EFFECT OF CULTURE CONDITION ON PROTEASE PRODUCTION OF PROTEOLYTIC FUNGI FROM BEAN CROP SOIL AND PADDY MILL SOIL

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Abstract

The present study is based on the characterization of protease producing fungi from the bean crop soil in Vegetable and Fruit Research Development Centre (Hlegu Township) and paddy mill soil in Daik-U Township. Screening of fungi was done by employing skim milk agar (SMA) plating technique at room temperature and the zone of proteolysis was noted. The two isolates that gave high positive protease activity results in screening identified as Penicillium sp. (V2) and Cladosporium sp. (D12) by analyzing the macroscopic and microscopic morphological criteria. These fungi were used for protease production via submerged fermentation (SmF). Effects of four different fermentation media (Medium I, Medium II, Medium III and Medium IV), different fermentation period, 1% carbon sources, 1% nitrogen sources, different substrate and casein substrate concentration on protease production were investigated. The highest enzyme activity was obtained with production medium II at four days with 1% casein as best substrate, 1% glucose as carbon source and yeast extract as nitrogen source.

Keyword: Protease activity, Penicillium sp., Cladospordium sp., bean crop soil and paddy mill soil

Introduction

Protein is one of the three major food groups needed for proper nutrition. Proteolytic enzymes or proteinases are the group of enzymes whose catalytic function is to hydrolyze (break down) proteins. Microbial proteases are an important family of proteases and have an advantage over animal and plant proteases, as microbes can be cultured on a large scale in less time, and the growth conditions can easily be optimized in lab conditions. Fungi are an important component of the microbiota typically constituting more of the soil biomass than bacteria depending on soil depth and nutrient condition (Saxena et al., 2017). Carbon sources are very important for microbial growth as they provide carbon skeletons needed for synthesis of new organic molecules (anabolism). The growth of the microbes and enzyme production requires nitrogen sources. Most of the microorganisms require fixed nitrogen source to synthesize proteins, nucleic acids and other cellular components (Suseela et al., 2017).

Proteases are also having extensive applications in the development of environmentally friendly technologies as well as in several bioremediation processes. Several classification systems currently available, provides rich and information about each and every identified protease (Pansuriya and Rekha., 2010). Proteases constitutes large and complex group of enzymes that plays important role in nutrition and various applications in medical and industrial field. A variety of organisms such as bacteria, fungi, yeast, actinomycetes are known to produce these enzymes (Kalpanadevi et al., 2008). Molds of genera Aspergillus, Penicillium and Rhizopus are especially useful for proteases (Sandhya et al., 2005).

Protease is the important industrial enzyme invests an accounting for about 60% of the total enzyme market in the world (Niyonzima and More., 2013). Microorganisms are the most common

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source of commercial enzymes due to their physiological and biochemical properties, cultural conditions and ease of cell manipulation. The growth of industrial enzyme market has expanded to nearly 85 enzymes, which are currently in commercial production. The protease enzyme constitutes two third of total enzyme used in various industries. Furthermore, study was undertaken the assessment of culture conditions on protease production from fungi isolate by submerged fermentation and to evaluate the effect of different media, fermentation period, carbon sources, nitrogen sources, different substrates and substrate concentration on protease activity.

Materials and Methods

Sample collection and isolation of fungi

Soil samples used from different environmental sources like bean crop soil in Vegetable and Fruit Research Development Centre (Hlegu Township) and paddy mill soil in Daik-U Township were collected aseptically from 6 inches depth in sterile zip lock plastic bag. One gram of each soil sample was serially diluted in sterile condition. From diluted suspension 100µL was spread on potato dextrose agar (added 1% casein) medium, incubated at room temperature for three to five days and was monitored every day for the growth of fungal colony. Further these fungi were sub-cultured on potato dextrose agar (added 1% casein) to obtain the pure strain (Sharma et al., 2015). Among them those showing maximum zone of proteolysis were selected for this investigation, named as isolated V (bean crop soil) and D (paddy mill soil).

