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SUBSTRATE SERVING FOR CULTURE OPTIMIZATION AND PROTEASE PRODUCTIVITY BY *Penicillium Notatum* STRAIN ON DEPROTEINISED FOLIAGE EXTRACT FROM LUCERNE AND BEET

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ABSTRACT

When Penicillium notatum and Aspergillus niger grown on Lucerne DPJ by serving 1 % and 2 % of the substrates, viz., casein, starch and carboxymethyl cellulose (CMC), it showed the promising results of mycelial biomass and hydrolytic enzyme activities. Present attempt was on growth of species Penicilium notatum and Aspergillus niger on beet and lucerne foliage DPJ broth medium as compared with control glucose nitrate (GN) medium. Aspergillus niger not showed its growth on DPJ when enriched with casein and starch but there was growth on DPJ alone when substrates were not added. Therefore DPJ itself has the efficacy in initiating fungal growth. Beet DPJ inhibited the growth of Penicillium notatum when substrates were added, and only 1% of CMC was found responsible to initiate its growth by addition in DPJ. Penicillium notatum thrieved well on lucerne DPJ. Increasing concentration of casein in Lucerne DPJ enhanced rate of enzyme protease. Beet DPJ found having its antimicrobial influence. The objective of research was optimization of enzyme productivity by DPJ for industrial use.

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Contribution/Originality: This study is one of few studies performed by the influence of deproteinised leaf extract from lucerne and beet leaves by adding the substrates in it and subjecting it for the estimation of enzymes. The secondary metabolites can be having the quality of specific herbal influence along with the fungal effects like antibiotics or the vitamins.

1. INTRODUCTION

Study assesses sutability of cabbage as substrate of solid state fermentation for production of cellulase enzymes by *Penicillium notatum*, optimises fermentation condition for maximum yield of enzymes, and presents characterisation of enzymes and enzyme kinetics. Cellulase concentration was estimated for carboxymethyl cellulase (CMC) [1]. In the past few decades, starch degrading enzymes like α-amylase by fungi like *Penicillium notatum* etc have received a great deal of attention because of their perceived technological significance and economic benefit [2, 3]. The synthesis of a cellulolytic enzyme from *Penicillium notatum* assay is based on the increase in reducing groups following the incubation of carboxymethylcellulose (CMC) with the enzyme [4].

As DPJ from beet leaves is utilised for the mycelial growth of *Penicillium*, it is found that endophytic fungi like *Alternaria* found in leaves of beet [5]. This fungi gets sterilised in autoclave before inoculation. DPJ from lucerne was consumed for the growth of *Penicillium notatum*, it was searched that Lucerne

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(*Medicago spp.*) is an important forage crop grown worldwide. Lucerne plants contains appreciable amount nutrients found in leaf protein research. In Beet leaves, the presence of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids was investigated. Among the SFA, pentadecilic (15:0), palmitic (16:0), and stearic (18:0) acids were identified. The MUFA identified were pentadecenoic (15:1n-9), palmitoleic (16:1n-7), oleic (18:1 n-9), and vaccenic acids (18:1n-7). The commonly PUFA present in the lipid fraction of leaves are linoleic (LA, 18:2n-6) and alpha-linolenic (LNA, 18:3n-3) acids, which belong to the ω-6 and ω-3 families, respectively [6]. The chlorophyllase of sugar-beet leaves was characterized and partly purified by gel-filtration and ion-exchange chromatography on various types of Sephadex [7].

