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# Optimizing of *Trichoderma viride* Cultivation in Submerged State Fermentation

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**Abstract: Problems statement:** A study in Malaysia had shown a strain of *Trichoderma viride* was isolated from the soil. Questions were raised whether *Trichoderma viride* submerged state fermentation affected by parameters and which of them is effective? **Approach:** To investigate the effects of the submerged state fermentation parameters; concentrations of carbon g $L^{-1}$; (10, 45 and 80), glucose, nitrogen g $L^{-1}$; (0.10, 0.35 and 0.60) ammonium sulfate, temperature; (20, 30 and 40), PH; (4.0, 6.0 and 8.0) rpm. Experiments were performed in triplicate and the results were statistically analyzed using computer software Response Surface Methodology (RSM) using a Box-Behnken design was applied to batch cultures of *T. viride*, for identifying the effects of process variables (carbon sources, nitrogen sources, temperature, RPM and PH). The fermentation was carried out in shake flasks using a complex medium fungal biomass the mycelium was filtered through filter paper (Whatman No. 40). It was washed first with distilled water tow times. The washed mycelium was dried at 105±1°C to constant mass. It was placed in the desiccators and then the mass was determined. **Results:** The fermentation pattern was studied to investigate the effects of the submerged state fermentation parameters; concentrations of carbon g $L^{-1}$; (10, 45 and 80) glucose, nitrogen g $L^{-1}$; (0.10, 0.35 and 0.60) ammonium sulfate, temperature; (20, 30 and 40), PH; (4.0, 6.0 and 8.0) rpm; for *Trichoderma* biomass production for biotechnological uses (biocontrol agent). Optimum parameters and maximum biomass production were studied. The maximum biomass production of 13.6 g $mL^{-1}$ mycelium was noted after 5 days. Although maximum fungal biomass presented maximum growth rate that observed between the 3rd and 4th days of fermentation. At 3rd day 13.2 g $L^{-1}$ fungal dry mass was present, after that there was a slight decrease in the mycelial dry mass. A Box-Behnken experimental design was used to investigate the effects of five factors; concentrations of carbon g $L^{-1}$; (10, 45 and 80) glucose, nitrogen g $L^{-1}$; (0.10, 0.35 and 0.60) ammonium sulfate, temperature; (20, 30 and 40), PH; (4.0, 6.0 and 8.0), rpm; (100, 175 and 250) on the concentrations of biomass produced in batch cultures of *Trichoderma viride*. Optimal medium for maximizing the production of biomass in batch cultures of *T. viride* should contain 45 g $L^{-1}$ C, 0.35 g $L^{-1}$ N, 30 Temp, 175 rpm and Ph 6 for 5 days fermentation. Optimization of *Trichoderma* cultivation in submerged state fermentation to produce the optimum biomass as stage of biocontrol agent and biofertilizer production which made the production line more significant. **Conclusion:** Based on a statistically designed search, results indicated that an optimal medium for maximizing the production of biomass in batch cultures of *T. viride* should contain 45 g $L^{-1}$ C, 0.35 g $L^{-1}$ N, 30 Temp, 175 rpm and Ph 6. This composition can yield the optimum biomass 5 days of culture. The identified optimal medium is rich in carbon but provided a limiting level of nitrogen.

**Key words:** Fermentation, *Trichoderma*, biomass, submerged state

## INTRODUCTION

In the recent years, the environmental contamination caused by excessive use of chemical pesticides increased the interest in integrated pest management, where chemical pesticides are substituted by biopesticides to control plant pests and plant diseases. *Trichoderma*-Based Biocontrol Agents (BCAs) possess better ability to promote plant growth and soil remediation activity compared to their

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counterparts (virus, bacteria, nematodes and protozoa[3,4]. Their capability to synthesize antagonistic compounds (proteins, enzymes and antibiotics) and micro-nutrients (vitamins, hormones and minerals) enhance their biocontrol activity.

Like other fungal BCAs, conidial mass of *Trichoderma* is the most proficient propagule, which tolerates downstream processing (e.g., air drying). Despite the advantages, mass production of *Trichoderma* BCAs is less prevalent, owing to high-cost raw materials like Mendel's medium, molasses, corn steep liquor and other[8,9].

