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IN VITRO SCREENING OF CELL WALL DEGRADING ENZYME PRODUCTIVITY FROM FUNGAL CULTURE FILTRATES ON DEPROTEINISED PLANT FLUID BY CUP PLATE ASSAY

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ABSTRACT

The present study purpose is to evaluate the potentiality of the deproteinised juice made from selected plants to induce the enzyme productivity by growing the fungi on it. It is because, in earlier findings, the DPJ was found potential in enhancing fungi and the plant growth when used as the medium. The internal factors are responsible in DPJ which induces plants and fungi growth. During the process of Green Crop Fractionation (GCF), the deproteinised juice (DPJ) obtained from tissues of cabbage, beet, lucerne (Alfalfa), carrot and *Anathum* (Dill) forages left after leaf protein extraction employed as a medium for the cultivation of mycelial biomass of *Penicillium* and *Aspergillus* fungi. The mycelial growth in vitro was compared with the glucose nitrate medium. The culture filtrates were used to screen different secreted hydrolytic or cell wall degrading enzymes. The agar 'cup-plate' diffusion technique has been applied to the quantitative determination of enzyme activity, principally to amylase, cellulase and protease. With all enzymes so far examined, the relationship between diameter of zone over a wide range secreted quantitatively was examined. All fungi grew well on DPJ in comparison to their growth on glucose nitrate (GN) medium. Comparatively with GN medium, lucerne DPJ was found having more mycelial cellular proliferation. When the fungi grown on different concentrations of substrates, enriched with carboxymethyl cellulose, casein and starch in deproteinised leaf extracts and GN medium, it was found that there was the enhancement in the mycelial dry weight grown on DPJ as compared with glucose nitrate medium. *Penicillium* showed more yield of enzyme activities especially of cellulases and amylases as compared to protease by cup plate method. There was enhancement of mycelial biomass when the concentrations of substrates increased from 1% to 2%, while there was no change in the activity of all enzymes by increasing the concentrations of substrates. Activities of the enzymes in vitro can be indicative of the pattern of organogenesis in callus cultures in further studies by fungal culture filtrates cultured on deproteinised fluid medium.

Keywords: Deproteinised Juice; Lucerne *;* mycelia, *Penicillium chrysogenum ;* Glucose nitrate medium ; Casein ; Protease ; cellulase

INTRODUCTION

In view of using plant nutrients more efficiently**, Prof. Pirie (1942)** advocated the process of green crop Fractionation (GCF). The process of GCF involves extraction of protein from green leaves for use in human and animal nutrition. During GCF, fresh green leaf tissues are macerated and subsequently pressed. The leaf juice released due to the pressing is then heated to 95ºC, as a result of which, proteins in juice, coagulate to form a curd referred to as leaf protein concentrate (LPC). The LPC is isolated from remaining portion of leaf juice, called as deproteinised juice (DPJ), by filtration through cotton cloth.The juice liberated consists of enzyme proteases and amylases. When this juice was fermented for enhancing hours, it breaksdown the protein and carbohydrates, estimated by cup plate diffusion assay. The use of LPC as a protein and vitamin A supplement in human and animal nutrition is well recommended while the DPJ is considered as a byproduct of this process **(Pirie 1987).** To make the process more efficient and economical, and also to avoid local environmental biopollution, the DPJ has to be used in a proper way as it is disposed randomly because it can cause environmental biopollution. Lucerne DPJ used as a fertilizer for cowpea growth and citric acid produced by growing *Aspergillus* **(Makoto and Shigekata, 2017)**. It was found that *Trichoderma viride* cell proliferation was inhibited by the DPJ made from *Allium cepa* leaves. While on the other hand, DPJ from *Amaranthus* enhanced enzymes proteases and cellulases from the culture filtrates of *Trichoderma*. It was found when DPJ fermented by yeast, there was enhancement in mycelia dry weight of yeast as compared with Hansens medium used and decrease in enzyme invertase and alcohol productivity **(Jadhav and Deshmukh 2018)**. DPJ ehhances rhizosphere microflora. When It is used at high concentrations, it caused phytotoxicity in Pea plants during growth. Effect of high concentrations of Lucerne and *Eicchornia* DPJ experimented on mitosis, it caused chromosomal aberrations **(Jadhav 2009)**. Several uses of DPJ hae been suggested, which include its use in animal nutrition along with pressed crop residue and as a source of *Rhizobium* growth and nodulation inducer. Formerly, when DPJ fermented by yeast it enhanced mycelial biomass and secreted hydrolytic enzymes experimented by cup plates method. Deproteinised juice was used from plants for the rhizogenesis in vitro in tissue culture. DPJ also has the efficacy in enhancing the enzyme nitrate reductase in gramineae plants during growth (Jadhav and Gare, 2018).