The process of green crop fractionation (GCF) and its byproducts evaluation was rcommended Pirie [8]; Collins [9]. Ghewande [10] cultivated 5 fungal species viz., *Aspergillus Fusarium*, *Phoma*, *Penicilium* and *Helminthosporium* on the DPJ expressed during fractionation of hybrid napier grass. They also reported the possibility of utilizing this liquor for the production of microbial biomass. Mukadum, et al. [11] observed that the DPJ from some plants may inhibit germination of pathogenic fungal spores. It is now well established that byproduct of GCF, the DPJ is the potential broth medium for growing fungi as DPJ contains minerals, amino acids, phytohormones, vitamins etc and also enzymes like protease etc. [12-14]. In earlier findings, deproteinised juice (DPJ) without adding any substrates has the potential for growing economically important microbes. Usually fungi grows well on solid mediums. But liquid DPJ made from some plants induces mycelial growth as per the earlier findings by the reporters. Very few DPJ like *Allium cepa* and beet foliage DPJ found inhibitory for growth of fungi and its enzyme protease[15]. It was already experimented that many industrial hydrolytic enzymes like proteases got secreted when *Penicillium chrysogenum*, *Aspergillus flavus* and *A. niger* fungi grown on DPJ [16]. *Raphanus* DPJ was found to induce more production of organic acids as compared with PDB medium. As compared with cabbage and cauliflower DPJ, *Raphanus* DPJ was found more significant. It was also found that the yeast fermentation also secretes the hydrolytic enzymes at appreciable amount in the broth of DPJ made from various foliages. The diameter of zones of enzymes amylases and cellulases were maximum by Oat *(Avena sativa L.)* DPJ. Enzymes proteases expressed by almost all deproteinised leaf extracts like *Cucurbita maxima*, *Momordica charantia*, *Moringa* etc. [17]. These hydrolytic enzymes also are the cell wall degrading enzymes. 9 other fungi grew well on DPJ of Lucerne as compared with glucose nitrate medium and produced the protease, cellulase, amylase and lipase enzymes more as compared with the culture filtrates of GN medium also by adding the substrates of casein, carboxymethyl cellulose (CMC) and starch at 1 and 2 % level. *Penicillium notatum* is an antibiotic fungi and the extracellular proteinase also purified from it Yuri [18]; Makonnen and Porath [19]; Kamath, et al. [20]. Earlier report was of glucose oxidase has been isolated from a culture filtrate of *Penicillium notatum* [21].

During present investigation, attempts were made to use the lowest concentrations of CMC, casein and starch at 0.75 and 1 % for the strains growth of *Penicillium notatum* and *Aspergillus niger* by addition in beet and lucerne foliage DPJ and its enzyme protease. In earlier studies, the substrates CMC, casein and starch were used at high concentration. The present objective is to enhance the productivity of enzymes and subjected comparative characterization of various substrates with the DPJ.

2. MATERIALS AND METHODS

2.1. DPJ Broth Medium

During the process of green crop fractionation (GCF) of lucerne *(Medicago sativa L.)* and beet *(Beta vulgaris L.)*, the pulp is formed by fractionation which is squeezed to express the juice and pressed crop residue by filtration with the muslin cloth [6]. Deproteinised juice obtained by heating the juice at 95 °C temperature and filtered by thick muslin cloth. The residue obtained by heated juice filteration is called as

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Leaf protein concentrate (LPC). This LPC is beneficial for human or poultry as the protein and vitamin source.

2.2. Glucose Nitrate Medium and Substrates

Glucose nitrate medium (GN) was prepared by dissolving 10 g glucose, 2.5 g, KNO3, 1 g KH2 PO4 and 0.5 MgSO4 in 1 lit distilled water. The DPJ was used at 2% level as a culture medium after dissolving 20 g dry DPJ in 1 lit of distilled water. In order to study the mycelia growth and enzymes production enhancement, starch, casein or carboxy methyl cellulose (CMC) served as a substrate at concentration of 0.75 % and 1 % to either GN medium or % DPJ solution. Strain inoculation from isolates twenty five ml of either GN medium of DPJ solution, either alone or enriched with substrate, were placed in conical flasks. The flasks were plugged with non-absorbent cotton and autoclaved at 15 lbs for 30 min. The flasks were cooled in UV chamber and inoculated with either strains of *Aspergillus niger* or *Penicillium notatum*. These were incubated till sporulation 5 - 8 days) and filtered through whatman filter paper to harvest microbial biomass. It was dried in oven at 65°C and mycelia dry weight (MCW) obtained were recorded. The culture filtrates released during filtration, were considered as crude enzyme preparations and activity of protease was measured using cup plate method. Substrate casein is used in both i.e., the growth broth medium as well as in the agar substrate plate for enzyme activity. The enzyme production was expressed as diameter of the zone formed due to its activity on specific medium. The agar "cup plate" diffusion assay of Dingle, et al. [22] can be used to quantify the activity of a variety of enzymes. The gel was developed after incubation by flooding the assay plate. Samples pipetted into diameters of wells (mm) punched in the agarose with a cork borer. It is incubated at 33°C.

2.3. Enzyme Protease

The basal medium composed of 2 % agar, 4 % gelatin, 1 % peptone, 1 % casein and pH was adjusted to 6.8. In this assay, casein acts as a substrate [22].