*Trichoderma* spp. Have gained wide acceptance as effective BCAs against several commercial phytopathogens. These antagonistic fungi are most common among fungal biocontrol agents because of their multiple BCA characteristics, namely, antagonism and plant-growth stimulation[10]. Thus, mass-scale production of *Trichoderma* spp. would have great potential for commercial use. Micropropagules of Trichoderma spp. In the form of conidia are preferred over chlamydospores and mycelial biomass because of the viability and stability in field application. Therefore, there are several BCA products of *Trichoderma* spp. In the market containing conidia of *Trichoderma* spp. As active ingredients. Multiple BCA action renders the production of *Trichoderma* spp. Conidia of commercial and environmental interest. There is abundant literature on the use of conventional synthetic media like glucose, cellulose, soluble starch and molasses to produce *Trichoderma* spp.[5,6]. However, the cost of these raw materials for commercial production of BCAs is one of the major limitations behind the restricted use. To overcome the cost limitation, many researchers have successfully used substrates like corn fiber dry mass, sewage sludge compost and cranberry pomace. Despite the use of alternate sources, the cost of production was still high, as these raw materials need to be supplemented by othernutrients[10]. Dominguesa *et al.*[2] found that the influence of the size binoculum and the composition of the fermentation medium on the morphology and cellulase production were studied. Different inoculums sizes were studied but the significative change in fungus morphology was observed for spore's concentration between $10^5$ and $10^7$ spores $mL^{-1}$ (i.e., $10^2$ and $10^4$ spores $mL^{-1}$ in pre-culture medium). In the medium without Tween 80, at low inoculum size, the majority of the pellets were large and well individualized, in contrast, at higher inoculation densities small flocs were obtained, with higher production of soluble protein and higher filter paper activity. It was found that the average pellet size seems to be inversely proportional to the inoculum size. Medium composition, namely Tween 80, also influences the morphology of *T. reesei* Rut C-30 and enzyme production. The presence of Tween 80 in fermentation medium inhibited the pellet formation of this strain [2]. The optimum conditions for cellulase production were $(NH_4)_2SO_4$, 0.5 g $L^{-1}$ as nitrogen source, pH (5.0), temperature (28°C) and incoulum size ($10^8$ spores $mL^{-1}$). Tween 80 (0.1%) improved the overall cellulase production as under these optimized conditions[7].

*Trichoderma viride*, a mould of family Hypocreaceae, order Hypocreales and class Ascomycetes, is well known for the biological control and the production of cellulose and chitinase[1]. In this study we reported the existence of biological agent strain of *T. viride.*

The aim of this study is to evaluate and investigate the effects of the submerged state fermentation parameters; concentrations of Carbon g $L^{-1}$; (10, 45 and 80) Glucose. Nitrogen g $L^{-1}$; (0.10, 0.35 and 0.60) Ammonium Sulfate. Temperature; (20, 30 and 40). PH; (4.0, 6.0 and 8.0). Rpm; to obtain optimal submerged culture conditions for bio-by production from *Trichoderma*, to the best of environmental conditions for submerged culture of *Trichoderma viride*.

The purpose of this study is to optimize the submerged culture conditions to produce the suspension by *Trichoderma* with respect to several operating variables in shake flask fermentation for industrial biotechnological uses as biocontrol agent on large scale.

## MATERIALS AND METHODS

**Isolation of producer microorganism:** Fungal species was isolated from soil samples by using a selective medium (PDA). Samples were dusted over the plates and the plates were incubated at 30±1°C for four days. The microbial colonies were developed which were picked up and purified by streaking and incubated at 30±1°C for 3 days. A green colonies forming culture with was selected and identified to be *Trichoderma viride* [1]. The culture was maintained on potato dextrose agar slants.

**Fermentation:** The fermentation was carried out in shake flasks using a complex medium consisting of (g $L^{-1}$) ammonium tartrate, 2.0; magnesium msulphate heptahydrate, 4.0; dipotassium phosphate, 14.0; calcium chloride, 0.2, NaHPO4,4.0, yeast extract, 3.0, trace elements, 2.0 mL and glucose 6.0 The flasks containing 200 mL fermentation medium were inoculated by 6 days old vegetative inoculums. The vegetative inoculums were developed from spore suspension prepared from 6 days old culture slants.