Primary screening for proteolytic fungi by casein hydrolysis plate assay (Sethis and Gupta., 2015)

Preliminary screening was done by inoculating the fungal isolates on skim milk agar medium containing casein as protein source at room temperature for one to seven days. After incubation, the plates were observed for clear zone of hydrolysis surrounding the colony. The protease was exhibited by a zone of proteolysis, which was exhibited by a zone of proteolysis which was demonstrated by clear area surrounding the fungal growth. The zone diameter was measured in cm and results are recorded (Warcup,1950 and Abe et al.,2015). The enzyme index (EI) expressed as R/r, which R is the degradation zone diameter and r are the colony diameter (Hankin and Anagnostakis,1975, Abe et al.,2015). The species that exhibits maximum clear zone was selected for further identification.

Identification and characterization of protease enzyme

The two fungi were identified according to the Barnett 1960 and Dube 1983.

Fermentation condition (Anson, 1938)

The 100ml of synthetic media containing casein 0.5g, dextrose 0.5g, yeast extract 0.5g, magnesium sulfate 0.02g, dipotassium hydrogen phosphate 0.1g and sodium carbonate 0.01g was taken separately into 300ml one Erlenmeyer flasks and adjusted to pH 8.0. The cotton flasks were then autoclaved at 121°C for 20 mins and allowed to cool to about at room temperature. The contents of the flasks were inoculated with one loop of fungi and incubated at room temperature in a rotary shaker operated at 250 rpm for 4 days. At the end of fermentation period the contents were centrifuge at 10000 rpm (4°C) for 10 mins at room temperature, and the cell free supernatant was used of crude enzyme for enzyme activity assay.

Protease specific activity assay

Protease specific activity assay was performed according to the method of Colowick and Kaplan, 1955. Briefly, 1.3ml of 1% casein dissolved in 50mM potassium phosphate buffer (pH 7) was added to both sample and blank tubes and incubated at room temperature for 30 mins. Subsequently, 0.7ml of crude protease extract was added to only the sample tube and incubated for 30 mins at room temperature. The reaction was stopped with the addition of 3ml of 5% trichloroacetic acid (TCA) solution in both the sample and blank tubes. The mixture was then incubated for 30 mins at room temperature. UV-visible spectrophotometer was used to determine the absorbance value at 275nm. The more tyrosine that is released from casein, the more chromophores are generated and the stronger the activity of the protease. Absorbance values generated by the activity of the protease are compared to a standard curve, which are generated by reacting known quantities of tyrosine with the amount of tyrosine in microgram. From the standard curve the activity of protease samples can be determined in terms of Units, which is the amount in microgram of tyrosine equivalents released from casein per mL minute under assay condition described by Abdilbar et al., 2021.

Effect of different media (Sarker et al., 2013) and fermentation period on protease enzyme activity (Naik et al., 2013)

In this experiment, production of protease by submerged fermentation was study in five different basal medium (medium I, medium II, medium III and medium IV) the composition of fermentation Medium I was casein 10g, yeast extract 10g, glucose 20g, K2HPO4 1g, KH2PO41g, MgSo40.2g, pH 7.0, Medium II was casein 5g, glucose 10g, yeast extract 5g, K2HPO41g, MgSo4.7H2O 0.2g, Na2CO3 0.1g, pH 8.0, Medium III was casein 5g, K2HPO41g, MgSO4.7H2O 2g, NaNO32g, FeSO4.7H2O 0.01g, KCL 2g, pH 7.0 and Medium IV was casein 5g, glucose 25g, yeast extract 0.5g, K2HPO42g, (NH4)2SO41.5g, MgSO4.7H2O 0.3g, CaCl2 0.3g, urea 0.3g, pH 9.0 . The effect of fermentation periods on protease production was determined by incubation for 1 to 7 days. The enzyme activity was measured under assay condition.

