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Optimization of biomass and pigment production by Penicillium species isolated form virgin forest floor

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ABSTRACT

Extracellular pigment-producing ascomycetous filamentous fungi belonging to the genera *Penicillium* was obtained from soil and its optimal culture conditions investigated. The effect of nutrient culture media, pH and temperature on growth, sporulation and pigment production of three *Penicillium* species isolated from virgin forest floors of North east India was studied to optimize the mycelial growth and secondary metabolite production. Culture media significantly affected the growth, sporulation and conidial discharge of the species. The average colony growth of the three *Penicillium* species was higher in Malt Extract Agar (MEA) medium (4.1±0.59cm) whereas spore production was higher in Potato Dextrose Agar (PDA) medium (87.3±3.1). The optimal culture conditions for pigment production were as follows: inoculum's age, 7 days; temperature, 25 °C; pH, 6 and medium, PDA. Mycelial growth had also a significant correlation with the media pH and incubation temperature together with the pigment production. Under the optimal conditions obtained in the flask culture tested, the isolates secreted coloured pigments into the culture medium which was dependent on the pH of the solution. The high concentration of pigments produced by *Penicillium* species may be utilized for commercial production of pigments or the metabolites having industrial importance.

KEYWORDS

Culture medium;
Penicillium species;
PH;
Pigment production;
Temperature.

INTRODUCTION

Penicillium is an ascomycetous fungal genus with widespread occurrence in most terrestrial environments and about two hundred species are well described. Most of the species are soil inhabitants, food borne contaminants or food ingredients used in the preparation of cheese and sausages[18,30]. Many isolates produce diversified active secondary metabolites, including antibacterial [10,31], antifungal substances[19,23], immunosuppressants and also potent mycotoxins[9-10].

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Thousands of Penicillium isolates have probably been screened in bioprospecting programs, and new bioactive metabolites continue to be discovered[23,26], indicating their current importance as sources of high amounts of novel bioactive molecules to be used by pharmaceutical industry.

There is worldwide interest for the production of pigments from natural sources due to a serious safety problem with many artificial synthetic colorants, which have widely been used in foodstuff, cosmetic and pharmaceutical manufacturing processes[20]. Other than Penicillium, there are number of micro-organisms which have the ability to produce pigments in high yields[15]. The red pigments, produced in solid-state cultures by several species of the genus Monascus, have been traditionally used in many Asian countries for colouring and securing a number of fermented foods[7, 8]. For industrial applications of microbial pigments, higher production of pigment yield, chemical and light stability are essential features. Isolation of new strain is still of particular interest because of necessity to obtain microorganisms with suitable characteristics for submerged cultivation [33]. We targeted to isolate and screen the efficient pigment producers as a potential role in food, cosmetics and pharmaceutical industry. For commercial application, optimization of fermentation condition in order to produce more yield and stability of pigment from Penicillium is necessary.

Although there are a number of natural pigments, only a few are available in sufficient quantities to be useful for industry because they are usually extracted from plants[24]. Therefore, it is advantageous to produce natural pigments from micro-organisms. There are a number of microorganisms which have the ability to produce pigments in high yields, including species of Penicillium[14,15].

Amongst them, many species of Penicillium have attracted special attention because they have the capability of producing different coloured pigments showing high chemical stability and economic importance[5,15]. Penicillium species produce a much diversified array of active secondary metabolites, including antibacterial[31, 25] antifungal substances[28], immunosuppressants, cholesterol-lowering agents[22], and also potent mycotoxins[9,10]. We have been screening a collection of filamentous fungi isolated from virgin forest soils of Brahmaputra valley of Indo Burma Biodiversity hotspot region for novel fungal natural products targeting at metabolites with biotechnological applications for the pharmaceutical industry. Three most active extracts producing coloured secondary metabolites was obtained from Penicillium isolates.