Table 1: Factor values and the responses of box design runs

| Run | Carbon g $L^{-1}$ | Temperature | Rpm | PH | Nitrogen g $L^{-1}$ | Biomass g $L^{-1}$ |
|---|---|---|---|---|---|---|
| 1 | 10 | 20 | 250 | 4 | 0.10 | 7.550 |
| 2 | 80 | 20 | 250 | 4 | 0.10 | 8.210 |
| 3 | 80 | 20 | 250 | 8 | 0.60 | 7.800 |
| 4 | 45 | 30 | 175 | 6 | 0.35 | 12.50 |
| 5 | 10 | 20 | 100 | 4 | 0.60 | 7.650 |
| 6 | 45 | 30 | 175 | 6 | 0.35 | 13.50 |
| 7 | 80 | 20 | 100 | 8 | 0.10 | 8.800 |
| 8 | 10 | 40 | 100 | 8 | 0.60 | 8.900 |
| 9 | 80 | 40 | 100 | 8 | 0.60 | 13.40 |
| 10 | 45 | 30 | 175 | 8 | 0.35 | 10.50 |
| 11 | 45 | 30 | 100 | 6 | 0.35 | 11.32 |
| 12 | 10 | 20 | 250 | 8 | 0.10 | 7.230 |
| 13 | 45 | 30 | 175 | 6 | 0.35 | 13.60 |
| 14 | 80 | 40 | 250 | 8 | 0.60 | 8.200 |
| 15 | 10 | 20 | 250 | 4 | 0.60 | 7.800 |
| 16 | 10 | 20 | 100 | 4 | 0.10 | 7.500 |
| 17 | 45 | 30 | 175 | 4 | 0.35 | 13.40 |
| 18 | 10 | 20 | 100 | 8 | 0.60 | 7.000 |
| 19 | 45 | 30 | 175 | 6 | 0.60 | 13.20 |
| 20 | 45 | 40 | 175 | 6 | 0.35 | 13.20 |
| 21 | 80 | 20 | 250 | 4 | 0.60 | 12.90 |
| 22 | 10 | 40 | 250 | 8 | 0.10 | 7.890 |
| 23 | 80 | 40 | 250 | 8 | 0.10 | 7.800 |
| 24 | 10 | 40 | 100 | 4 | 0.60 | 8.200 |
| 25 | 45 | 20 | 175 | 6 | 0.35 | 9.200 |
| 26 | 10 | 20 | 250 | 8 | 0.60 | 9.100 |
| 27 | 10 | 20 | 100 | 8 | 0.10 | 7.800 |
| 28 | 80 | 40 | 100 | 8 | 0.10 | 7.500 |
| 29 | 45 | 30 | 175 | 6 | 0.35 | 13.00 |
| 30 | 10 | 40 | 100 | 4 | 0.10 | 8.320 |
| 31 | 80 | 20 | 100 | 8 | 0.60 | 12.90 |
| 32 | 80 | 20 | 100 | 4 | 0.10 | 7.800 |
| 33 | 80 | 40 | 250 | 4 | 0.60 | 7.500 |
| 34 | 10 | 30 | 175 | 6 | 0.35 | 13.00 |
| 35 | 45 | 30 | 250 | 6 | 0.35 | 13.20 |
| 36 | 80 | 30 | 175 | 6 | 0.35 | 12.89 |
| 37 | 45 | 30 | 175 | 6 | 0.35 | 11.00 |
| 38 | 80 | 20 | 250 | 8 | 0.10 | 8.200 |
| 39 | 80 | 40 | 100 | 4 | 0.60 | 12.90 |
| 40 | 10 | 40 | 100 | 8 | 0.10 | 7.500 |
| 41 | 10 | 40 | 250 | 4 | 0.60 | 8.100 |
| 42 | 45 | 30 | 175 | 6 | 0.35 | 13.00 |
| 43 | 10 | 40 | 250 | 4 | 0.10 | 9.500 |
| 44 | 45 | 30 | 175 | 6 | 0.35 | 12.80 |
| 45 | 45 | 30 | 175 | 6 | 0.35 | 13.00 |
| 46 | 10 | 40 | 250 | 8 | 0.60 | 9.600 |
| 47 | 80 | 20 | 100 | 4 | 0.60 | 12.89 |
| 48 | 80 | 40 | 100 | 4 | 0.10 | 7.200 |
| 49 | 45 | 30 | 175 | 6 | 0.10 | 9.000 |
| 50 | 80 | 40 | 250 | 4 | 0.10 | 7.220 |

The inoculums development and the fermentation were carried out at 30 ±1°C, pH 7.0 with orbital shaking at l50 rpm.

**Parameters:**

- Carbon g $L^{-1}$ : (10, 45 and 80) Glucose
- Nitrogen g $L^{-1}$: (0.10, 0.35 and 0.60) ammonium sulfate
- Temperature : (20, 30 and 40)
- PH: (4.0, 6.0 and 8.0)
- Rpm: (100, 175 and 250)

**Dry cell mass determination:** To determine the fungal biomass the mycelium was filtered through filter paper (Whatman No. 40). It was washed first with distilled water tow times. The washed mycelium was dried at 105±1°C to constant mass. It was placed in the desiccators and then the mass was determined.

**Specific growth rate:** To determine the specific growth rate (m), natural log of biomass (ln X) was plotted against time (t). The slope of the line at any moment gives the specific growth rate at that moment.