Effect of carbon sources on protease enzyme production (Prabhakaran et al., 2015)

The medium was prepared with composition same as the fermentation media but the carbon sources is replaced by different other carbon sources on protease production was investigated. The protease activity was determined as mentioned above.

Effect of nitrogen sources on protease enzyme production (Akcan, 2012)

The influence of different nitrogen sources 1% of beef extract, yeast extract, peptone, KNO3 and NaNO3 were determined for protease production, used incubated the culture at room temperature for the best fermentation period. The cell free filtrate was used to assay for protease activity.

Effect of different substrates on protease enzyme production (Shivakumar Srividya, 2012 and Bijay et al., 2017)

Protease activity was carried out with 1 % various protein substrates including gelatin, casein, wheat bran and soya powder. These substrates present in selected production medium were substituted with 1% of each substrate. After incubation at room temperature for the best fermentation period with each of the substrates separately, enzyme activity was measured under enzyme assay.

Effect of substrate concentration on protease enzyme production (Saxena et al., 2017 and Hariharan et al., 2018)

The effect of casein substrate concentration ranging from (0.5%, 1%, 1.5% and 2%) at room temperature for the best fermentation period using the method described above.

Results

Isolation and screening of fungi

Eleven and twelve soil fungal isolates were isolated on potato dextrose agar (added 1% casein) medium from bean crop soil in Vegetable and Fruit Research Development Centre, Hlegu Township and paddy mill soil in Daik-U Township respectively. These isolated fungi by using serial dilution of pour plate method at room temperature for three to five days.

Preliminary production of proteolytic enzyme on skim milk agar (SMA) medium

The fungal isolates were screened for the ability to produce protease enzyme on skim milk agar (SMA) medium by streak plate method. Among all isolated fungi; two strains were showed clear zones formations around the fungal growth indicating the property of protease. Although only two fungi V2 and D12 showed maximum proteolytic activity with the zone of about 2.5cm for five days and 2cm for six days respectively as shown in figure (1 and 2). Pure colonies were obtained with distinct morphological features.

Morphological characteristics of genus level on prominent fungi

In the present investigations, the selected fungal strains were done by identification of macroscopic and microscopic characters using standard method in table (1). The fungal strain was identified as Penicillium sp. (V2) and Cladosporium sp. (D12) as shown in table (1) and figure (1 and 2).

Table (1) Characters of proteolytic fungi isolated from bean crop soil and paddy mill soil

No	Sources	Macroscopic characters of proteolytic fungi	Microscopic characters of proteolytic fungi	Species
1	Bean crop soil in Vegetable and Fruit Research Development Centre (Hlegu Township)	The mycelium color green color inside, white color peripher and yellow in reverse view.	Conidiospores arising from the mycelium singly, branched near the apex to form a brush-like, conidia-bearing apparatus, ending in a group of phialides, conidia brightly colored in mass, ovoid, in dry basipetal chains.	*Penicillium* sp. (V2)
2	Paddy mill soil (Daik-U Township)	The mycelium color is brown in surface view and black in reverse view.	Conidiophores dark, branched variously near the apex, clustered, conidia dark, lemon-shaped, pigment present.	*Cladosporium* sp. (D12)

Forward view | Reverse view | Proteolytic activity | Micrograph of Penicillium sp. (X400)

Figure. 1. Morphological and microscopical characters of isolated fungi from V2

Forward view | Reverse view | Proteolytic activity | Micrograph of Cladosporium sp. (X400)

Figure. 2. Morphological and microscopical characters of isolated fungi from D12

Optimization parameters of two selected fungi

The present study of four different media, Penicillium sp. and Cladosporium sp. were showed Medium II as shown in figure (4). The protease production by microorganisms is greatly influenced by incubation period. The optimum protease activity was found four days for both species as shown in figure (5). Further increase and decrease in incubation period lead to decline in enzyme activity. The production media was supplement with 1% carbon and nitrogen sources were obtained glucose and yeast extract as shown in figure (6 and 7). The effect of 1% various substrates Penicillium sp. and Cladosporium sp. was exhibited the best activity in casein as shown in figure (8). The range of substrate concentration (0.5% to 2%) which were used in this study to check the optimum protease activity of both species were found 1% concentration as shown in figure (9).