Several reports have indicated that pigment production in submerged culture was affected by numerous environmental factors, particularly the nitrogen source and medium pH[3,4,16,17]. The growth medium, its pH and temperature had strong influences on the growth, sporulation and conidial discharge of the fungal species[36]. Molds are affected by all the environmental factors; (chemical and physical). Physical and chemical factors have a pronounced effect on diagnostic characters of fungi. Fungal growth (spore germination, vegetative growth and sporulation) has a specific set of conditions that is optimal. Important conditions in this set are nutrient types and concentrations, light, temperature, oxygen and water availability[21]. However, the effect of environmental factors on growth of fungi is generally less specific and restricted than the effect on secondary metabolite production[29].

Temperature, water activity (aW) and pH were considered to be some of the most important factors in fungal groth and differentiation[27]. In the present study, optimal culture conditions for the production of red pigment by Penicillium species

were investigated in shake flask and batch fermenters. Therefore, the objectives of this study were to provide information on the effects of culture media, its pH and temperature on mycelial growth, conidial discharge and pigment production of three Penicillium species collected from the natural virgin soils.

MATERIALS AND METHODS

Inoculum preparation

Penicillium spp. (P001, P002 and P003) were isolated from virgin forest floor of Assam (India) and identified according to[32]. The stock culture isolates were maintained on a potato dextrose agar (PDA) slant. The isolates were initially grown at 25°C on a PDA plate for 7 days for inoculum preparation. A loop full cells from the outer zone of the colony was punched with a sterile cutter ($1cm^2$) and transferred to 25 ml potato dextrose broth medium in a 250 ml flask, and grown at 25°C under basal conditions (static) for 7 days[14].

Effects of agar medium on colony growth and conidial discharge

The eight agar media used were: Potato Dextrose Agar (PDA), Czapek Dox Agar (CDA), Oat Meal Agar (OMA), Yeast Extract Agar (YEA), Malt Extract Agar (MEA), Czapek Yeast Agar (CYA), Corn Meal Agar (CMA) from Himedia brand (TABLE 1).

TABLE 1 : Agar base solid and liquid media (Himedia).

Agar Base Culture media (Himedia)	Without agar base (Liquid media)
Potato Dextrose Agar (PDA)	Potato Dextrose (PD)
Czapek Dox Agar (CDA)	Czapek Dox (CD)
Oat Meal Agar (OMA)	Oat Meal (OM)
Yeast Extract Agar (YEA)	Yeast Extract (YE)
Malt Extract Agar (MEA)	Malt Extract (ME)
Czapek Yeast Agar (CYA)	Czapek Yeast (CY)
Corn Meal Agar (CMA)	Corn Meal (CM)

Media were prepared with deionised water to 1L, and 10 ml dispensed into 9-cm plastic Petri dishes. Agar plugs of 8 mm diameter taken from the margin of starter cultures were placed at the centre of test agar medium plates, arranged randomly, and stored in an incubator at 25±2°C in the dark. Colony diameters were measured at 7 days after incubation. For each medium type, four replications were used, and the test was repeated once.

Effects of liquid medium on mycelial mass and pigment production

The eight liquid media were used for mycelial production test were: Potato Dextrose (PD), Czapek Dox (CD), Oat Meal (OM), Yeast Extract (YE), Malt Extract (ME), Czapek Yeast (CY) and Corn Meal (CM). The media were amended with deionized water to 1L in volume. Eight mm agar disks from PDA starter cultures were used to seed 250 ml Erlenmeyer flasks containing 100 ml liquid media. Seeded flasks were incubated in the growth chamber as described above on a rotary shaker at 200 rpm for 7 days. Mycelia were harvested by filtration using Buchner funnel. Then the mycelia were washed thoroughly with distilled water and the excess of water was removed by plotting with filter papers[37]. The mycelia of water were removed by plotting with filter papers. The myclia were then either directly weighted for fresh weight and dried at 80°C till constant weight obtained for mycelial dry weight. The growth rate of each isolate on each type of medium was calculated as the mean mass of mycelial growth.

Extracellular red pigments production was indirectly evaluated by measuring the absorbance of the culture filtrate at 530 nm in spectrophotometer[14]. Each treatment comprised four replicate flasks, and the experiment was repeated once.