**Statistical analysis:** Experiments were performed in triplicate and the results were statistically analyzed using computer software Response Surface Methodology (RSM) using a Box-Behnken design was applied to batch cultures of *T. viride*, for identifying the effects of process variables (Carbon Sources, Nitrogen Sources, Temperature, RPM and PH). And the significant difference among replicates has been presented as least significant tests in the form of probability values (Table 1).

## RESULTS

The rate of growth and biomass production by microorganisms is quite important in understanding their fermentation pattern. (Table 2) shows the rate of production of fungal biomass during liquid media fermentation by *T. viride* at 30±1°C for 5 days of incubation. Maximum growth rate was observed between 3 and 4 days of fermentation. At 3d 13.2 g $L^{-1}$ fungal dry mass was present. After 3 d, there was a slight decrease in the mycelial dry mass.

The experimental runs and results for the Box-Behnken design are shown in Table 1. The 50 runs in a single block were used to study the effects of five factors on one response. Biomass concentration ranged from 7.00-13.60 g $L^{-1}$. The ANOVA tables (Table 3) give the statistical significance of the effects for biomass. With regard to biomass concentration, four effects had P-values of less than 0.05 (Table 3), indicating that they were significantly different from zero at the 95% confidence level. These effects were the nitrogen concentration, carbon concentration, the quadratic effect of carbon concentration and the interaction between carbon and nitrogen concentrations.

Considering the F-ratio statistic (Table 2), it was concluded that a change in nitrogen concentration caused the major variation in biomass concentration. This was because nitrogen was the limiting nutrient. The effect of fermentation pH was not statistically significant as biomass was harvested at the stationary growth phase.

The R2 statistic (Table 3) indicated that the model as fitted explained 81.96% of the variability in biomass concentration. The adjusted R2 statistic, which is more suitable for comparing models with different numbers of independent variables, was 69.53% (Table 3). The standard error showed the standard deviation of the residuals to be 1.36. The Durbin-Watson (DW) statistic tested the residuals to determine if there was any significant correlation based on the order in which they occurred in the data file. There was probably no significant autocorrelation in the residuals.

Table 2: Specific growth rate

| Time (day) | Biomass g $L^{-1}$ | $Ln_x$ |
|---|---|---|
| 1 | 07.5 | 2.00 |
| 2 | 11.2 | 2.42 |
| 3 | 13.2 | 2.60 |
| 4 | 12.7 | 2.54 |
| 5 | 12.4 | 3.20 |

Table 3: Analysis of variance for biomass (R-squared = 0.8196, Adj R-squared = 0.6953 Std. Dev = 1.36)

| Source | SS | MS | DF | F.v | F. prob |
|---|---|---|---|---|---|
| Model | 242.830 | 242.830 | 12.140 | 6.590 | <0.0001* |
| A-carbon | 13.560 | 13.560 | 13.560 | 7.360 | 0.0111 |
| B-temperature | 0.200 | 0.200 | 0.200 | 0.110 | 0.7449 |
| C-rpm | 2.810 | 2.810 | 2.810 | 1.530 | 0.2265 |
| D-pH | 0.600 | 0.600 | 0.600 | 0.330 | 0.5723 |
| E-nitrogen | 32.070 | 32.070 | 32.070 | 17.410 | 0.0003 |
| AB | 6.270 | 6.270 | 6.270 | 3.400 | 0.0754 |
| AC | 11.830 | 11.830 | 11.830 | 6.420 | 0.0169 |
| AD | 0.180 | 0.180 | 0.180 | 0.099 | 0.7549 |
| AE | 16.100 | 16.100 | 16.100 | 8.740 | 0.0061 |
| BC | 0.650 | 0.650 | 0.650 | 0.350 | 0.5572 |
| BD | 0.880 | 0.880 | 0.880 | 0.480 | 0.4939 |
| BE | 0.036 | 0.036 | 0.036 | 0.020 | 0.8891 |
| CD | 0.580 | 0.580 | 0.580 | 0.310 | 0.5798 |
| CE | 6.140 | 6.140 | 6.140 | 3.330 | 0.0782 |
| DE | 6.612 | 6.612 | 6.612 | 3.589 | 0.9526 |
| A2 | 0.170 | 0.170 | 0.170 | 0.091 | 0.7652 |
| B2 | 5.450 | 5.450 | 5.450 | 2.960 | 0.0960 |
| C2 | 0.450 | 0.450 | 0.450 | 0.240 | 0.6263 |
| D2 | 1.340 | 1.340 | 1.340 | 0.720 | 0.4015 |
| E2 | 6.210 | 6.210 | 6.210 | 3.370 | 0.0766 |
| Residual | 53.430 | 53.430 | 1.840 | 3.390 | |
| Lack of fit | 48.850 | 48.850 | 2.220 | | 0.0512ns |
| Pure error | 4.580 | 4.580 | 0.650 | | |
| Cor total | 296.260 | 296.260 | | | |

