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INDUCTION OF THE DEXTRINASE AND OTHER HYDROLYZING ENZYMES IN THE SUSPENSION CULTURE OF *AZADIRACHTA INDICA* A. JUSS.

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ABSTRACT

Amylase expression was present in callus of *Azadirachta indica* A. Juss. We studied the amylase activity in the callus culture to check the effect of starch on enzyme expression. Starch addition exogenously into MS media, increases the enzyme production. 0.5% of starch in the medium induced a higher amylase activity as compared to 1% of starch. This suggests that the higher concentration of starch, down regulate the expression of amylase in the neem callus. Besides amylase, protease and lipase were also detected in the suspension culture of *Azadirachta indica*. In addition, effect of respective inducers (dextrin, casein, tween-80, CMC, gelatin and olive oil) on the hydrolytic enzyme production capability of *Azadirachta indica* suspension culture was also studied. Dextrinase was produced by the culture, using 1% inducer. The protease was also induced in a similar way as amylase. However, no effect of inducer was observed on the lipase, this is because the MS media contain sucrose as its component which is preferable for cells to utilize than lipid polymer. Therefore, the culture condition was manipulated to force cells to hydrolyse lipid polymer. This is the first report of dextrinase from *Azadirachta indica* cell suspension culture and presenting an alternate way of producing hydrolytic enzymes.

Key words: Dextrinase, Hydrolytic enzyme, Suspension Culture, Inducer.

INTRODUCTION

Hydrolases are the enzymes, which breaks down the bonds in substrate, to produce its monomers and dimers. Two types of hydrolases are reported 1) endo-hydrolase and 2) exo-hydrolase (Gupta *et al.,* 2003). Amylases are most important hydrolases due to its starch hydrolytic property, which convert starch into glucose, maltose and limit dextrin. In nature three different types of amylases are found: α-amylase, β-amylase and **γ**-amylase (Sundarram and Murthy, 2014).α-amylases hydrolytically catalyse the α-1, 4 glyosidic bonds of starch and produce glucose and dextrin, which is dependent on calcium for their catalytic activity. β-amylase also act on α-1, 4 bonds of starch so, the hydrolysis is incomplete with these enzymes. γ-amylase however can break α-1,4 and α-1,6 glyosidic linkages which leads to complete hydrolysis, producing only glucose units.

In the world enzyme market, amylase contributes approximately 25%, due to its starch hydrolytic property (Raul *et al.,* 2014).Purified amylases are used for following purposes;

- Production of glucose and fructose syrup.
- Baking Industry: to ferment starch in dough making.
- Detergent industry: to remove starch containing stains from fabrics and utensils.
- Textile and Paper industry: as a sizing agent.
- In fuel alcohol production process.

Because of the increasing demand of amylase, different sources are utilized industrially for production such as microbial, animal, fungal and plant. Microbes are most concerned today as they are more commercially feasible than other sources (Burhan *et al.,* 2003). Plant cell culture studies, reported that the callus of higher plants shows the extracellular production of different enzymes in cultured media. Plant cells have potential to produce dyes, medicines, enzymes and other secondary metabolites commercially (Simmons *et al.,* 1991).Amylases have been isolated and purified from different plants such as barley and rice plants (Oboh, 2005) and according to the Pandey *et al.* (2000), it is proved that the amylase which is active on wide spectrum of pH and temperature is present in the cassava mash waste water.

Apart from these studies, amylases are produced from suspension of various plants. The α-amylase was found to be expressed in cultured cells of French bean. The synthesis of α-amylase is related to sugar starvation and the exogenous addition of gibberellin has no effect on the enzyme production. The enzyme was also found to be up regulated under osmotic stress by providing high concentrations of mannitol (Kim *et al.,* 1997). Simmons *et al.,* (1991) reported the expression of α-amylase in rice seed callus. The callus secreted about 840µg α-amylase with 10.9 x $l0^3$ units of activity per gram dry weight callus per day, indicate α-amylase the predominant protein in the

culture medium. Masuda *et al.* (1988) detected that glucoamylase and α-amylase are present in callus and suspension cultures of sugar beets (*Beta vulgaris* L.) as well as in mature roots as soluble fraction. They also reported the method of enzymes purification.