Many microbial enzymes are commercially exploited and successfully used on industrial scale to catalyze several chemical processes. These enzymes proved to be better, cheaper and environment friendly compared to the use of chemicals. Recently, enzymes have also been exploited in bioremediation of complex waste substances **(Whiteley and Lee, 2006)**. Therefore, enzyme production now became a multi-billion dollar business. Plant biomass is one of those natural complex materials, containing lignin, cellulose and hemicelluloses, found in abundance and regarded as promising chemical feedstock **(Kang et al., 2004)**. Microbial enzymes, involved in the degradation and transformation of plant cell-wall polysaccharides, have found many biotechnological applications **(De Vries and Visser 2001**). Ligninases, hemicellulases, cellulases, pectinases and amylases are the enzymes which are required to degrade not only the plant biomass to its completion but also have found applications in different industries and pharmaceutical preparations. Cellulose is the most abundant biopolymer present on this planet and can be degraded to glucose when different types of cellulases act in synergy **(Yang et al**., **2003)**. Cellulases include endo-/3-1,4-glucanase, P-glucosidase, and cellobiohydrolase, which cooperate in the complete hydrolysis of cellulose to Glucose. Starch is a reserve source of glucose in plants and readily hydrolysed by amylases produced by almost all living organisms. However, microbial amylases are of great industrial importance. Beside these carbohydrolases, proteases are another important group of industrial enzymes that are widely used in detergent, baking and some other industries **(Balkan and Ertan 2005)**. Fungi are ecologically involved in the degradation of a variety of complex materials, a property that is attributed to a battery of enzymes produced by these microorganisms. Fungal enzymes have been used in enzyme-technology industries for decades **(Dalboge 1997)** and hence there is an ever-increasing demand for the isolation and screening of new fungal isolates. Though recombinant DNA technology has provided some new methods to obtain and develop microorganisms of industrial importance nonetheless, classical techniques are still in use **(Kuznetsova et al., 2005)**.

There is the view that, dried mycelium of *Penicillium chrysogenum* is antibiotic and can be substituted as a protein source for soybean meal, provided it is made more palatable and less disagreeable in odor. *Aspergillus niger* is cultured for the industrial production of many substances. Various strains of A. niger are used in the industrial preparation of citric acid and gluconic acid and have been assessed as acceptable for daily intake by the World Health Organisation. Toxic *Aspergillus flavus* used in the present investigation for comparison.

Lucerne, a valuable plant grown mainly for animal feed, is rich in protein, minerals (Ca, Cu, Fe, Mg, Mn, P, Zn, Si), vitamins (A, B, C, D, E, K, U), phytochemical substances (carotene, chlorophyll, coumarins, isoflavones, alkaloids, saponins), contains secondary metabolites of plants (phytoestrogens: isoflavones and coumestrol), and antinutritional components (phytates, L-canavanine, saponins).

It may be used as a dietary supplement in human nutrition. The proteins found in lucerne are comprised of numerous exogenous and semi-exogenous amino acids which are desirable for human body. Cabbage leaves consists of phytochemical compounds include sulforaphane and other glucosinolates. Cabbage is a source of indole-3-carbinol. (*Beta vulgaris* L.) leaf is a good source of natural antioxidants such as betalains, flavonoids, polyphenols, vitamins, and folic acid. Total phenol content is the highest. Betalains comprise betacyanins (red-violet pigments) and betazanthins (yellow pigments). Carrot leaves have omega-3 and omega-6 fatty acids, which are scarce in nature. Other compound present such as pyrrolidine.

Green leaves of cabbage, beet, Carrot and Lucerne (Alfalfa) were utilised in the experiment during present investigation. These were fractionated to obtain the main fraction Leaf protein Concentrate (LPC). External covered cabbage leaves usually thrown in the market or given as a feed to the animals. Usually beet and Carrot leaves are thrown or given to ruminants as fodder.Therefore such crops are utilised in the research for proper prevention and consumption of organic waste.Present study was initiated to screen the indigenous fungal strains to explore their secreted hydrolytic and cell degrading potential by deproteinised leaf juice for their possible future applications and its employment for commercial production and consumption in tissue culture research of enzymes viz., amylase, protease and cellulase.