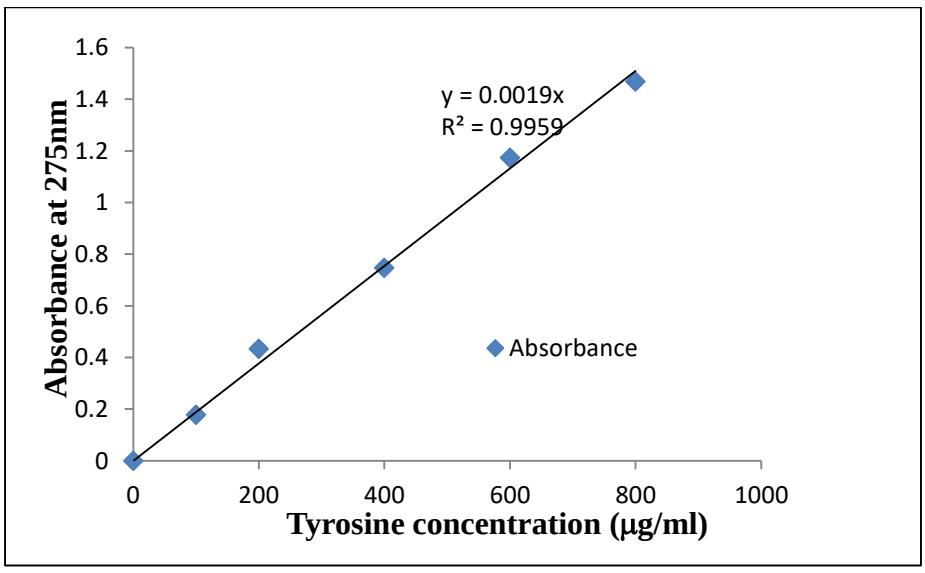

Figure.3. Plot of absorbance as a function of standard tyrosine concentration

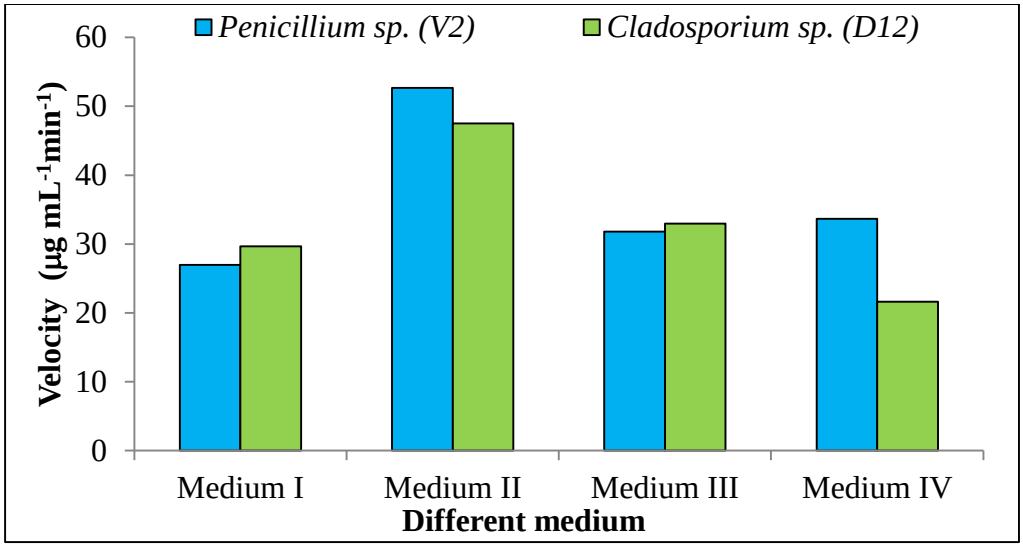

Figure. 4. Plot of velocity as a function of different medium for *Penicillium* sp. and *Cladosporium* sp.