The yields of desired products are very low or sometimes not detectable in dedifferentiated cells such as callus tissues or suspension cultured cells. To obtain products in concentrations high enough for commercial manufacturing, therefore, many efforts have been made to stimulate or restore biosynthetic activities of cultured cells using various methods. Most significant method used is enzyme induction through chemical interactions. (Kedderis, 2010). Enzyme induction can be defined as the increased synthesis (higher amount) or decreased degradation (increased activity) of enzymes that occurs because of the presence of an exogenous substance (Alsanosi *et al.*, 2014).

During every process of enzyme induction, amino acids are coded in a fashion to form single specific protein with repetitive response as compared to other proteins in the cell. For the regulation of enzyme synthesis some steps need to be considered; 1) mRNA formation 2) mRNA function, its degradation and movement 3) tRNA function 4) polypeptide synthesis. Reports are submitted on the above described possibilities in various plants. (Filner *et al.*, 1969). Inducers used are often substrates or they could be substrate analogues.

Lijavetzky *et al.* (2008) reported that the use of methyl jasmonic acid combined with cyclodextrin gives the synergistic effect on resveratrol production in grapevine cell cultures. In the aleurone cell of cereal seeds, hydrolases production is enhanced when gibberellic acid is used (Filner *et al.*, 1969).Three forms of α-glucosidase have been isolated from suspension-cultured rice cells. The three enzymes readily hydrolyzed maltose, maltotriose, maltotetraose, amylose, and soluble starch. The three enzymes produced panose as the main α-glucosyltransfer product from maltose (Yamasaki and Konno, 1985). Enzyme activity of the cellulase type was isolated from the culture medium by Zemekt *et al.,* (1992) through cultivating Spruce callus culture (*Piceaabies* (L.) Karst) on the hydrophilic surface of a gel prepared from crosslinked-hydroxylethyl cellulose as the only source of carbon in the growth medium. Medora *et al.*(1984) examined protease activity and total protein in callus cultures of *Carica papaya* L. The basic Wood and Braun medium produced good growth and high protease activity. Reduction of macro and/or microelements in Murashige and Skoog basic medium increased protein content and enzyme activity of the callus. In some instances, inclusion of peptone enhances the proteolytic activity. Protease activity was examined by Carlberg *et al.* (1984) in embryogenic and non-embryogenic strains of carrot cells on variety of substrates. The non-embryogenic strain shows 5-10 times higher activity when compared with embryogenic strain. The activity was found to be more pronounced in differentiated cultures.

An esterase which hydrolyses the pyrethroid insecticide cyfluthrin, was isolated from tomato cell suspension cultures and purified 10-fold. The apparent molecular weight of the enzyme was estimated to be 32, 000 Dalton, the pH-optimum 8-10, and the temperature optimum 35°C (Preiss *et al.*, 1988).

It is considered that like other plant cultures, *Azadirachta indica* A. Juss (Neem) callus cells are also capable of producing enzymes in suspension cultures supplied with proper nutrients and plant hormones. However, it is reported that parts of neem plant possess enzymatic activity. Neem exudate gum possess proteolytic activity when tested by Nayak *et al.* (1979) against casein and albumin as substrates. The enzyme was found to be stable at high temperature and wide pH range but no hydrolytic activity was detected on synthetic substrates. Manjula *et al.* (2017) worked on the utility of *A. indica* oil seed cake for lipase production. *A. indica* seed cake is a low-value by-product resulting from bio-diesel production. Solid State Fermentation (SSF) technique was used for extracellular lipase production through penicillin species. The lipase activity in the crude was found to be 2.74 U/ml/min. Among different saturations the maximum enzyme was recovered at 80% saturation. Several studies have been carried out to obtain lipases by solid state fermentation using different agro-industrial residues, such as wheat bran, gingelly oil cake, rice husks, castor bean waste (Toscano *et al.*, 2011).

In this study, we investigated the neem callus for production of dextrinase/amylase and other hydrolytic enzymes in cell cultures and the production can be up-regulated by incorporating inducers in the media.

