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Research Article

Study of Fungi to Evaluate Their Potential for Metabolites Production under Submerge Condition by Fermentation Technology

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Abstract: *Aspergillus* is filamentous achlorophyllus fungi and was isolated from agriculture crop fields of Mandsaur then identified by microscopic method using cotton blue and lacto phenol. Biomass and metabolites i.e. protein and enzyme were produced by *Aspergillus* under submerge fermentation technology during various incubations. After observation it was found that maximum biomass, protein and invertase was shown as 1.10gm, 4.575mg/ml and 21.80µg/ml after 10 and two days incubation time respectively by *Aspergillus* spp. These metabolites were estimated using standard protocols.
Keywords: *Aspergillus* spp., Biomass, Dinitro salicylic acid Submerge Fermentation technology, Invertase, Incubation time, Lowry reagent, Protein.

INTRODUCTION

Generally fungi are filamentous non-photosynthetic organism and nutritionally multiplied on dead organic materials or living cells as saprophyte or parasite. In growth phase, fungi produced their primary and secondary metabolites and available in their growth medium [9]. Protein is a nitrogenous compound and made up of 20 different amino acids. All living organisms required protein for their growth. Generally fermentation technology was used for commercial protein production using many microorganisms including fungi, yeasts, bacteria and actinomycetes. Proteins are nitrogen or amino acid supplements and most of the enzymes are made of proteins. Besides energy source, protein required for fungi, bacteria, actinomycetes and other unicellular and multicellular cells for their membrane and enzyme synthesis [10].

Filamentous fungi have been widely used in the fermentation industry as it becomes a principal source of protein, enzymes and other metabolites. Therefore, fungi have been widely investigated by various researchers due to low cost and high productivity which attracted many other researchers to improve fungal strains by molecular techniques and also bioprocess [5, 16].

Many cells membrane made of lipoprotein, glycoprotein and other compounds where protein associated with them. Therefore, proteins are produced by fermentation technology and used in a lot of industries such chemical, detergent and food as nutrient supplements for human diet [24, 31].

Invertase catalyze the hydrolysis of sucrose to produce an equimolar mixture of d-glucose and d-fructose at concentrations lower than 10% sucrose, thus making these enzymes suitable for biotechnological applications [40]. Invertases (EC.3.2.1.26) are members of glycoside hydrolases family [1] and have numerous beneficial favorable functional properties [17].

The maximum rate of invertase enzyme production in presence of sucrose by *A. niger* during submerged culture condition was found as 3.67 U/L/h at 12.5 g/L while in the case of glucose-sucrose mixture; it was 13.95 U/L/h at 25 g/L. It was observed that the enzyme yield was 1.25 times more in presence of sucrose than with combined action of glucose-sucrose [38].

Invertases have been characterized in many filamentous fungi such as *Penicillium*, *Neurospora* and *Aspergillus spp.* [4, 19-20, 36]. The enzymatic activity of invertase has been characterized in other microorganisms i.e. *Thermomyces lanuginosus* [8], *Candida utilis* [7], *Penicillium chrisogenum* [33], *Saccharomyces cerevisiae* and *S. carlsbergensis* [21, 23]. *Saccharomyces cerevisiae* synthesizes two invertases i.e. a glycosilated periplasmic protein and a cytosolic non glycosilated protein [37, 46].

Dworschack and Wickerham [14] demonstrated that extracellular invertase was also produced by yeast and some physiological factors influence the production of extracellular and intracellular invertase by *Saccharomyces uvarum.* Invertase production by *P. chrysogenum* in penicillin fermentation broth was studied by Damle *et al.* [11] and reported that invertase produced extracellularly.

Elorza *et al.* [15], Trimble and Maley [43] reported that *Saccharomyces cerevisiae*-136 produced external invertase in culture filtrate supplemented with

50% sucrose and maltose. Olutiola and Cole [34] described that an extracellular invertase production in *Aspergillus flavus* induced by sucrose containing liquid medium. The biosynthesis of invertase was repressed when repressive hexose sugars (glucose or fructose) were added to sucrose metabolizing cells.