MATERIAL AND METHODS

Fractionation

Fresh green leaf tissues of Lucerne (*Medicago sativa Linn*.), Beet root (*Beta vulgaris. L*), Carrot *(Daucas carota.Linn*) and Cabbage (*Brassica oleracia L.c.v.capitata*) were collected from field/Botanical Garden. The leaves were pulped. The pulp was pressed (Davys et.al. 1969) and the juice released due to the pressing was collected in stainless steel container. It was heated to 95ºC with constant stirring and filtered through cotton cloth to isolate leaf protein concentrate (LPC) from deproteinised leaf Juice (DPJ). The DPJ released as filtrate, was carefully dired in hot air oven at 65ºC and dry samples were stored for subsequent use.

In vitro preparation of culture media

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 disolving 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) were aded as a substrate at concentration of 1% and 2% 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 *Penicillium chrysogenum, Aspergillus niger or A. flavus*.

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 mycelial dry weight (MCW) obtained were recorded. The culture filtrates released during filtration, were considered as crude enzyme preparations and activities of amylase, protease, and cellulase were measured using cup plates method**.** The enzyme production was expressed as diameter of the zone formed due to its activity on specific medium.

Enzyme assays

The agar "cup plate" diffusion assay of Dingle et al can be used to quantify the activity of a variety of enzymes. The gel was developed after incubation by flooding the assay plate. Samples were pipetted into diameters of wells (mm) punched in the agarose with a cork borer. It is incubated at 33°C.

For amylase

Soluble starch 10 gm, Na2HPO4 20 g, D.W. 1000 ml at pH 6.9. Autoclaved 15 ml medium was poured in petriplate. After solidifying the medium, cavity was made in centre of petridish. It is filled with culture filtrate and incubated. It is flooded with bugols iodine solution as an indicator after diffusion.

For 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.

For cellulolytic activity

Test carried out with sodium citrate buffer at pH 5.5. Substrate CMC (1%) mixed with melted 2% agar under aseptic conditions.The enzyme activity zones were developed by covering the plates with 3% lead acetate. After washing, diameter of zones were measured **(Dingle et al. 1953).**

RESULTS AND DISCUSSION

Mycelial dry weight of fungi

The deproteinised leaf juice (DPJ) samples supported cell proliferation of three fungi under investigation. This might be attributed to the presence of soluble nutrients of cell, released in DPJ during tissue fractionation.

Table 1 The yield of fungal cells biomass on Glucose nitrate (GN) medium and DPJ in vitro from 5 plant species.

Medium	Yield of Mycelial Dry weight (MDW) of *Penicillium chrysogenum*	Yield of MDW of *Aspergillus niger* (mg)	Yield of MDW of *Aspergillus flavus* (mg)
GN	91	353	125
Alfaalfa DPJ	313	358	169
Beet leaves DPJ	150	408	160
Carrot leaves DPJ	177	400	181
Cabbage DPJ	168	330	192
Dill DPJ	93	104	131
C.D- (P=0.05)	58	42	29

Table 1. Gives an account of mycelial dry weight (MDW) production by the three fungi on GN as well as DPJ medium. The cell proliferation of all fungi, was significantly higher on DPJ than that recorded on GN medium. Suitability of DPJ as a medium for fungal growth was thus confirmed as was observed that when different flous were utilized by mixing in DPJ there was enhancement in enzymes **(Sayyed 2014).** Among all plant species, the DPJ from Lucerne, was found more suitable for microbial growth as showed in figure 1, 2, 3 and 6.