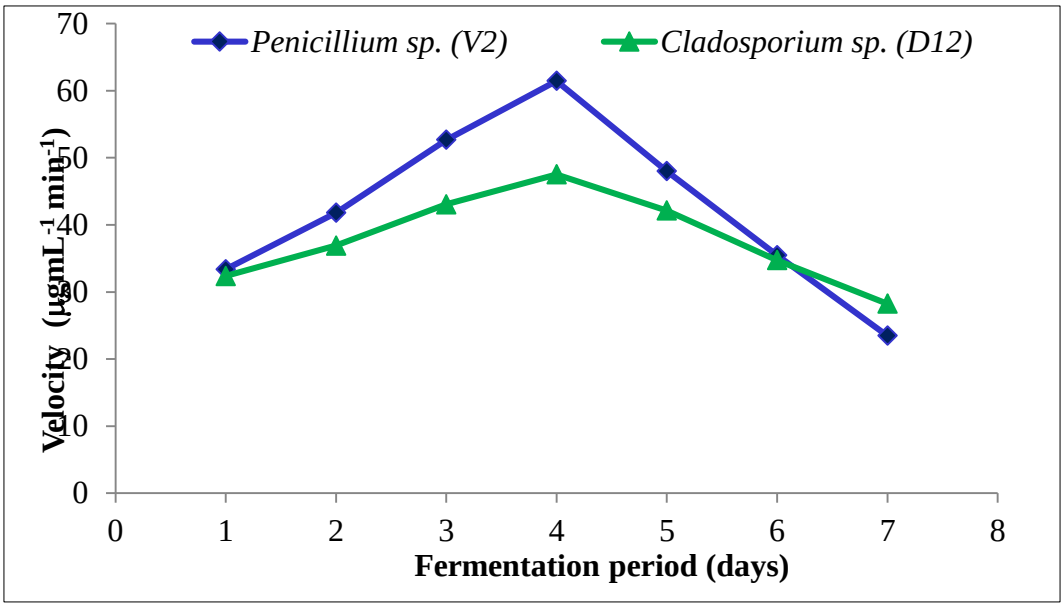

Figure. 5. Plot of velocity as a function of fermentation period for *Penicillium* sp. and *Cladosporium* sp.