Fungal spore production

The numbers of fungal spores were estimated by transferring 9 mm disc of fungal cultures into

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10 ml test tube containing 5 ml distilled water. The tubes were shaked vigorously using vortex mixer for 5 min. One-tenth ml of fungal spores suspension was transferred into a haemocytometer and then the spores was counted. Accordingly, the number of fungal spores in 5 ml was calculated. The same procedures were repeated three times and the mean number of spores was considered.

Optimization of culture condition

The culture conditions examined were culture media, media pH, incubation temperature and inoculum age. To determine the effect of pH on mycelian growth and pigment production PD broth (PDB) was used as base medium[6]. The PDB media pH was adjusted from pH 3-7 and the cultural isolates were incubated at different temperature from 10-40°C in the shaker incubator. Experiments were conducted in shake flasks, fungal growth and pigment production were monitored and all experiments were performed in duplicate[5].

Data analysis

All experiments were performed twice with replicates and data were recorded. Analyses of variance and regression analysis were performed using the statistical software package of MS Excel and Origin Pro (version 8.0).

RESULT AND DISCUSSION

Effects of agar medium on colony growth and conidia discharge

The growth rates of the isolates of Penicillium on seven agar media varied significantly (r^2 = 0.5, 0.36, colony growth; 0.89, 0.90, conidia production; P=001). P001 grew better on CDA and MEA and than other media; while for P002 YEA was the best agar medium for colony growth. CMA and CYA were not suitable for the growth of either isolates. OMA and PDA were better for P003 and CYA was not suitable for the growth of the isolates (Figure 1A). The effect of agar media on colony growth of these three isolates was significant (P < 0.001). The average colony diameter of P001 was 3.8±0.61cm with the maximum on CDA i.e. 4.5±0.20cm. However, MEA was the best medium for overall colony growth for the three isolates (mean colony diameter 4.1±0.6cm).

The three Penicillium isolates were cultured on different growth media and incubated at 25 ±2°C for 7 days for solid media, and 14 days for liquid media. Results revealed that the culture characteristics and secondary metabolites profiles of the isolates were greatly affected by the type of

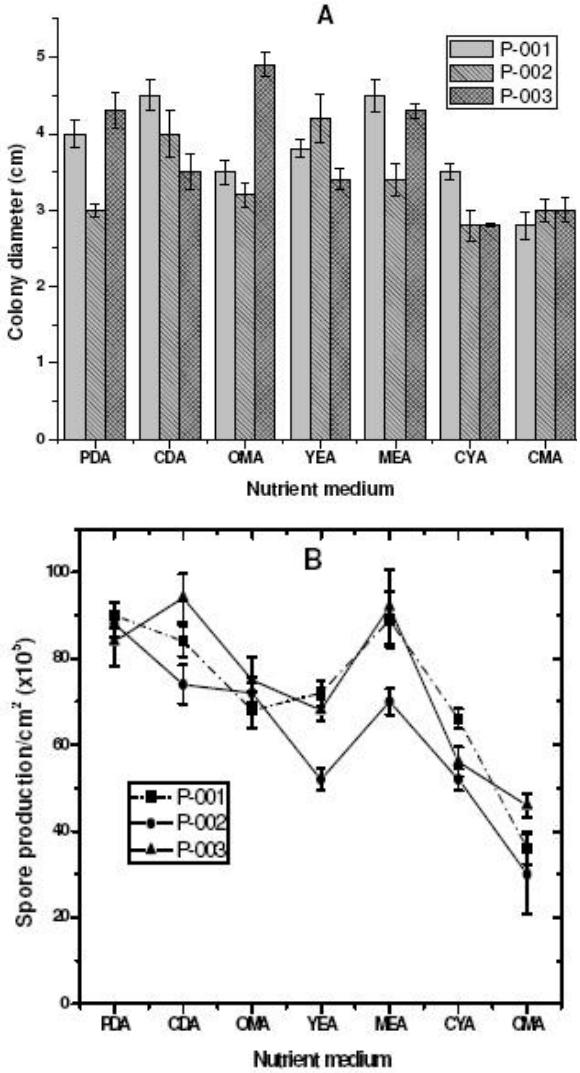

Figure 1: Effects of agar media on colony radial growth (A) and conidia production (B) for three isolates (P001, P002 and P003) of Penicillium sp. The seven agar media used were: PDA, CDA, OMA, YEA, MEA, CYA, and CMA. Petri dishes with fungal cultures were incubated at 25°C in the dark for 7 days. Vertical bars above the means represent the STDV.