MATERIALS AND METHODS

EXPLANT PROCESSING

PLANT MATERIAL COLLECTION

The leaves which are young and apparently healthy were collected from the tree located in Department of Biotechnology, University of Karachi.

SURFACE STERILIZATION OF PLANT MATERIAL

Fifty percent sodium hypochlorite was used as sterilant, to surface sterilized neem leaves. 100 mL of fifty percent NaOCl was prepared by mixing 50mL commercial Robbin bleach (100%) in 50mL of autoclaved distilled water. All laboratory equipment (tools, jars and petri dishes) and distilled water was autoclaved at 15 psi and 121 °C for 20 minutes. Neem leaves were prewashed in running tap water for 15-30min to remove dust from surface. The leaves were then dipped in 50% NaOCl solution for 20 minutes, followed by rinsing then in autoclaved distilled water two times with five minutes of interval with invert mixing. After surface sterilization, under laminar flow hood, the leaves were transferred into the sterilized petri plate with the help pf forceps.

CALLUS DEVELOPMENT

MEDIA PREPARATION

Neem callus was initiated and maintained in MS media (Murashige and Skoog, 1962). Separate stocks of macro salts (10X), Micro salts (100X), vitamins solution (10X) and Iron-EDTA (10X) were prepared and stored in fridge. Hormonal stocks (IBA: 20mg/20mL and BA 10mg/10mL) were also prepared and refrigerated.. These stocks were used throughout the experiment. Sucrose was used as main carbon source in the media.

The pH of the media was adjusted to 5.85 using either 0.1 N NaOH or 0.1N HCl. The media was boiled to dissolve agar completely and then 15-20mL of media was dispensed in approximately 300ml of jars before autoclaving at 121°C for 15psi for 15 minutes (with no dry time). The media was stored at room temperature until used.

CALLUS INDUCTION

Followed by surface sterilization, the edges of leaves were trimmed off and excised into four pieces and transferred onto MS medium supplemented with IBA (4mg/L) and BA (1mg/L). The cultures were kept at room temperature in darkness. The callus was maintained by subculturing them in every 30 days.

AMYLASE INDUCTION STUDIES

AMYLASE INDUCTION THROUGH STARCH

SUSPENSION MEDIA PREPRATION

The liquid MS media was prepared in the same manner as callus induction media with the depletion of BA and agar.1% starch as inducer was incorporated in media and the media for positive control (without inducer) and negative control (only MS media) was prepared .The pH of the media adjusted to 5.85. The media was then autoclaved without dry time at 121°C and 15psi for 20min.

SUSPENSION CULTUR INITIATION

For the development of suspension culture, friable off-white callus was inoculated into the media, aseptically. The cultures were placed on platform shaker for about 15 days with a speed around 90-100rpm under 16:8 hours of light and dark period.

FILTERATION

The suspension obtained was subjected to filtration with the aid of filter paper to separate the cell mass from the medium. The enzyme produced is present in the filtrate, stored at 4°C.

BIOASSAY FOR AMYLASE

SUBSTRATE AGAR PLATE PREPRATION

Media containing 2% agar and 1% starch was prepared in distilled water and then autoclaved. Plates were poured with autoclaved media and the wells were made using sterilized borer. 150 µL of induced CFE (cell free extract), positive control and negative control was poured in the wells.

PLATE INCUBATION

Plates were incubated for 24hat 37°C for proper absorption and lysis.

RESULT AND OBSERVATIONS

Diluted gram's iodine solution was used to flood the starch agar plates for zone analysis. Hydrolysed starch shows a clear zone on blue-black background of unhydrolyzed starch.

OPTIMIZATION OF STARCH CONCENTRATION

SUSPENSION MEDIA PREPRATION

Four-hundred mililiter of liquid MS media was prepared and the pH was adjusted to 5.85 and dispensed into four different 250mL flasks. Four different concentrations of starch 0.5, 1, 1.5 and 2% were poured and then autoclaved.

SUSPENSION CULTURE INITIATION

Each flask was inoculated with friable off-white callus aseptically and then cultures were placed on shaker in same fashion as done for the induction studies.

FILTERATTION

Filtration was carried to separate CFE from the media with the aid of filter paper.