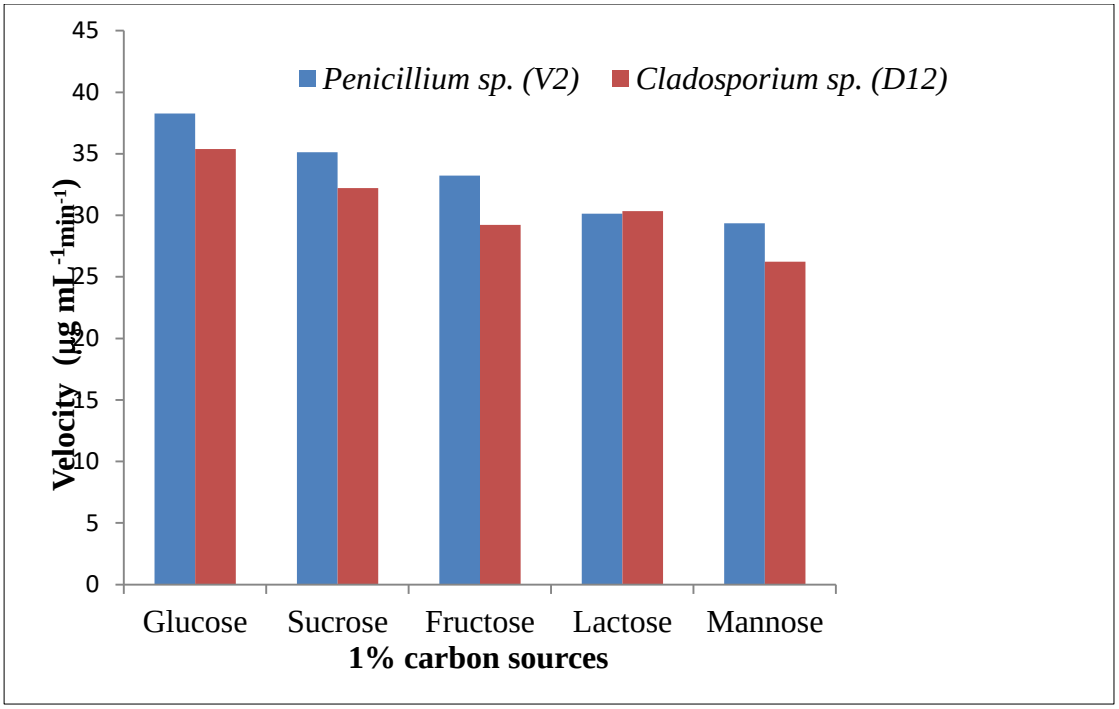

Figure 6. Plot of velocity as a function of carbon sources for *Penicillium* sp. and *Cladosporium* sp.

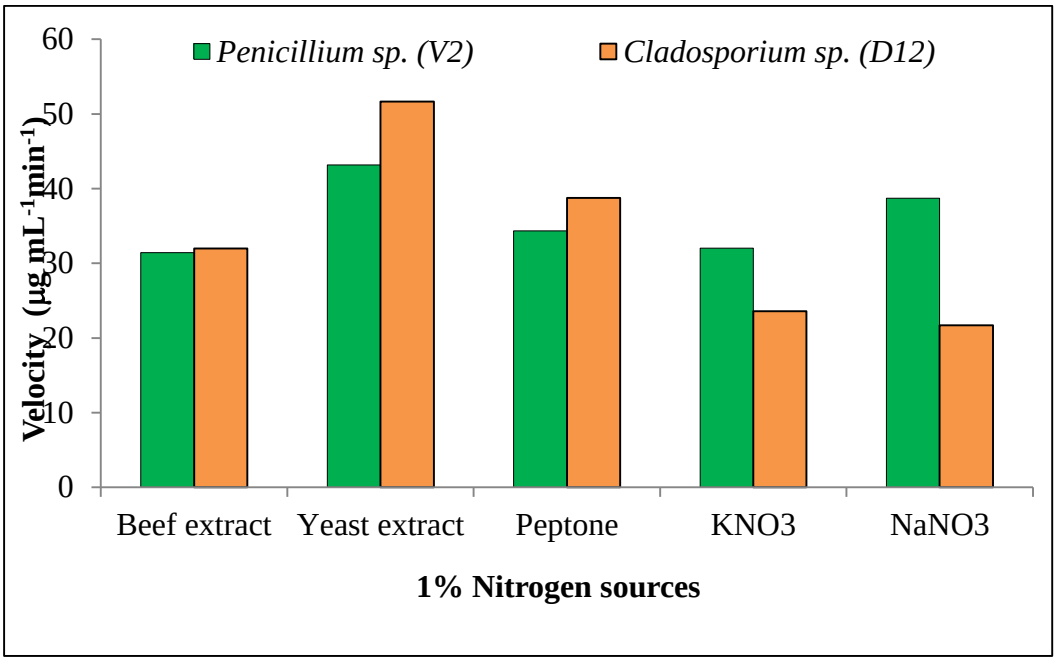

Figure 7. Plot of velocity as a function of nitrogen sources for *Penicillium* sp. and *Cladosporium* sp.