Vainstein and Peberdy [45] investigated that *Aspergillus nidulans* produced an extracellular invertase when cultured on a medium containing sucrose or raffinose and was found maximal on medium containing sucrose after 15 hrs of incubation at 28°C. The amount of invertase in the culture medium was declined after this incubation time.
Rubio and Maldonado [39] studied that invertase produced by *Aspergillus niger* strain showed the maximum enzyme activity at 60°C temperature and pH 5.0. Medium containing glucose (0.1 M), fructose (0.5 M) and sucrose (1.2 M) as compared to that with only sucrose (1.8 M) solutions produced 3-fold more oligosaccharides at pH 7.5 [42].

Herwig *et al*. [21] reported that *S. cerevisiae* invertase as hydrolyzing capacity of sucrose and was controlled by the presence of hexoses such as glucose and fructose in medium. Commercially, invertase is produced on large scale primarily by using submerged fermentation. The well-known methods for production of invertase are submerged and solid state fermentation [19-20, 37]. The conventional method for the production of invert sugar involves acid hydrolysis of sucrose and has low conversion efficiency, high energy consumption and thus high cost of production due to acid hydrolysis whereas microbial invertase splits sucrose into glucose and fructose (sugar syrup) with 100% conversion efficiency [28].

The product obtained by invertase has the advantage of being colorless as compared to that obtained through acid hydrolysis [3, 6, 12, 27]. Kotwal and Shankar [26] studied the importance of invertase for the sucrose hydrolysis that yielded an equimolar mixture of glucose and fructose called as inverted syrup, also commonly used in beverage and food industries. Robledo-Olivo *et al*. [38] studied invertase production by *Aspergillus niger* in submerged culture using different concentrations of glucose and sucrose and found that the initial concentration of sucrose was increased from 6.25 to 50 g/L then a higher biomass production (6.1 g/L) was achieved. The biomass production was increased four times more when a glucose-sucrose combination was used as substrate (26.31 g/L).

Uma *et al*. [44] produced high levels of invertase in *A. flavus* under optimized culture conditions on fourth day of incubation at an optimum pH (5.0), temperature (30^{o}C), and inoculums size (3%) in Czapek Dox medium using fruit peel waste as a substrate by submerged fermentation and also obtained improved enzyme when nutritional factors such as sucrose and yeast extract were added into the medium.

Invertase is extensively used in confectionaries, food industries and in pharmaceuticals [4]. Invertase is used for the inversion of sucrose in the preparation of invert sugar and syrup. It is one of the most widely used enzymes in food industry where fructose is preferred than sucrose especially in the preparation of jams and candies, because it is sweeter and does not crystallize easily [2]. The objectives of the present study are to produce fungal biomass, total protein and invertase enzyme during various incubations using sucrose and evaluated by standard protocols.

MATERIAL AND METHODS

Soil sample collection

Five to six soil samples were collected from agricultural crop fields around Ratlam (23.32ºN and 75.07ºE Longitude) in Malwa region, Madhya Pradesh, India and were mix to form one composite sample. Soil sample was crushed using Mortar and Pestle to clean by removing rocks particles and plant debris.

Isolation of fungi

Fungi were isolated from soil by serial dilution technique [13, 22] using 10 gm soil and were dissolved in 0.85% saline water in Erlenmeyer conical flask (500 ml capacity) having 95 ml saline water and beads then mix properly by shaking, mark as 10^{-1} dilution. Obtain 10^{-3} dilution by serially transferred 10 ml soil sample from 10^{-1} dilution to conical flasks having 90 ml saline water. 0.1ml sample was transferred from 10^{-3} dilution to sterilize Petri dish and poured melted Potato dextrose agar (PDA) medium. Plate was shaking gently to mix sample and medium then left at room temperature to solidify medium. The solidified plate was incubated at 30℃ for 5 days. After that plate was used for observation.

After incubation period, few spores were taken by wire loop and streaked on PDA medium plate by zigzag method and incubated at 30°C for 5 day. Colonies were observed by light microscope using 10 and 40X objective lens. Pure colonies were transferred to PDA plate and used for identification (Fig.- 1).