BIOASSAY FOR COMPARISION OF INDUCER CONCENTRATION

Media containing 2% agar and 1% starch was prepared in distilled water and then autoclaved. Plates were poured with autoclaved media and four wells were made using sterilized borer. 150 µLCFE of each percentage of starch was poured in individual wells. Plates were incubated for 24hours at 37°C for proper absorption and lysis. Diluted gram's iodine solution was used to flood the starch agar plates for zone analysis.

INDUCTION OF HYDROLYTIC ENZYMES OTHER THAN AMYLASE

SUSPENSION CULTURE PREPARATION

MS media deprived of agar and cytokinin was used to make suspension. To carry out the induction studies, specific inducers were used for respective enzymes. The autoclaved media was used as negative control while positive control have no inducers in the suspension media. The concentration of inducers added was 1%. The inducers used in experiment for different enzymes are described in **Table 1.** The pH of medium was adjusted to 5.85 and then autoclaved at 121°C and 15psi for 15min (with no dry time).

Under laminar flow hood, the portion of friable off-white callus was transferred into all flasks except for the negative control. These flasks were then incubated on plate-form shaker for about 16 days at 100rpm under 16:8 hours of light: dark period. The callus cells produce enzymes extra-cellularly into the medium. To check enzyme production level, media was separated from cell mass. After 16 days, suspension culture was removed from shaker to settle down the large clumps of cells in the bottom. The suspension was then filtered using filter paper under laminar flow hood.

To visualize the effect of inducers on enzymes production, substrate agar plate technique was used. One hundred and fifty µL of CFE was poured into the wells made by using sterilized borers in plate. After pouring the enzyme filtrate, the enzyme substrate reaction starts and to favour the enzyme substrate reaction plates were then incubated. Substrate lysis /precipitation on plate indicate enzyme production.

DEXTRINASE

Following the growth phase of cells, on stationary phase cells were filtered at day 16 and bioactivity was analysed on a media containing 2% agar and 1% dextrin. Media was autoclaved and then poured with dextrin induced CFE and positive and negative control CFEs. These plates were incubated at 37˚C for 24hrs.Diluted grams iodine was used to analyse the hydrolysis zone of dextrin.

CELLULASE

CMC agar plates were prepared by dissolving 1% carboxy-methyl cellulose (CMC) in distilled water on heating and then agar is dissolved and media was autoclaved. The plates were then poured and incubated with CFEs at 37˚C for 24hrs.CMC plates were flooded with Congo red, and after 30-40 min, the plates were followed with 1M NaCl solution.

GELATINASE

Gelatinase production was analysed on a medium containing 2% agar and 1%gelatin as substrate. Media was used after autoclaving and then CFEs was poured in the wells and incubated at 37˚C for 24hrs.Super saturated ammonium sulphate was used for flooding to enhance lysis. This effect was pronounced after 15min of flooding.

PROTEASE

Casein plate was prepared by dissolving 1%caesin with a few drops of 0.1N NaOH. Add 2% agar and dissolved, which is then autoclaved. The plates were poured, and were incubated at 37˚C for 24hrs after dispensing CFEs. Casein lysis was shown after flooding with super saturated ammonium sulphate. Casein makes the medium opaque and deflects light rays.

LIPASE

Lipase activity was checked on a medium containing 2% agar and 1% tween 80 along with 1% olive oil. 5mM of $CaCl_2.2H_2O$ was added to activate the hydrolysis of triglycerides. The media was autoclaved, poured and incubated at 35˚C for 24hrs along with CFEs. Lipase positive plate shows precipitation zone around the wells due to complex formation between calcium salts and free fatty acids released by lipase while no precipitation indicates negative results. Plates were incubated for 1 day in refrigerator to enhance lipid hydrolysis.

ESTERASE

Esterase activity was determined by utilizing the same media as for lipase activity except olive oil which is not used in esterase bioassay. Plates were incubated as for the lipase activity.

Table 1.Inducers used for the induction of hydrolytic enzymes.