Fungal strains of *Penicillium notatum* and *Aspergillus niger* were cultivated by prior inoculation under the laminar air flow hood under the aseptic conditions on glucose nitrate medium and another broth liquid medium called deproteinised leaf juice (DPJ).

3. RESULTS AND DISCUSSION

In earlier studies the fungi were grown successfully on glucose nitrate medium as well as deproteinised leaf juice broth. Later the growth of fungi was attempted on the lucerne DPJ and GN medium by adding the substrates of carboxymethyl cellulose, casein and starch at 1% and 2 %. There was enhancement in the mycelia yield as was activity of the enzymes amylases, proteases and cellulases.

During present investigation, in order to enhance mycelial weight and to prove DPJ as conventional nutrient source as a medium for fungal growth, studies were carried out. DPJ alone found to enhance microbial growth including yeast in previous studies. In the present study, CMC, casein and starch were added to confirm more enhancement in the mycelial yield on GN as well as another DPJ medium made from beet leaves at lowest 0.75 % and 1 % level.

Table 1 indicates *Aspergillus niger* thrieved well on both 0.75 and 1 % of the concentrations of CMC. It was added in glucose nitrate medium. Glucose nitrate medium was the control. Glucose nitrate liquid broth containing starch and casein of 0.75 and 1 % of concentrations were not successful in initiation of mycelial growth. The DPJ of beet foliage inhibited the mycelial cell proliferation of *A. niger* illustrated in Figure 1.

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Table-1. Mycelial growth of *Aspergillus niger* on Glucose nitrate medium by adding various substrates and Beet leaves DPJ by adding casein substrate.

No.	Concentration of various substrates	Mycelial dry weight (g)
1.	Glucose nitrate + 0.75 % CMC Glucose nitrate + 1% CMC	0.485 0.160
2.	Glucose nitrate + 0.75 % starch Glucose nitrate + 1% starch	-ve -ve
3.	Glucose nitrate + 0.75 % casein Glucose nitrate + 1 % casein	-ve -ve
4.	Beet foliage DPJ + 0.75 % casein	-ve

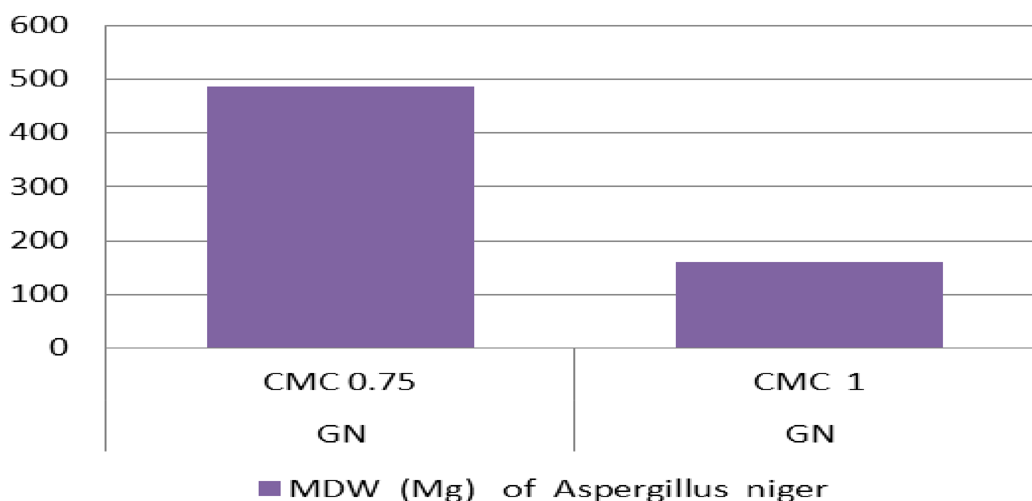

Figure-1. Mycelial dry weight (MDW) of *Aspergillu s niger* on beet foliage DPJ after the addition of carboxymethyl cellulose at various concentrations.

Figure- 2. Growth of *Penicillium* on GN medium and DPJ broth medium by adding 1% substrate casein.

In earlier research the DPJ made from *Allium cepa* also found inhibitory in growing the fungi *Trichoderma* and also inhibited the enzyme protease studied by cup plate method. While on the other hand *Trichoderma* grew well on few cruciferous and *Amaranthus* sp foliage DPJ.