Identification of fungi

After purification prepared microscopic slide for each pure culture. Fungal culture was taken from colony by forceps on clean glass slide and separated by needles. Culture was stain with Cotton blue and lacto phenol and covered with glass cover slip then observed in light microscope using low power (10X) and high power (40X) objective lens. Morphological characteristics of fungal culture were identified using laboratory manual for introductory mycology [18, 35, 41]. After purification and identification spores of pure colonies was transferred to PDA slant and incubate at

30ºC for 5 days then stored in refrigerator at 4ºC for 3-6 month.

Spore count

Sterilized water (5.0 ml) was introduced to culture slant and scraped by nichrome wire loop then 0.1ml spore suspension was used to count spore using heamocytometer.

Biomass production

Spores of *Aspergillus* spp. (1 ml) were transferred to steam sterilized production medium (Table- 1) and incubated at 30ºC for 2, 4, 6, 8 and 10 days under submerged condition.

Estimation of dry biomass

After incubation period, biomass was harvested by filtration method using Whatman No. 1 filter paper and dried in hot air oven at 60ºC for 24 hrs then weigh using electronic balance (Fig.- 2).

Protein production

Poured 5 ml sterilized water in culture tube and then spores were scraped by using nichrome wire loop. One milliliter spore suspension was transferred to five Erlenmeyer conical flasks having 100 ml production medium (Table- 1) and incubated at 30ºC for 2, 4, 6, 8 and 10 days under submerged condition for fermentation. After incubation time, culture filtrate was obtained by filtration using Whatman No. 1 filter paper and stored in refrigerator fro protein and enzyme estimation (Fig.- 3).

Estimation of total protein by Lowry method

Lowry reagent C mixes with 0.2ml of culture filtrate in glass test tubes [42]. The tubes were incubated at 37ºC for 20 min in water bath then cooled and added and 0.2 ml Folin phenol. The tubes were again incubated at room temperature for 30 min and recorded color density at 660 nm by Spectrophotometer. The absorbance difference was calculated using standard curve of protein (BSA 1 mg /ml).

Enzyme production

Spores of selected fungi i.e. *Aspergillus* spp. were transferred to PDA slants and incubated at 30ºC for five days. Five milliliter distilled water was introduced to culture slant and scraped by wire loop. One milliliter spore suspension was transferred to five Erlenmeyer conical flasks having 100 ml production medium (Table- 1) and incubated at 30ºC for 2, 4, 6, 8 and 10 days under submerged condition for fermentation. After incubation time, culture filtrate was obtained by filtration using Whatman No. 1 filter paper and stored in refrigerator for enzyme estimation.

Enzyme estimation by DNS method

Enzyme activity was assayed using sucrose as substrate [25]. The assay was performed on the basis of red brown color produced by DNS reagent [30]. In this assay, 1 ml of 2 mM sucrose solution in 0.05 mM sodium acetate buffer of pH 5 was taken in glass test tube and added 0.5 ml culture filtrate then mixed by using cyclomixer. All test tubes with reaction mixer were incubated in water bath at 30ºC for 15min then added 2 ml DNS reagent (DNS- 10 g Phenol- 2 g Sodium succinate- 0.5 g, sodium hydroxide- 10 g and Distilled water- 1 L) to stopped enzyme - substrate reaction and again incubated for 20 min at boiling water bath to developed red brown color. All test tubes were takeout from water bath and cooled then added 1 ml of 1.0% Rochella salt (Sodium potassium tartrate- 10 g and Distilled water- 1 L). All test tubes were left at room temperature for 20 min to stabilized red brown color. Color density was recorded by spectrophotometer at 550 nm using spectrophotometer and compared with standard curve of glucose (1 mg/ml).

RESULTS AND DISCUSSION

The biomass, protein and invertase estimation of various incubation extract was evaluated using standard protocols and have found following estimations given in Table- 2 to 4.