S.No	Inducers	Enzymes
01	Starch	Amylase
02	Dextrin	Dextrinase
03	Carboxy-methyl cellulose	Cellulase
04	Gelatin	Gelatinase
05	Casein	Protease
06	Tween 80 & olive oil	Lipase
07	Olive oil	Esterase

CULTURE CONDITION STUDIES

Metabolites production is related to the source of energy, nitrogen, phosphates, growth regulators and inoculum density. To force the neem callus for lipid hydrolytic enzyme production, modification in the media was studied as in following headings.

LIPASE

Liquid MS media was prepared. Other than gelling agent and cytokinin deprivation, sucrose was not added in the media so that callus cells utilize lipids as energy source for their metabolic reactions. The pH was adjusted to5.85. Tween-80 and olive oil of 1% was added as inducers in the media and the medium was then autoclaved. Suspension was made by adding fresh callus and incubate flask on shaker for 16 days with same culture condition as for induction studies of lipase. Lipase activity was tested in agar plates as we performed for induction studies with lipase.9

ESTERASE

Suspension media was prepared, which was sucrose deficient and no gelling agent and cytokinin was added. Tween 80 of 1% concentration was used, with 5.85 pH of the medium. Suspension was made aseptically and CFEs was prepared according to above mentioned protocol. Assay was performed like induction studies of esterase.

RESULTS AND DISCUSSION

CALLUS INDUCTION FROM NEEM LEAVES

Leaves were taken as explant used to induce callus. After surface sterilization they were cultured on the MS media and stored in dark. In the initial period, curling of leaves occurred and after few days of incubation, small white lumps are began to form from the cut edges of leaves. The leaves started to lose its differentiated property. At about 22^{nd} day of incubation, the leaves completely lost differentiated state and the whole explants converted into 100% callus.