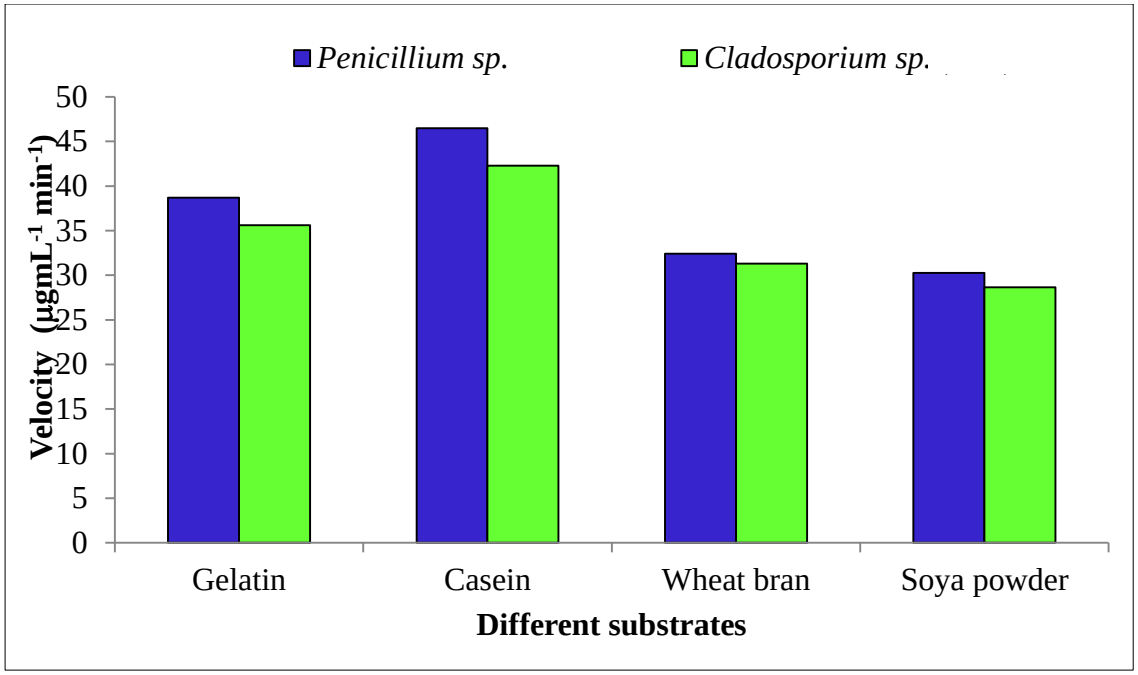

Figure 8. Plot of velocity as a function of different substrates for *Penicillium* sp. and *Cladosporium* sp.