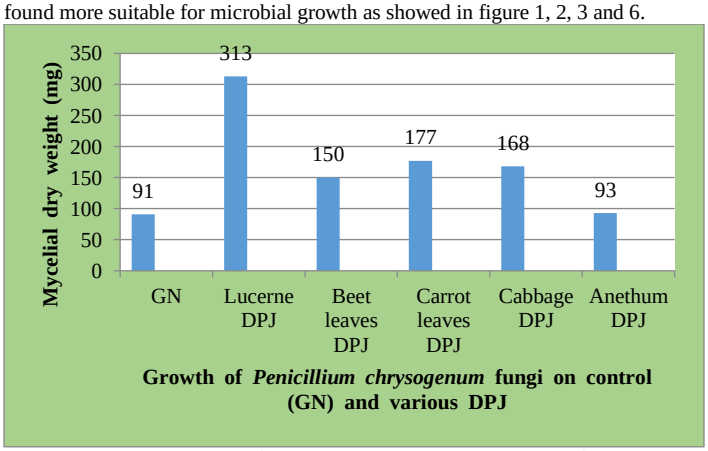

Figure 1 In vitro Yield of Mycelial dry weight (MDW) of *Penicillium chrysogenum* on the deproteinised juice medium used from various plant forages compared with Glucose nitrate (GN) medium**.**

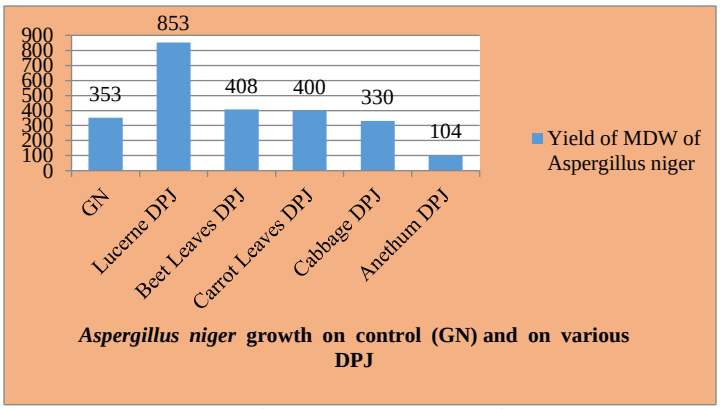

Figure 2 In vitro Yield of mycelial dry weights of *Aspergillus niger* on Deproteinised juice (DPJ) from various forages of plants as compared with Glucose nitrate (GN) medium**.**

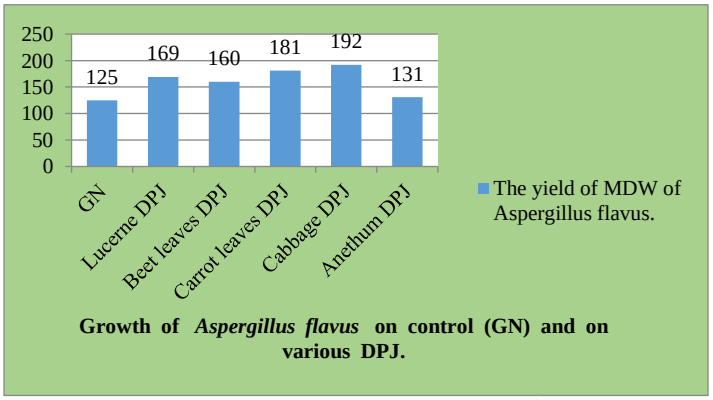

Figure 3 In vitro yield of Mycelial dry weight (MDW) of *Aspergillus niger* on deproteinised juice of various forages as compared with Glucose nitrate (GN) medium.

Studies of fungal enzymes by DPJ with addition of different substrates

It is previously concluded that the growth and enzyme production by fungi growing in vitro on DPJ can be increased by supplementation of DPJ with the suitable substrate for production of hydrolytic enzymes of *Aspergillus niger*. However, when the DPJ was supplemented with starch and casein, as a source of carbohydrate and protein respectively, the fungi gave very poor response. It is therefore felt that a specific fungus should be identified and used for the production of specific enzyme using the DPJ from suitable plant tissues. In order to enhance the production of enzyme amylase, protease and cellulase, the DPJ was enriched with either starch, casein or carboxymethyl cellulase. In this experiment these substrates were added to GN medium as well as the DPJ obtained from lucerne at either 1% or 2% level. *Penicillium chrysogenum* was grown on the medium and its mycelial dry weight was recorded after incubation for 7 days the results are presented in table 2.

