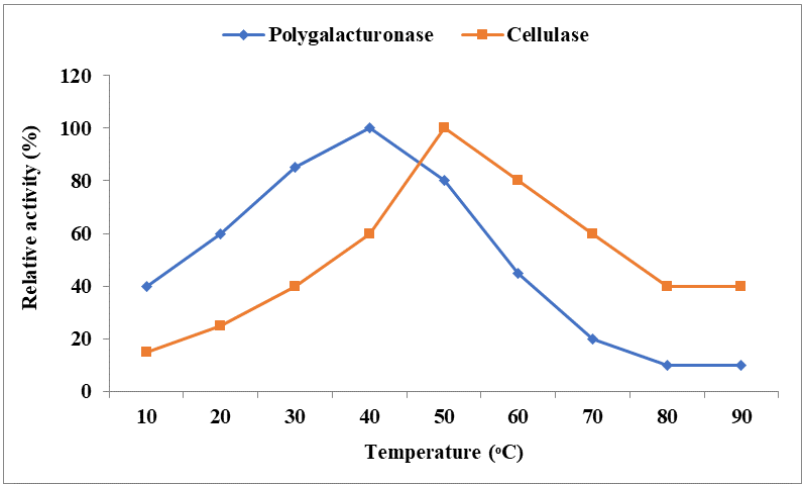


**Fig. 1:** Effect of temperature on polygalacturonase and cellulase enzymes of *R. oryzae* MT4489.

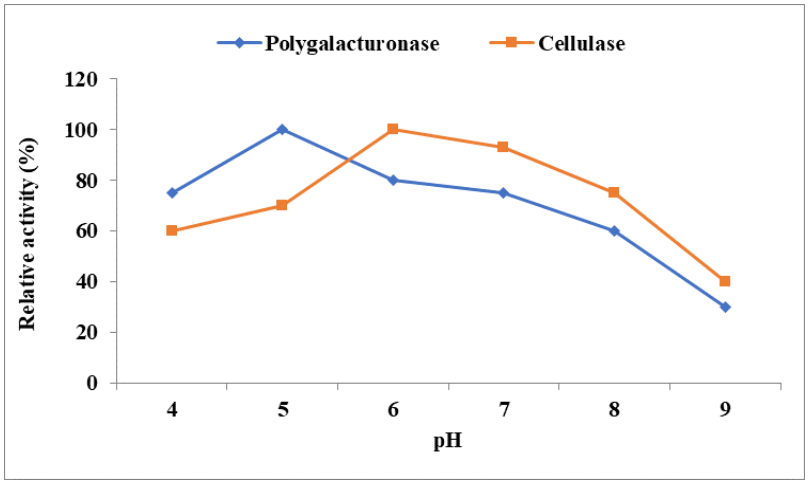


**Fig. 2:** Effect of pH on crude extract of polygalacturonase and cellulase enzymes of *R. oryzae* MT4489.

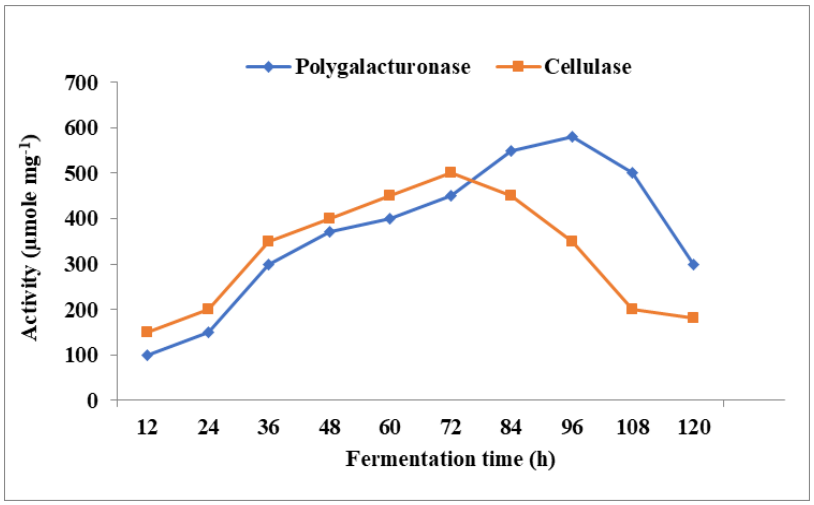


**Fig. 3:** Effect of fermentation time on crude activity of ploygalacturonase and cellulase enzymes of *R. oryzae* MT4489.

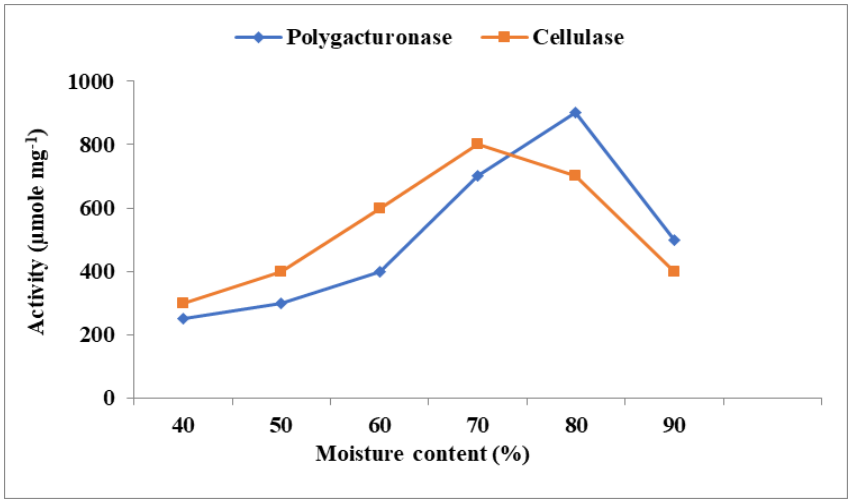


**Fig. 4:** Effect of moisture content on production of polygalacturonase and cellulase enzymes of *R. oryzae* MT4489.

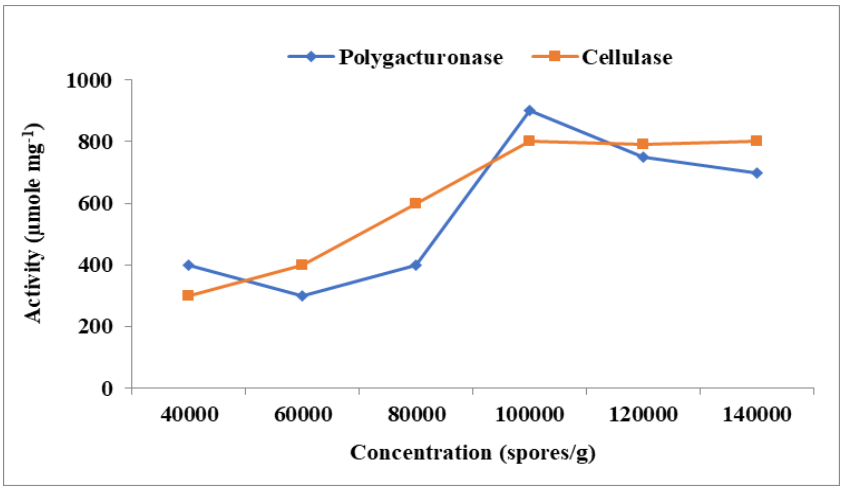


**Fig. 5:** Effect of inoculum concentration on the production of polygalacturonase and cellulase enzymes of *R. oryzae* MT4489.

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