## DISCUSSION

**Biomass concentration:** From the analysis of the data in Table 1 by the least squares method, the following second-order model was fitted:

Biomass (g $L^{-1}$) = +12.74+0.63 * A+0.076 * B-0.29 * C-0.13 * D+0.97 * E-0.44 * A * B-0.61 * A * C-0.076 * A * D+0.71 * A * E-0.14 * B * C+0.17 * B * D-0.034 * B * E-0.13 * C * D-0.44 * C * E-0.014 * D * E+0.2 * $A^2$-1.48 * $B^2$-0.42 * $C^2$-0.73 * $D^2$-1.58*$E^2$

The positive effect of nitrogen concentration on biomass produced was quantified through its regression coefficient, 13.6. A pictorial representation of the effect is shown in Fig. 1 where the biomass concentration is plotted for various values of the five factors (Table 1). The interactive effect of carbon and nitrogen concentrations on biomass is clearly shown in Fig. 1 and 2. At the lowest nitrogen concentration, 0.1 g $L^{-1}$, an increase in carbon supply had little effect on biomass production as cultures were nitrogen-limited. In contrast, at 0.8 g $L^{-1}$ of nitrogen, the biomass concentration increased with increasing carbon in the culture medium because now carbon limited growth (C/N increased).these results agreed with results found by Dominguesa *et al*.[2] and Kocher and Kalra[7].

An independent experiment was used to verify the optimum conditions identified in the Box-Behnken experiment. The verification consisted of triplicate runs comparing the control fermentation the optimized growth medium with 45 g $L^{-1}$ C, 0.35 g $L^{-1}$ N, 30 Temp, 175 rpm and Ph 6. The biomass concentrations obtained under the optimal conditions 13.6 g $L^{-1}$ (Fig. 2).

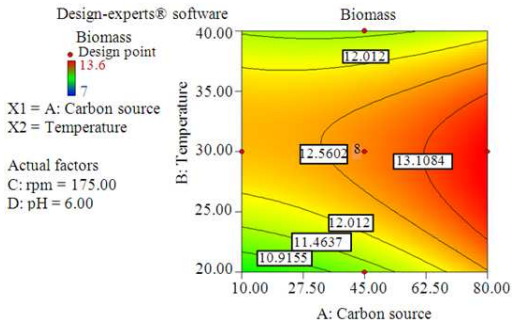


Fig. 1: Main effects plot for biomass concentration. Range of variables: Carbon g $L^{-1}$; (10-80) glucose. Nitrogen g $L^{-1}$; (0.10-0.60) ammonium sulfate. Temperature; (20-40). PH; (4.0-8.0). Rpm; (100-250)

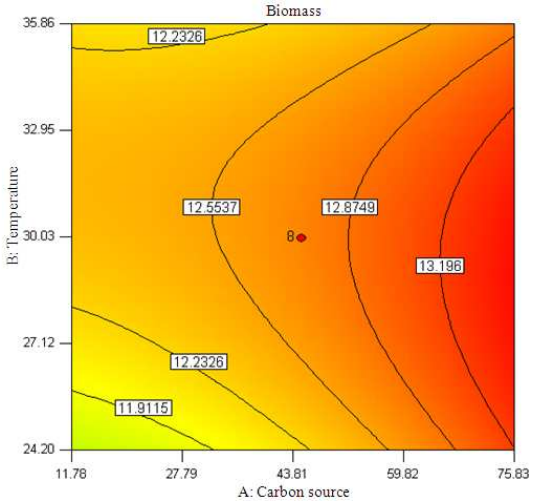


Fig. 2: Main effects plot for maximum biomass concentration

## CONCLUSION

Based on a statistically designed search, an optimal medium for maximizing the production of biomass in batch cultures of *T. viride* should contain 45 g $L^{-1}$ C, 0.35 g $L^{-1}$ N, 30 Temp, 175 rpm and Ph 6. This composition can yield a biomass 13.6 g $L^{-1}$ within 5 days of culture. The identified optimal medium is rich in carbon but provides a limiting level of nitrogen. Maintaining a good level of rotary shaker rpm which may returned to the important of dissolved oxygen maximum values of the biomass yield on nitrogen are obtained when the C/N mass ratio in the medium is high.

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