The same experiment was conducted to observe the results in case of fungi *Penicillium notatum* illustrated in Table 2. *Penicillium notatum* was grown on both DPJ of lucerne and beet leaves. In order to enhance its mycelial biomass attempts were made to add the substrates viz., starch, casein and CMC depicted in Figure 2.

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Table-2. Mycelial biomass of *Penicillium notatum* on various concentrations of substrates by adding it in glucose nitrate medium, lucerne and beet foliage DPJ.

No.	Concentration of substrate (%)	Substrate	Mycelial dry weight (g) on broth medium		
			Glucose nitrate	Lucerne DPJ	Beet DPJ
1.	0.75	Starch	-ve	0.260	-ve
2.	1.00	Starch	0.190	0.280	-ve
3.	0.75	Casein	0.095	0.210	-ve
4.	1.00	Casein	0.140	0.200	-ve
5.	0.75	CMC	-ve	0.485	-ve
6.	1.00	CMC	-ve	0.160	0.740

The differences in the mycelial biomass of *Penicillium notatum* obtained on the DPJ from beet and Lucerne foliages as compared with glucose nitrate broth medium is illustrated in Table 2. Lucerne DPJ was successful in inducing mycelial growth in presence of all substrates viz., starch, CMC and casein at both 0.75 and 1 % level. It enhanced as per the increased concentration of starch. Lucerne DPJ reduced the mycelial growth when the concentrations of casein and CMC increased. In case of carboxymethyl cellulose as the substrate for mycelial growth, beet foliage DPJ inhibited the fungal growth on casein and starch substrates at both concentrations while on the other hand, it initiated the fungal growth at only 1 % CMC concentration. As this concentration of CMC at 1% was found appropriate for the growth of *Penicillium notatum* fungi on beet DPJ showed in Figure 3.

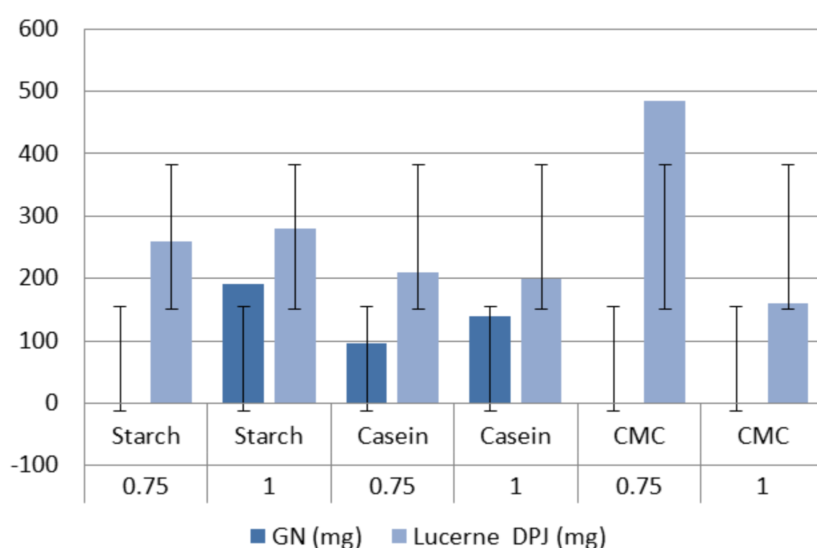

Figure-3. Illustration of mycelial weights on Glucose nitrate (control) and Lucerne and beet foliage DPJ after the addition of various substrates in the broth medium.

The mycelial biomass of *Penicillium* on Beet DPJ was more as compared with Lucerne DPJ i.e. 0.740 g. *P. notatum* grew well on increasing concentrations of starch and casein in glucose nitrate broth medium but got inhibited on carboxy methyl cellulose. Therefore it is proved that lucerne DPJ had the potential in inducing the commercial fungal growth. When the substrates are added in both medium, lucerne DPJ initiated the mycelial growth. Both the fungi *Aspergillus niger* and *Penicillium* showed reduced mycelial biomass if the carboxymethyl cellulose percent concentration increased from 0.75 to 1 % level illustrated in Table 1 and 2. *Penicillium* couldnt grow on both the concentrations of CMC if added in control GN medium.