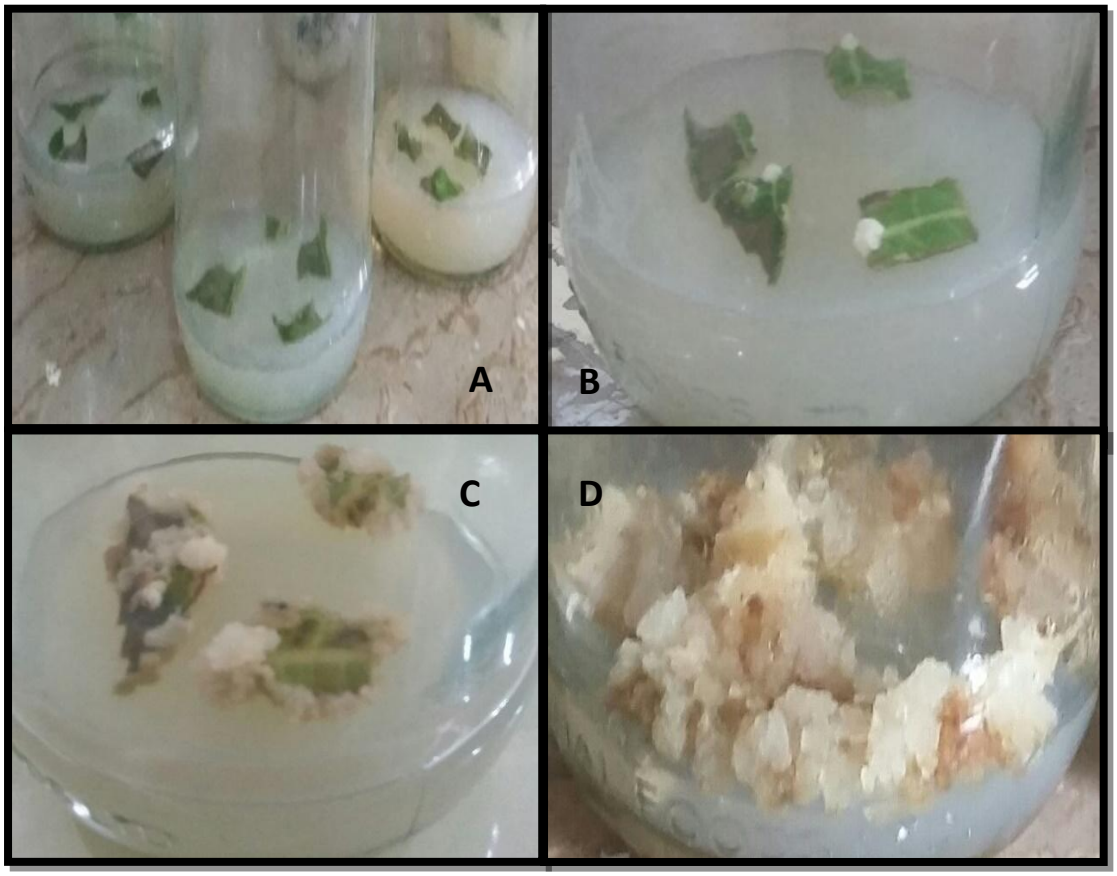

Fig. 1.Induction of the callus culture from explants initiation (**A**) beginning of callus formation (**B**), callus production (**C**) and whole explants covered with callus cells (**D**).

EFFECT OF STARCH ON AMYLASE PRODUCTION

Starch was used as inducer to up-regulate the production of amylase. Starch is the substrate of amylase so, if starch is present in the microenvironment, transcription of amylase gene is switched on to hydrolyse its substrate. This is positive enzyme induction. This induction occurred only when the medium become sucrose depletive, sucrose repressed the amylase production, as it is more favourable to utilize. The results of cell free extract of induced sample, positive control and negative control compared on the agar-starch plate. The inducer has pronounced effect on amylase production, a cleared zone is observed on the plate.

Fig. 2. Amylase activity on starch agar plate after 1 day incubation at 37 °C, flooded with diluted gram's iodine.

OPTIMIZATION OF STARCH CONCENTRATION

Four different concentrations of starch were compared on substrate agar plate (0.5%,1%,1.5%,2%). On comparison, 0.5% starch is more inducible concentration for amylase and as the concentration increases the amylase production is repressed (Figure 3). The zone of 1% inducer is narrow compared with previous plate due to the fluctuations in the incubation temperature, or it may be due to the thickness of the media in the plate. However, it was observed that, when 0.5% or below 0.5% starch was used in *Azadirachta indica* suspension culture, the amylase production was significantly enhanced.

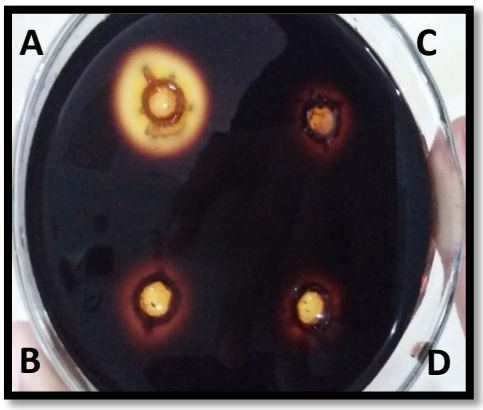

Fig. 3. Comparison of different starch concentration effect on amylase production.0.5% (**A**), 1.0% (**B**), 1.5% (**C**), and 2.0 % (**D**).

EFFECT OF DEXTRIN AND CARBOXY-METHYL CELLULOSE ON CARBOHYDRATE DEGRADING ENZYMES

Fig. 4 shows dextrin hydrolysis on agar plate. Comparatively dextrin induced CFE have higher dextrinase level than positive control. Due to amylase capability to hydrolyse dextrin as substrate, this hydrolysis could be the result of amylase production in the suspension because dextrin is made from starch by combination of slight depolymerization (hydrolysis) and trans glycosylation (molecular rearrangement) (Be Miller *et al*, 2003). Purification and characterization of these enzymes will lead to the confirmation. Zemekt *et al.* (1992) reported that the presence of cellulose and its derivative in the medium enhanced the production of cellulase. Splitting products of cellulose (cellobiose and higher oligosaccharides) were detected, when spruce callus was cultivated on media containing hydroxyethyl cellulose as only carbon source. In Fig. 5 no zone of hydrolysis is observed from the suspension. This may be result of poor suspension culture after 16 days of incubation although all the growth parameters were standardized. Hormonal treatment regulates the cellulase activity in tobacco as reported by Truelsen and Wyundaele (1991). Auxin indirectly regulate the endo-1,4-β-glucanase synthesis and utilize cellulose as its substrate in sucrose added media (Ohmiya *et al.*, 2000).