Table 2 In vitro production of enzymes by *Penicillium chrysogenum* when cultivated on either GN medium of Lucerne DPJ solution enriched with 1% substrate

Medium	Mycelial Dry Weight (mg)	Diameter of enzyme activated zone (mm)
GN + 1% starch	430	12, for amylase
DPJ + 1% starch	815	18, for amylase
GN + 1% casein	290	12, for protease
DPJ + 1% casein	500	14, for protease
GN + 1% CMC	500	14, for cellulase
DPJ + 1% CMC	1490	19, for cellulase

When rhizosphere fungi *Penicillium chrysogenum* was cultivated on GN medium containing starch the yield of MDW was 410 to 430 mg/25 ml. The yield increased to 815 and 955 mg when the fungus was cultivated on lucerne DPJ with either 1 or 2% starch.The addition of starch to the DPJ was thus found beneficial in giving higher yields of *Penicillium.* Addition of starch enhanced the production of amylase in both media but, its production was enhanced with more intensity in the DPJ medium supplemented with starch and casein, as a source of carbohydrate and protein respectively, the fungi gave poor response.

When casein was added to the GN medium, the fungus yielded 290 to 380 mg MDW/25 ml. The yield of *Penicillium* increased to as high as 910 mg /25 ml when the DPJ along with 2 % casein was used as a medium for its growth. The production of enzyme protease was enhanced due to the addition of casein, particularly in the medium containing DPJ showed in figure 7. When the protease we are testing digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments.

Luxurient growth of *Penicillium* was noticed in vitro when carboxymethyl cellulose (CMC) was added to the medium. The fungus produced 1510 mg MDW when it was cultivated on GN medium enriched with 2% CMC. When the CMC was used along with DPJ the yield of MDW was as high as 1800 mg.The cellulase production was maximum in DPJ which received CMC at 2% level. The overall results presented in table 3. Indicate that the DPJ can be used as a medium for the growth of fungi and production of enzymes. The enzyme production can be enhanced by supplying suitable substrates. Further studies in this respect are however, needed to evaluate the use of DPJ for the production of enzymes on commercial level.

Table 3 In vitro production of enzymes by *Penicillium chrysogenum* when cultivated on either GN medium or Lucerne DPJ solution enriched with 2% substrate

Medium	Mycelial Dry Weight (mg)	Diameter of enzyme activated zone (mm)
GN + 2% starch	410	12, for amylase
DPJ + 2% starch	955	18, for amylase
GN + 2% casein	380	12, for protease
DPJ + 2% casein	910	14, for protease
GN + 2% CMC	1510	14, for cellulase
DPJ + 2% CMC	1800	19, for cellulase

Table 3 gives information on growth and enzyme production in vitro by *Penicillium chrysogenum* when growth on either GN medium or Lucerne DPJ solution in the presence of substrate i.e. Starch for amylase, Casein for Protease and Carboxymethyl Cellulose for cellulase and for to the addition of substrate to (starch, Casein and Carboxy methyl cellulose (CMC) at 2% each respectively,showed in figure 4). On the basis of diameter of enzyme activated zones, it can be concluded that the production on enzymes was more pronounced on DPJ based medium rather than GN based media. Many of the amylolytic strains belonged to the genus Aspergillus, as they are dominant amylolytic genera in nature **(Norouzian et al., 2006).** The overall results indicated that lucerne DPJ was suitable for enzymes production through the cultivation on *Penicillium* showed in figures 5, 7, 8 and 9.

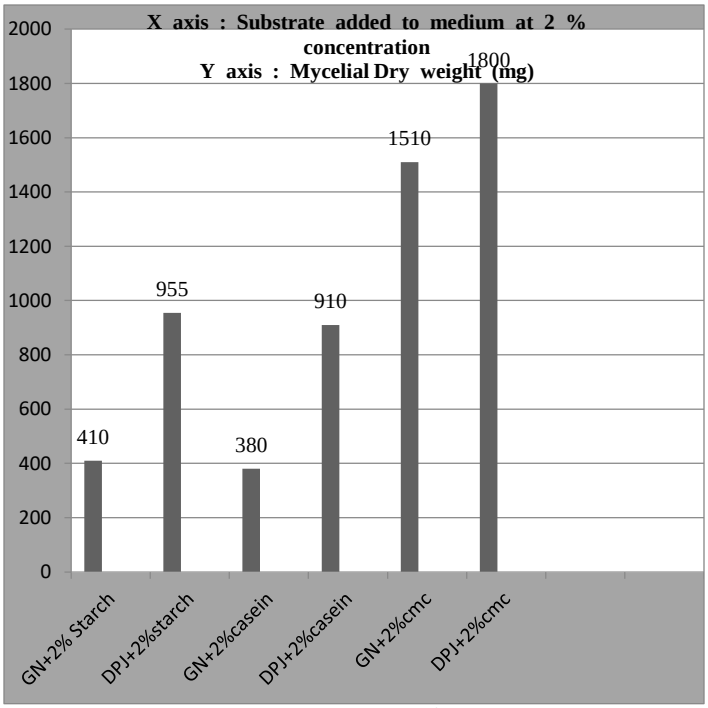

Figure 4 In vitro mycelial dry weight (gm) of *Penicillium chrysogenum* on Glucose nitrate medium and deproteinised juice added with the substrates of starch, casein and carboxymethyl cellulose at 2 % level.