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growth medium. The surface and reverse culture color, mycelial weight, colony diameter, and number of spores of the isolates were varied and coined to the type of the growth medium. The highest number of spores per cm^2 was obtained in P-003 on CDA ($94\pm5.6x10^5$ /cm^2), in P001 on PDA ($90\pm2.8\ x10^5$ /cm^2) and in P002 also on PDA ($88\pm2.9x10^5$ /cm^2) media. The CYA and CMA medium showed the lowest number of spores ($30\pm9.2x10^5$ /cm^2) (Figure 1B). Interestingly, the surface and reverse culture color of the isolates were noticeably changed from one medium to another. Surface color was gradually altered from light red to dark red or brownish (Figure 2).

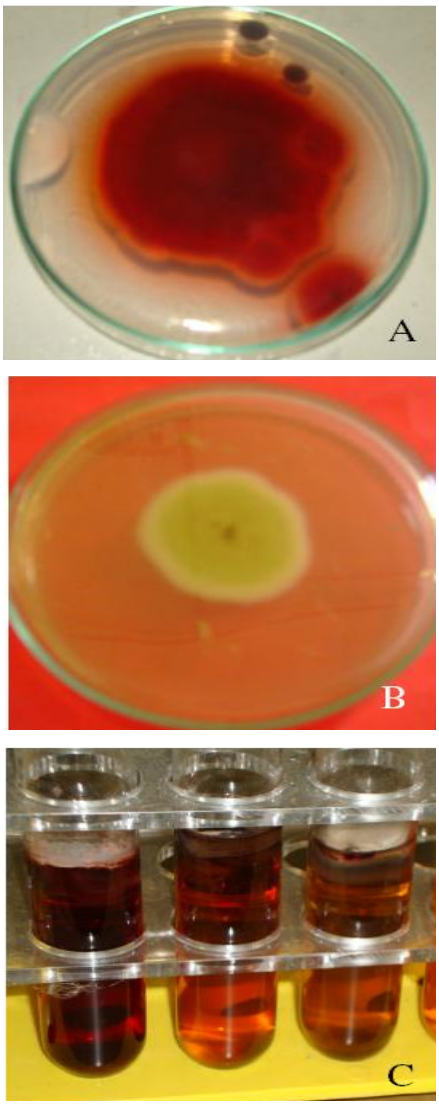

Figure 2: Penicillium isolates gworm on different media and its pigment production. A. Pigment production on the riverside of the colony; B. Colony diameter; C. Cultural extract of the Penicillium isolates.

Effects of liquid medium on fungal biomass and pigment production

The effects of liquid media on mycelial growth were significant ($P<0.001$) for the isolates. Maximum mycelial growth of P001 was observed in CD broth medium. Culture characteristics and secondary metabolites of Penicillium isolates were significantly affected by the type of the growth medium. CD medium showed the best mycelial fresh, dry weight for P001, P002 and P003 (1.00±0.05, 0.90±0.01 and 0.79±0.02 g/100ml medium respectively) followed by PDB (0.75 g, n=3) and OM (0.72g, n=3) medium (Figure 3A). The inoculum age on mycelial biomass production of the isolates was also recorded on PDB medium with different inoculum ages from 2 to 7 days at 25°C in shake flask cultures. The optimal inoculum age for biomass production was 6-7 days and an increase in inoculum age will not increase the mycelial growth and the the pigment production as well. A similar result was observed in submerged culture of P. japonica[2]. Amongst several fungal physiological properties, the inoculum age usually played an important role in fungal development[12].