Fig. 4.Dextrinase activity on dextrin agar plate flooded with diluted gram's iodine.

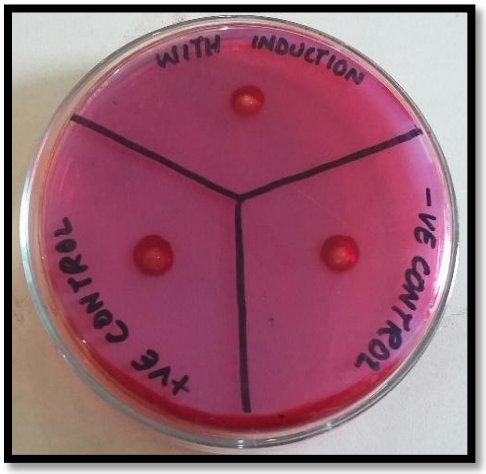

Fig. 5.CMC agar plate flooded with Congo red Followed by NaCl for cellulase activity.

EFFECT OF GELATIN AND CASEIN ON PROTEOLYTIC ENZYMES

Proteolysis was observed when casein is used as inducer in *A. indica* suspension culture (Fig. 6) and no activity was observed in gelatin induced suspension (Fig. 7). The protease activity can be due to the protein degraded amino acids tryptophan which acts as precursor for auxin. The proteolysis occurs to maintain cellular responses by eliminating abnormal proteins, activating non- active proteins and through programmed cell death of unwanted cells.

Fig. 6.Protease activity on casein agar plate, flooded with super-saturated ammonium sulphate.

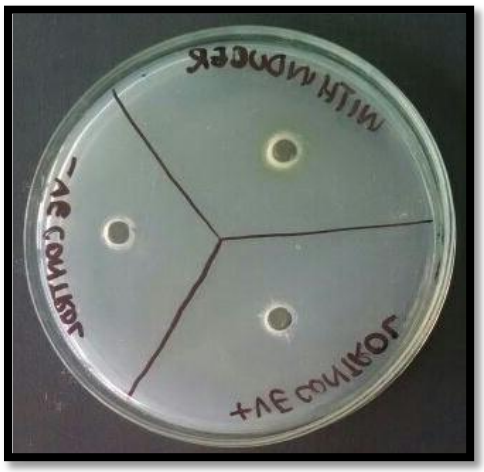

Fig. 7.Gelatin agar plate flooded with super-saturated Ammonium sulphate for gelatinase activity.

EFFECT OF TWEEN 80 AND OLIVE OIL ON LIPOLYTIC ENZYMES

Boekema *et al.* (2007) described that compounds like tween 80 and olive oil can induce lipase gene expression in different bacterial species. During induction studies, no lipolytic activity was observed either for lipase and esterase in *A. indica* suspension culture (Fig. 8 & 9). For callus cells, the preferred energy source is sucrose, and 3% of sucrose was used in the MS media. The presence of sucrose inhibits lipid hydrolysing enzymes production and breakdown of exogeneous lipids.

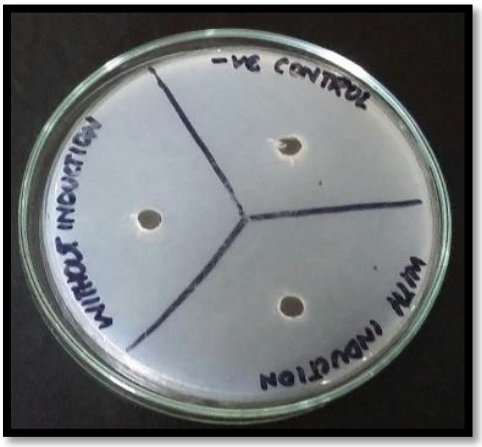

Fig. 8.Lipase activity agar plate.