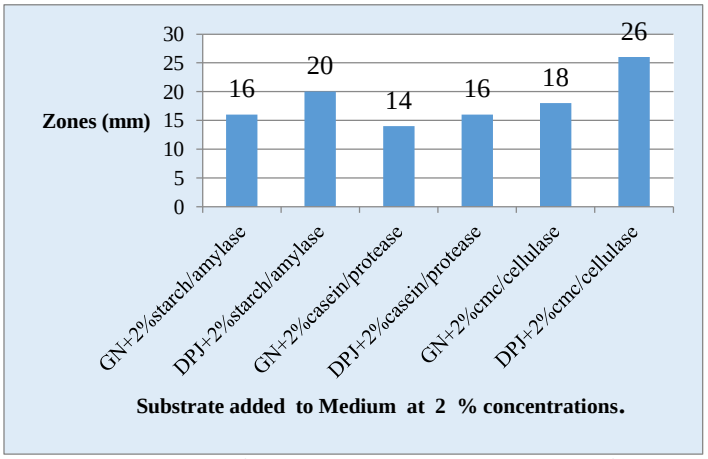

Figure 5 Measurement of cell wall degrading enzyme zones of amylase protease and cellulase by in vitro cup plate diffusion assay method by *Penicillium chrysogenum* grown on glucose nitrate medium compared with deproteinised juice from Lucerne *(Medicago sativa. Linn)* by the addition of the substrates starch, casein and carboxymethyl cellulose

During the process of GCF, after leaf protein extraction, DPJ formed, is rich in cell soluble nutrients. It contains high proportion of non-protein nitrogen, minerals, lipids vitamins, phosphorus, water soluble carbohydrates and hormones. Dry DPJ contains 40% carbohydrates. These values may fluctuate with the species used for fractionation. The dominant monosaccharides in DPJ are glucose and fructose. However content of these reducing sugars in DPJ is subject to great change, depending upon the species and maturity of the plants used. The presence of carbohydrates in this product makes it suitable for microbial biomass production.

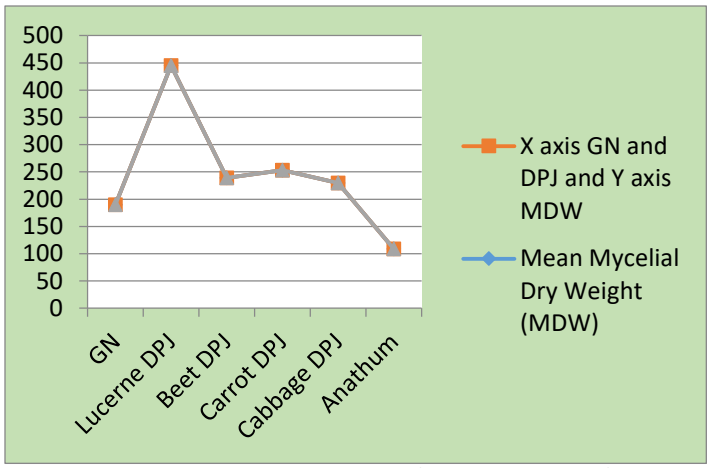

Figure 6 The graph shows statistical mean of cellular biomass of Fungi on glucose nitrate medium and deproteinised juice from various forages.

Fungi constitute a group of microorganisms that are widely distributed in environment especially in soil. Since they produce wide variety of hydrolytic enzymes and hence exist in nature in saprophytic mode. The action of enzymes is so important, for instance, wood-rotting fungi are classified on the basis of enzymatic deterioration of wood; white-rot fungi can degrade lignin and brown-rot fungi can degrade cellulose. Fungal hydrolytic enzymes share 40% of global enzyme market and have many industrial applications **(Archer and Peberdy, 1997)**. Therefore, there is a need for screening these enzymes for improved characteristics. Keeping in view this objective, a total of 3 local fungal isolates either were isolated from environmental sources or obtained from departmental culture collections, were subjected to screening for the ability to elaborated extracellular hydrolases. The filamentous fungus *Penicillium chrysogenum* is well-known by its ability to synthesize β-lactam antibiotics as well as other secondary metabolites. Like other filamentous fungi, this microorganism is an excellent host for secretion of extracellular proteins because of the high capacity of its protein secretion machinery.