For the determination of a suitable cultural medium together with carbon source for the red pigment production, the isolates Penicillium was cultivated in the seven different liquid basal medium. Out of the seven liquid media examined, CD, PD, OM and YE broth media were relatively favourable to the mycelial growth of the Penicillium isolates although the pigment production was quit different from one another. The maximum mycelial growth (1.00±0.05g/100ml) was achieved in CD medium in P001 and also the maximum pigment production (840±98.3) was obtained in the same medium. However, the reverse results were recorded in case of P003 where pigment production was higher in PD medium whereas myceliu growth was maximum in CD medium (Figure 3B). The pigment production on CY medium however low compared to other me-

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dium and no pigment production was observed by the isolate P003. There was significant correlation between the mycelial growth and pigment production in different medium (r^2=0.9, P<0.001) although pigment production depends on the growth medium (Figure 3B).

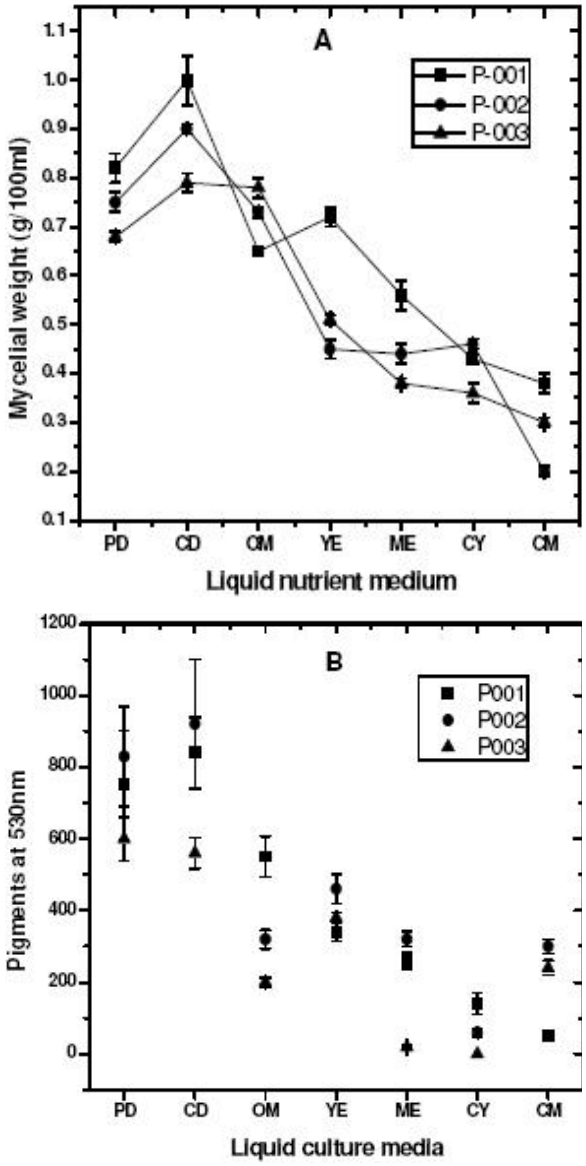

Figure 3: Effects of liquid culture media on mycelial biomass (A) and pigment production (B) for three isolates (P001, P002 and P003) of Penicillium sp. The seven media used were: PD, CD, OMA, YE, ME, CY, and CM broth. Conical flasks with fungal cultures were incubated at 25°C in the dark for 7 days. Vertical bars above the means represent the STDV.

Effect of pH and temperature on mycelial biomass and pigment production

The pH of the culture medium has already been reported to play a key role in pigment synthesis. Penicillium isolates was cultivated at different initial pH values (3·0–7·0) in shaker flask cultures on PD broth medium. The mycelial biomass and pigment production was affected by initial pH of the medium. The highest fungal biomass (0.82±0.02g/100ml) and pigment production (P001, 890±50.6) was observed when initial pH of culture mediumset at pH 6.0 in the PD medium (Figure 4). However, the pigment