Results obtained to evaluate total dry biomass are shown in Table- 2 and Fig.- 4 that dry weight of biomass was increased from 0.45 to 1.10 g with incubation time. Further Table 2 shows that 0.45 and 0.80 g biomass produced after 2 and 4 days incubation time and was increased expontially as 1.10 gm after 10 days incubation period. Figure 4 reveals that fungal hyphae utilized nutrient then produced more biomass but after 6 days biomass production was slowly due to late growth phase. The above results for biomass production were supported by [38] that reported higher biomass production of fungi with sucrose used as carbon source.

It is evident from Table 3 and Fig.- 5 that 4.575 mg/ml protein was produced by *Aspergillus* spp. after 2 days and also 1.11 mg/ml protein was recorded after 8 days incubation time. Further Table 3 indicates that fungi produced extracellular protein that used for their building blocks. Hence, the amount of protein was decreased 1.850, 1.0 and 0.905 mg/ml after 4, 6 and 10 days incubation periods respectively. Figure 5 shows that initially amount of protein was produced maximum then decreased because fungal mycelium production utilized these produced protein. Therefore, amount of protein was decreased after 4 to 6 days. After 8 days incubation time, protein amount was increased slightly then again decreased as same. The proteins are nitrogenous compounds and helps in synthesis of enzymes as well as energy source for living organisms. These observations are supported by Finkelstein and Ball [16], Moreira *et al*. [31], Banerjee *et al*. [5] and Karthiresan and Manivannan [24] which were reported that fungi have been used for their metabolite production.

Table- 4 and Fig.- 6 reveals that maximum enzyme activity was occurred after 2 days while shows minimum after 4, 6 and 10 days incubation periods. Further Table 4 indicates that *Aspergillus* spp. invertase production was obtained maximum on the basis of their assay after 48 hrs (21.83µg/ml) and also found minimum after 4 days incubation time. The amount of invertase in culture filtrate was slightly increased after 4 and 6 days incubation time. Initially sucrose was present in medium, function as inducer. Hence, amount of invertase was increase up to maximum but after growth of fungi on medium sucrose was metabolize then produced monosaccharide as glucose and fructose which play a role as inhibiter. Therefore concentration of invertase was decreased. These monosaccharides were utilized by fungi then again remaining sucrose was metabolizing so that invertase concentration was increased after 8 days incubation time.

These results are supported by Elorza *et al*. [15], Trimble and Maley [43], Olutiola and Cole [34], Vainstein and Peberdy [45] reported that fungi produced extracellular invertase by supplemented with sucrose. Rashad *et al*. [37] and Guimaraes *et al*. [19-20] also observed that invertase production was occurred in submerged condition at 30°C temperature and pH 5.0 after 4 days incubation time [44].

Table- 1 Composition of production medium.

S. No.	*Ingredients*	Quantity (gm/L)
1	Sucrose	20
2	Yeast extracts	10
3	Ammonium sulphate	1
4	Magnesium sulphate	0.75
5	Potassium dihydrogen phosphate	3.5
6	pH	5.0