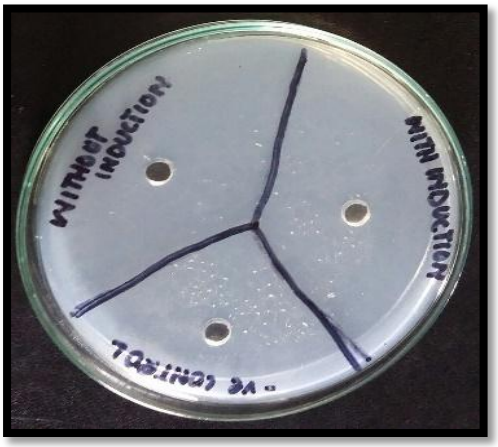

Fig. 9.Esterase activity agar plate.

CULTURE CONDITION STUDIES FOR LIPASE AND ESTERASE

To *et al.* (2002) reported that the presence of sugar in the media inhibits the cytosolic development and lipids breakdown and hence lipolytic enzyme production. Sugar starvation has been reported for varied plant species and its effects on their metabolism was analysed in cell cultures, callus cultures and organ cultures. Plant cells under sugar starvation, gradually shift towards protein and lipid metabolism due to the lack of carbohydrates is described by Morkunas *et al.* (2012).

In this study, the MS media used, was sugar starved and respective inducers for lipase and esterase was added in the media. The precipitation of lipid hydrolysis was observed, confirmed that sugar may regulate the lipase production. Zone of precipitation was not much clear for lipase activity and no precipitation was occurred in esterase activity after incubating plates in fridge (Fig. 10 and 11, respectively). During sugar starvation in maize root meristems, lipids and other carbon sources utilized by cells, this process could be reversed by supplying sugar in the media (Brouquisse *et al.*, 1992).

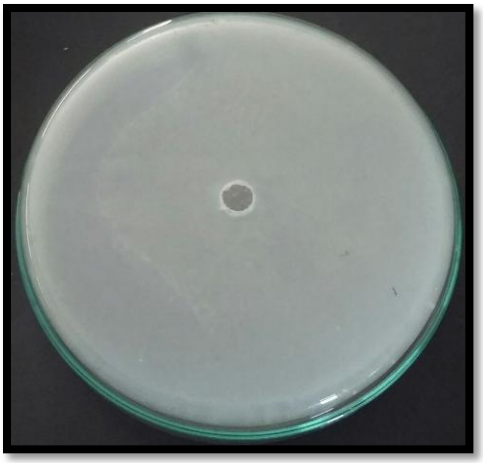

Fig. 10.Lipase activity on tween 80, olive oil and agar in sugar starved suspension culture.

Fig. 11.Esterase activity on tween 80 agar plate in sugar starved suspension culture.

CONCLUSION

This study was conducted to carry out induction studies for hydrolytic enzymes by utilizing different inducers in suspension culture. *A. indica* callus was induced on MS media at about 15 days of culturing, when the callus become off-white and friable it was used to make suspension culture to study the amylase induction in the callus. After 15 days of incubation, suspension was analysed for enzyme activity, which prove the positive effect of starch on amylase production and dextrin for dextrinase activity. However, further optimization of starch and dextrin was done to make the enzyme production process economically cheap in search of inducer concentration intended for higher concentration of enzyme. Lipase and esterase were not detected. However, sugar starvation regulates the expression of lipase production. If the media contains carbohydrates the callus cells don't breakdown lipid reserves for their metabolism. Proteases activity was up-regulated when casein was incorporated in the media. For cellulase and gelatinase the results were same as lipase and estrase. Cellulase activity was not observed due to the poor suspension culture, the density of the suspension was also increased due to the incorporation of CMC in media. In brief,induction studies concluded that the neem cultured cells have potential to produce some hydrolytic enzymes not all and this may be analyzed further with different experimental approaches. So, neem cells can be used for industrial scale production of these enzymes. This is the first report of dextrinase from cell suspension culture of *A. indica*.

To induce these enzymes other than induction different strategies should be used such as effect of elicitors, cell line selection and immobilization techniques for efficient production. Another objective for future research is the purification and characterization of these enzymes. The amino acids residue will deduce about the substrate variability of enzymes. The purification and characterization of these enzymes from neem callus is the object of further research and our experimental work would be significant for future.

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