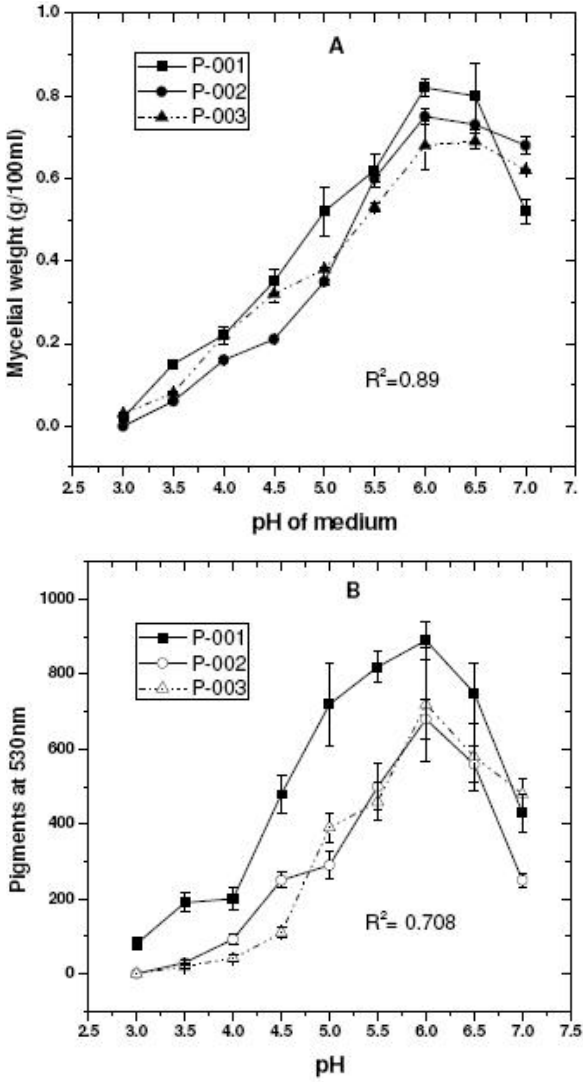

Figure 4: Effects of PD broth medium pH on mycelial biomass (A) and pigment production (B) for three isolates (P001, P002 and P003) of Penicillium sp. Vertical bars above the means represent the STDV.

production of an Penicillium species isolated from alkaline soil and marine sources was maximum in pH 9.0 which was reported to be the best for growth and pigment production[14,35]. The pigment

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production and mycelin growth was become lower in higher pH (>6.5). The nonlinear regression analysis curve showed significant relationship with mycelial biomass production and PD media pH (Figure 5).

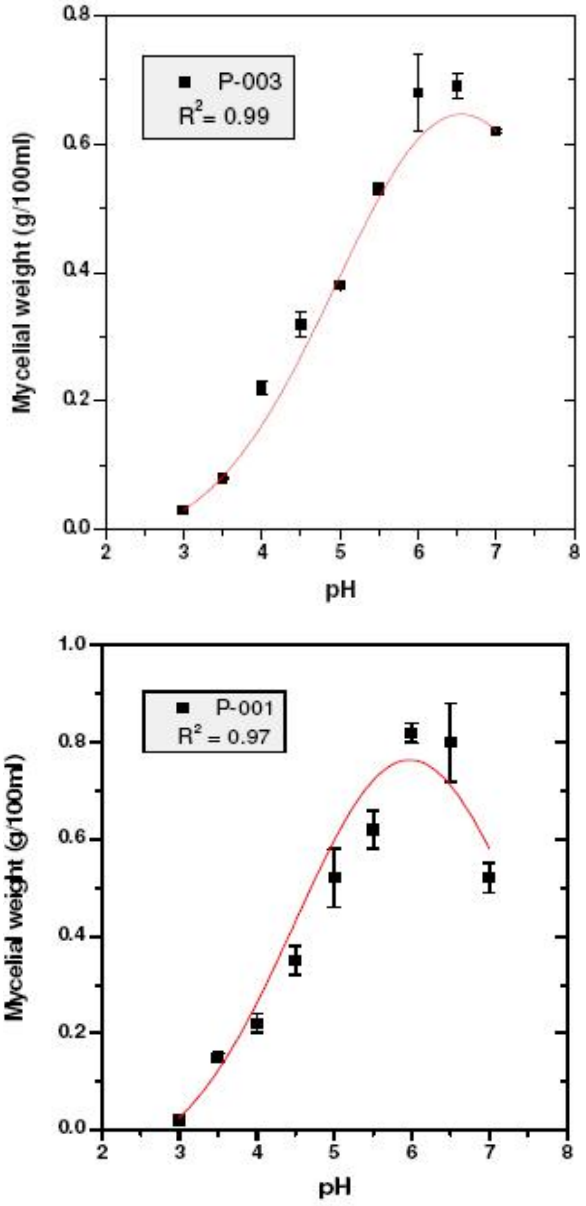

Figure 5: Regression analysis curve on the pH and mycelial biomass production of the two isolates of Penicillium spp. (P≤001). Vertical bars above the means represent the STDV.