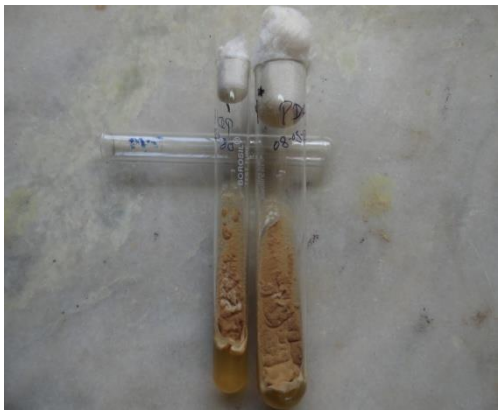

Figure- 1 (A): *Aspergillus* spp. culture on agar slant

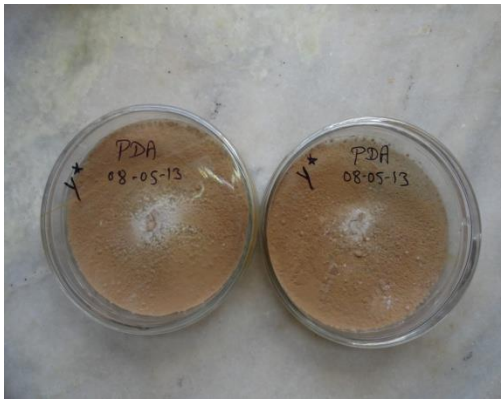

Figure- 1(B): *Aspergillus* spp. culture on agar plate

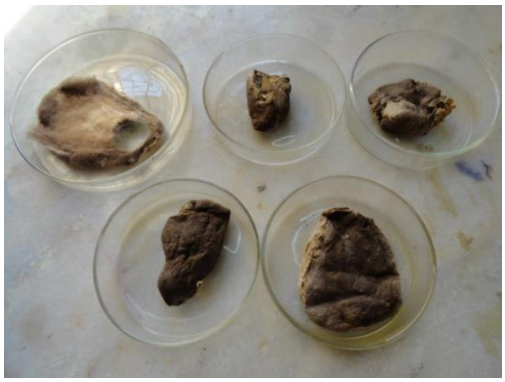

Figure- 2: Total dry biomass of *Aspergillus* spp. in varying incubation time (2 to 10 days).

Figure- 3 (A): Production media

Figure-3(B): Growth of *Aspergillus* spp. in production media

Table- 2 Estimation of total dry weight of biomass of *Aspergillus* spp.

S. No.	*Incubation days*	Dry biomass (gm)
1	2	0.45
2	4	0.80
3	6	1.00
4	8	1.02
5	10	1.10

Table- 3 Total protein estimation from culture filtrates of *Aspergillus* spp.

S. No.	Incubation days	Total protein(mg/ml)
1	2	4.575
2	4	1.850
3	6	1.000
4	8	1.110
5	10	0.905

Table- 4 Enzyme activity in culture filtrates of *Aspergillus* spp.

S. No.	Incubation days	Enzyme activity (µg/ml)
1	2	21.80
2	4	19.65
3	6	19.92
4	8	20.30
5	10	19.76

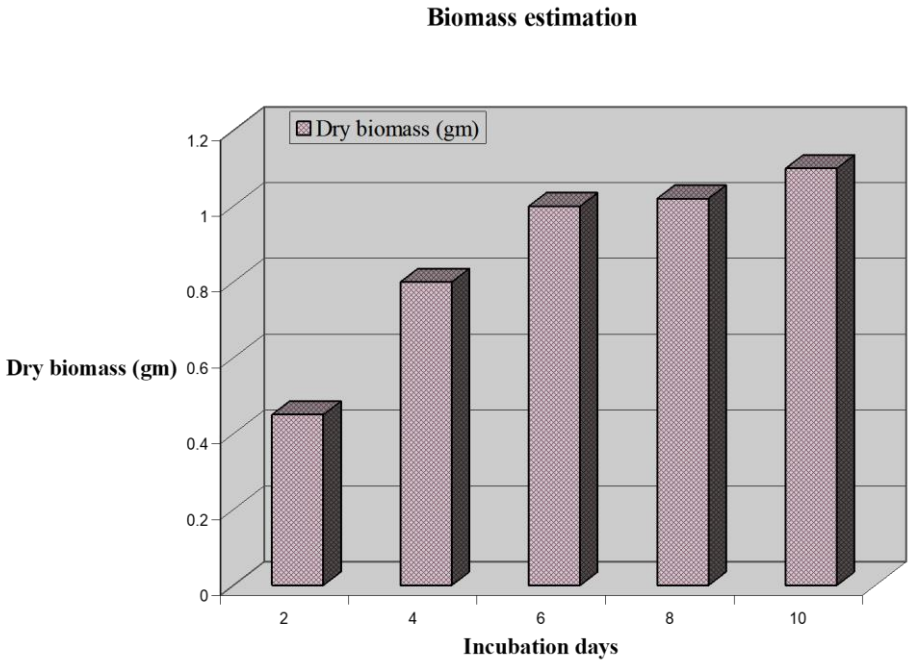

Figure- 4: Total dry biomass estimation of *Aspergillus* spp.