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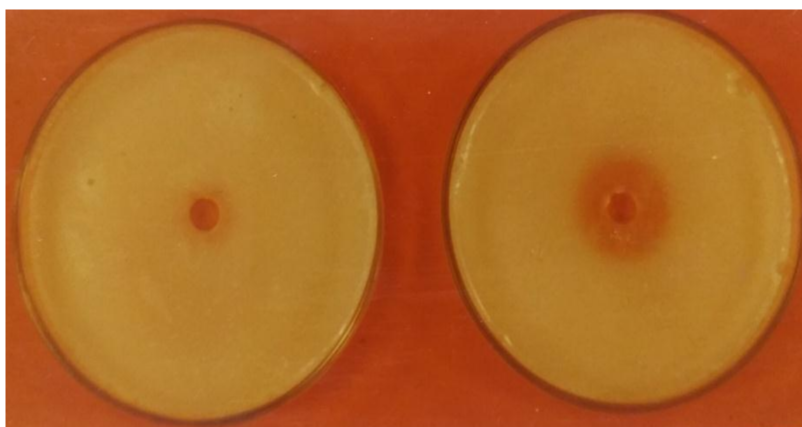

Figure-4. Production of enzyme protease on 1% casein added in DPJ before growth of *Penicillium chrysogenum* antibiotic fungi.

0.75 % starch when used in GN medium, there was no fungal growth. When 0.75 % of starch was added in beet DPJ, then also there was fungal growth of Penicillium. But when Lucerne DPJ was used by adding 0.75 % of starch there was growth. Same when 1 % CMC when used in GN medium, there was no fungal growth. When 1 % of CMC was added in beet DPJ, then there was fungal growth of *Penicillium.* But when Lucerne DPJ was used by adding 1 % of CMC there was growth. This indicates the more efficacy of Lucerne in inducing the mycelial growth as compared with beet foliage DPJ. The significant enhancement of the enzyme protease zone (mm) by the antibiotic Fungi *Penicillium notatum* illustrated in Table 3 by cup plate assay method and depicted in Figure 4.

Table-3. Secretion of the enzyme protease by *Penicillium notatum* on Lucerne DPJ broth after adding the substrate casein.

No.	Broth medium	Concentration of casein for fungal growth (%)	Enzyme protease zone (mm)
1	DPJ	0.75	15
2	DPJ	1.00	20

The colouration of the zone was slightly brownish. It was because of the increase in the concentration of the substrate casein dose at 1 % level in the DPJ broth of lucerne to optimize the cultural conditions. The DPJ broth was responsible to induce the enzymic zones as it already contains the quantity of protease as per the earlier investigation.

Therefore it reveals that DPJ have the potential in initiating the fungal growth as compared with GN medium in presence of substrates. Sometimes substrates becomes responsible to inhibit the mycelial growth, as it was already proved the successful growth of *Aspergilllus niger* and *P. notatum* growth on DPJ without adding any substrates. DPJ do not need any substrate for mycelial growth as it already contains the essential elements.

In earlier research DPJ induced the hydrolytic enzymes by fungi as well as yeast. DPJ itself contains presence of phytohormones responsible to activate enzymes. The fermented leaf juice from cauliflower also found activating the hydrolytic enzymes. When the specific substrates responsible to induce enzyme activity added in DPJ it showed striking influence. It was because deproteinised leaf juice still contains presence of few hydrophilic soluble amino acids after protein separation. Starch and casein at 0.75 and 1 % level in addition to the GN medium showed antifungal influence of *Aspergillus niger* growth. The DPJ used prepared from beet leaves in addition with casein showed antifungal influence of both *Aspergillus niger and Penicillium* fungi, perhaps may be because of presence of the nanoparticles in it. Earlier investigation showed presence of nanoparticles in *Colocasia*, cabbage and radish foliage DPJ.

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4. CONCLUSION

Therefore the research concludes that DPJ alone has the potential in having the mycelial growth. If it is inhibiting the growth of fungi, definitely the substrates when added, can induce mycelial growth and secrete appreciable amount of enzymes. The activity of enzymes indicates the presence of soluble proteins in DPJ or enhancement of solubility of amino acids in DPJ by fungi. The amino acids were hydrophilic. Enzyme activity already found at appreciable amount in earlier research without adding any substrates when various fungi were grown on DPJ broth medium for industrial purpose. Inhibition of fungal growth might be because of few fatty acids and nanoparticles content of beet leaves DPJ. Till date it is revealed that DPJ from Lucerne has the potential, as it initiated the mycelial growth and hydrolytic protease activity and balanced nanoparticles content. At high concentration specific DPJ can cause chromosomal abberations.

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