Penicillium isolates were cultivated under various temperatures (10–40°C) for mycelial growth and pigment production on PD broth medium (basal medium). The optimal temperature for mycelial growth and pigment production was found to be 25°C for all the isolates (Figure 6) which was also reported by several investigator time to time[1,5]. Optimal temperature was regarded as favourable for growth and pigment production of Penicillium sp. although it requires long periods for submerged culture which probably expose the culture to contamination risk. This was similar to penicillin production by P. chrysogenum[34]. Nonlinear regression curve was fitted with the dependent parameters i.e. pH and temperature with pigment production and the results was highly significant for all the three isolates (P=0.001, Figure 7).

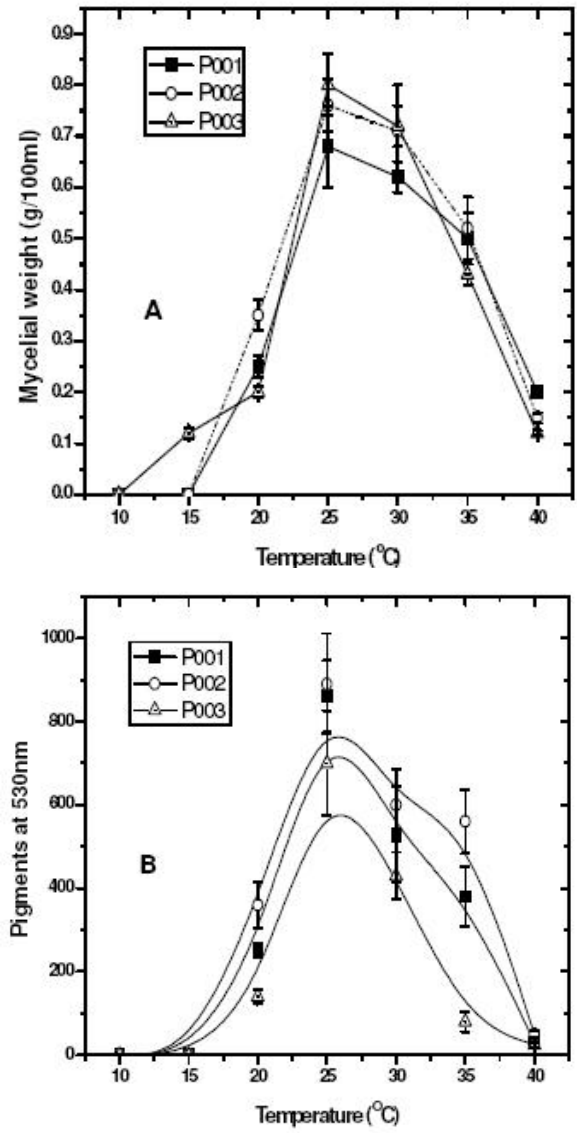

Figure 6: Effects of incubation temperature on mycelial biomass (A) and pigment production (B) of three isolates (P001, P002 and P003) of Penicillium sp. Vertical bars above the means represent the STDV.