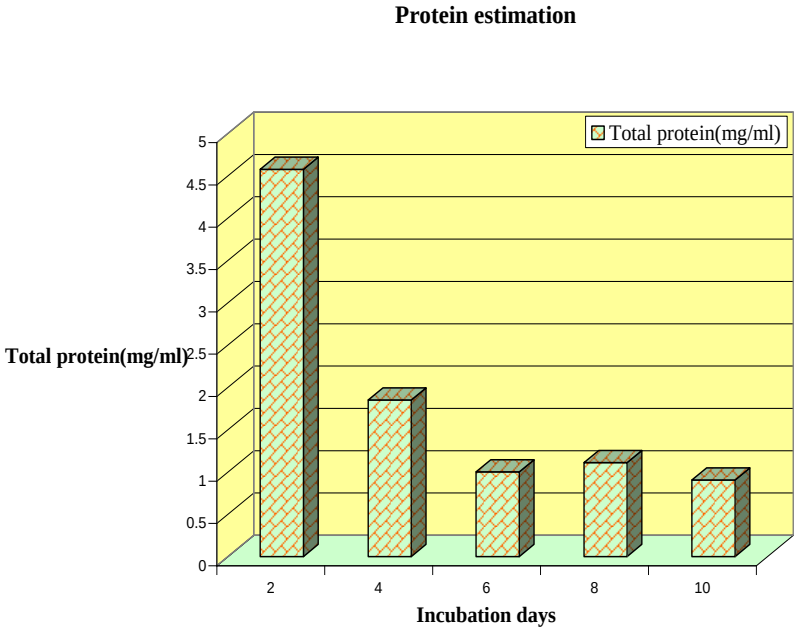

Figure- 5: Total protein estimation from culture filtrates of *Aspergillus* spp.

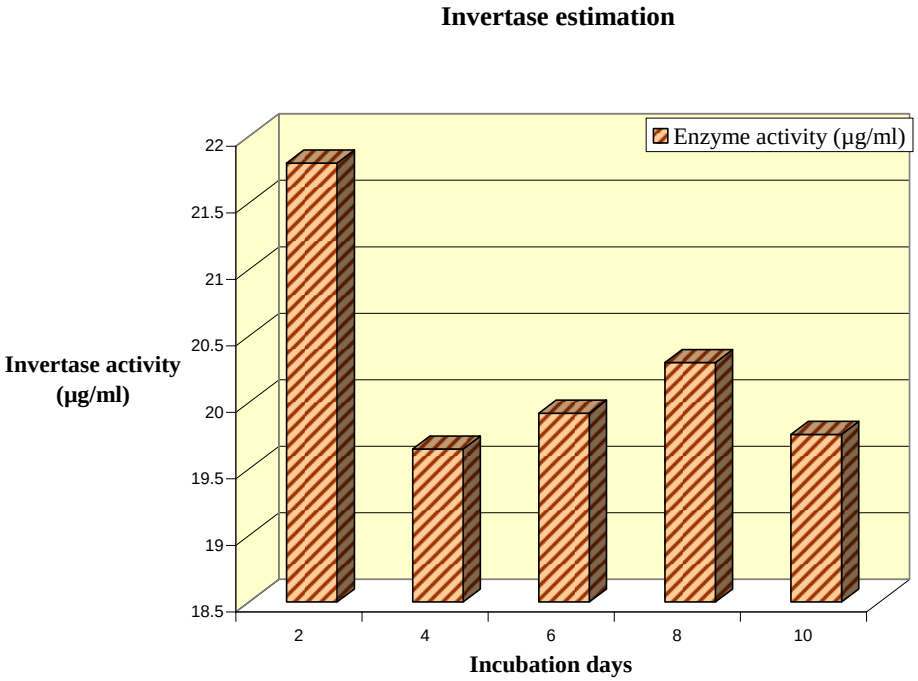

Figure- 6: Invertase estimation from culture filtrates of *Aspergillus* spp.

CONCLUSION

By evaluating it was found that *Aspergillus* spp. metabolites were produced maximally at 30ºC temperature and pH 5 after two day incubation times. These metabolites can be used in food and chemical industries for human diet and also preparation of glucose crystals and syrup as energy source.

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