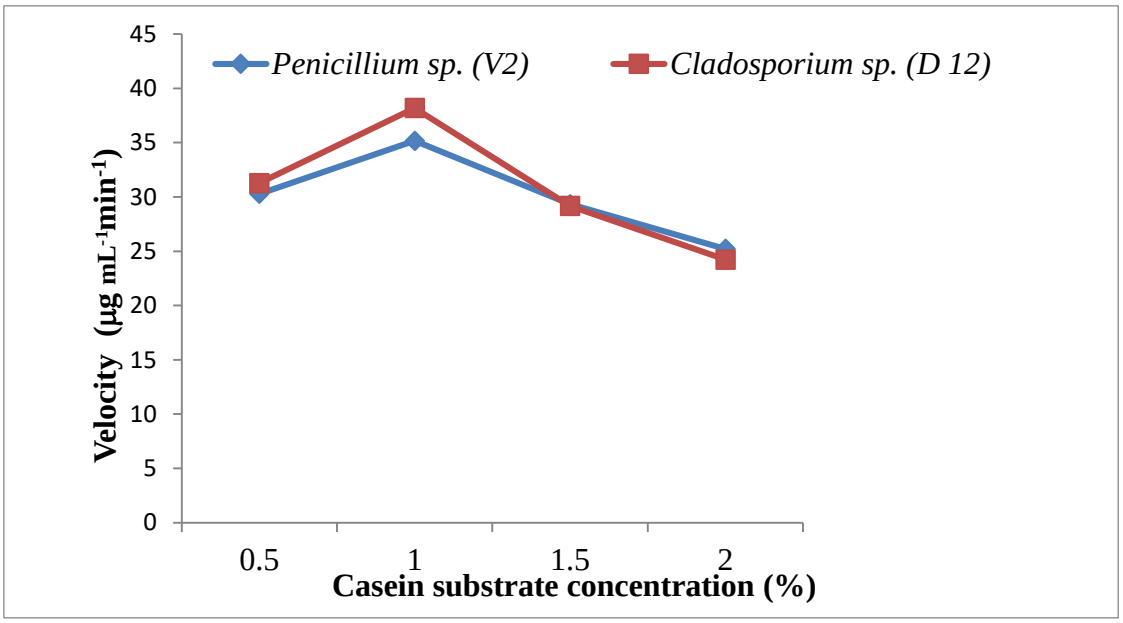

Figure 9. Plot of velocity as a function of casein substrate concentration for *Penicillium* sp. and *Cladosporium* sp.

Discussion and Conclusion

The present study investigated the protease fungi obtained from bean crop soil in Vegetable and Fruit Research Development Centre (Hlegu Township) and paddy mill soil in Daik-U Township. The two strains Penicillium sp. (V2) and Cladosporium sp. (D12) were shown as a maximum clear zone of hydrolysis on skim milk agar (SMA) medium for preliminary study of protease enzyme as shown in figure (1 and 2). Casein is decomposed by the protease to produce

degraded stages of protein products which include tyrosine among them (Haldane, 1965). The enzyme activity of the culture supernatant was determined by measuring the amount of tyrosine released from casein. Therefore, casein was chosen as the basal substrate in the experiment of enzyme-catalyzed reactions in this research works. This result is in accordance with the earlier report of Pelczar and Reid, 1972.

Enzyme properties of some parameter such as different fermentation media, culture period, 1% carbon sources, 1% nitrogen sources, different substrate and casein substrate concentration which were determined using spectroscopic method Wiseman 1975 and Oser 1976. In the present work, the Penicillium sp. and Cladosporium sp. were inoculated into four different submerged fermentation media and different fermentation period at room temperature to obtain the best protease enzyme production. In the present data, the optimum fermentation medium for two strains prominently showed medium II as shown in figure (4). During the fermentation period of Penicillium sp. and Cladosporium sp. prominently showed that 4 days fermentation period provide optimal result as shown in figure (5). In similarly, study was carried out by Gupta et al., 2002. Among the monosaccharides and disaccharides used in suitable 1% carbon sources such as glucose, sucrose, fructose, lactose and mannose, only glucose clearly exhibited in the selected strain as shown in figure (6). Wang and Lee., 1996; EI-Shore et al., 1997; Feroz, 2013 and Benluvankar et al., 2015 also reported that glucose proved to be the best carbon source for improving the productivity of the protease by Conidiobolus coronatus, Aspergillus niger and Penicillium sp. LCJ228. In the case of suitable 1% nitrogen sources (organic and inorganic) such as beef extract, yeast extract, peptone, KNO3 and NaNO3 only yeast extract clearly exhibited in the two selected strains as shown in figure (7). Which is in agreement with the reported of Phadatare et al., 1993, Ashour et al., 1996 and Mohammed, 2015. Different substrates were used for the production of proteolytic enzyme such as gelatin, casein, wheat bran and soya powder. The best substrate for obtaining maximum enzyme activity was found to be casein as shown in figure (8). Similarly result had been reported by Mulimani et al., 2002, Shankar et al., 2011 and Kumar et al., 2012. As casein is a protein, its role in induction of protease synthesis is evident from these results.

The effect of different concentration of casein ranging from 0.5% to 2% were used to determine the activity of protease enzyme. It was found that the higher concentration 1% of casein significantly enhanced maximum protease activity by Penicillium sp. and Cladosporium sp. as showed in figure (9). This result of present value has already been agreed with the literature of Battaglino, 1991; Kamath et al., 2010 and Saxena et al., 2017.

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