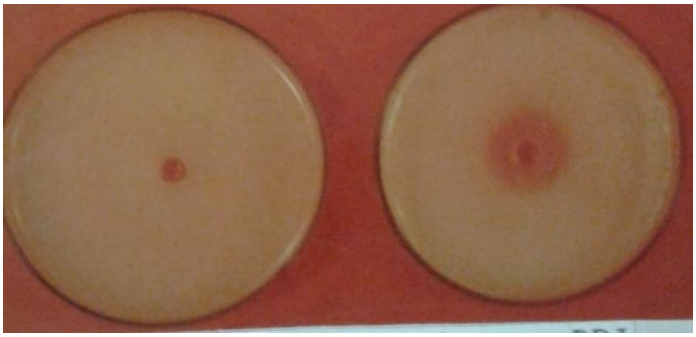

Figure 7 In vitro protease enzyme activity zones secreted by fungi performed by cup plate assay diffusion method grown on glucose nitrate medium (slow) compared with deproteinised juice from culture filtrates (substrate casein).

It is thus concluded that the deproteinised juice, left after leaf protein extraction from green foliage, can be employed for growth of economically important fungi and for commercial production of enzymes as it has the potentiality. The result varies when different forage deproteinised leaf extracts are utilised. Further investigation on production of enzymes, toxins, antibiotics and single cell protein (SCP) by growing microorganisms are in progress. The cell wall-degrading enzymes polygalacturonase and pectate lyase have been suggested to be crucial for penetration and colonization of plant tissues by some fungal pathogens in tissue culture. It was found that *Aspergillus nidulans*, a saprophytic Ascomycete, produces levels of these enzymes **(Dean et al. 1989).** Starch, total sugars, reducing sugars and protein contents and the specific activities of hydrolytic enzymes such as amylase, Phosphorylase, soluble acid invertase, wall-bound acid invertase, sucrose synthetase, acid and alkaline phosphatases and ribonuclease were determined in root forming, shoot forming and non-organ-forming callus cultures of tobacco. Organ-forming cultures not only showed higher amounts of the above metabolites but also higher enzyme activities compared to non-organ-forming cultures. The activities of these enzymes in relation to organogenesis is discussed **(Naidu and Kavikishor, 1995).**

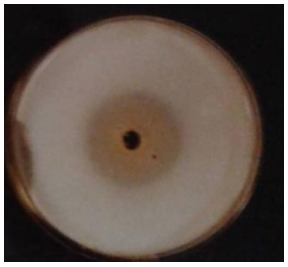

Figure 8 In vitro cellulase production by *Aspergillus niger* from culture filtrates grown on DPJ with 2% substrate CMC**.**

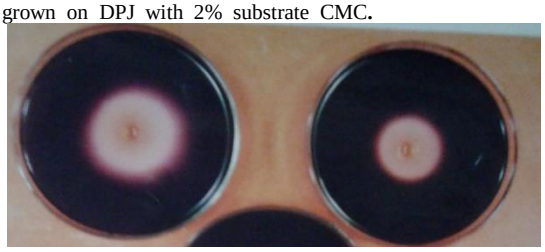

Figure 9 In vitro production of Amylase (substrate starch) by *Penicillium chrysogenum* on DPJ and Glucose nitrate medium by cup plate assay from culture filtrate.

DISCUSSION

Therefore our findings indicate that DPJ as a media made in vitro from specific plant forage tissues was responsible to induce the enhancement of cultured mycelial cell proliferation and secrete more fungal enzymes in media as compared with the fungi cultured on laboratory artificial glucose nitrate medium. The cellulase enzyme was found highest. Enzymes to isolate higher plant protoplasts can be done by the applications of culture filtrates of a cellulolytic fungus to release protoplasts in plant tissue culture. The production of these cell wall degrading enzymes enhancement by DPJ should have the novel approach of the tissue culture and industrial relevance.

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