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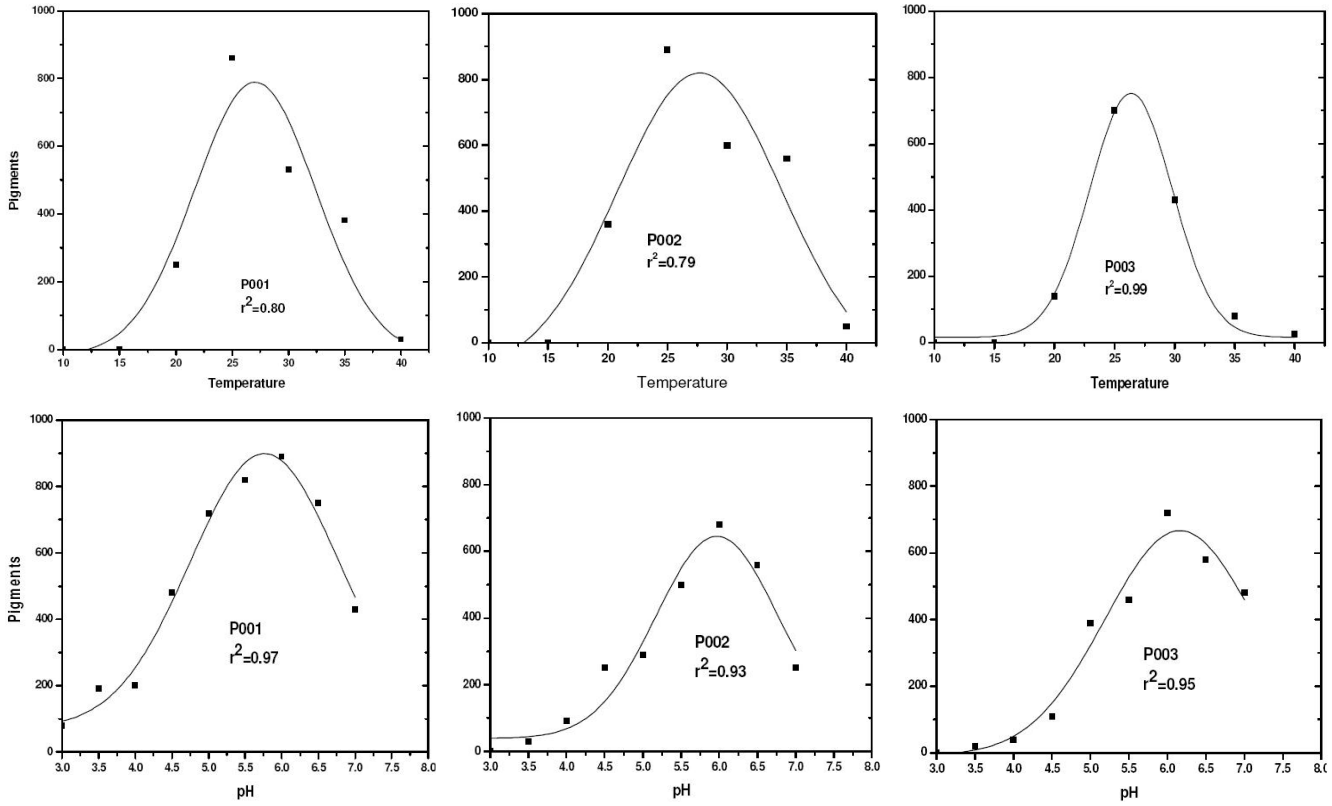

Figure 7 : Regression analysis curve on the PD broth pH and incubation temperature on pigment production of the three isolates of Penicillium spp. (P≤001).

Northolt and Bullerman[29] reported that the growth of fungi depends on the composition of the growth media, water activity (aw), pH, temperature, light, and the surrounding atmospheric gas mixture. Devi et al.[6] reported that PD medium at pH 6.5 and temperature 27±2° C, P.chrysogenum produces citrinin as secondary me olites under batch culture. Optimum concentration of pigment was produced during the stationary period of growth phase. It is therefore essential to monitor the growth of the organism simultaneously with the metabolic production for optimal yield of secondary metabolites. However, the results of this study revealed that both the secondary metabolites and culture characteristics including mycelial weight, colony diameter and number of spores of these three Penicillium isolates were significantly affected by the type of the growth medium. Guarro et al.[13] reported that because the production of secondary metabolites or the pigments was affected by environmental conditions and the production is restricted to certain conditions.

The effect of environmental factors on growth of fungi is generally less specific and restricted than the effect on secondary metabolite production. By optimization of culture conditions of Penicillium isolates, red pigment production can be improved by seven fold under submerged fermentation as reported by[14]. The higher concentration of pigment production by Penicillium sp. favours for commercial production of pigments.

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