Optimization of process parameters on the viable inocula of entomopathogenic fungi by response surface methodology for the fungal consortium preparation

S. Nivash Kumar, S. Karthick Raja Namasivayam*, T. Mohammed Kamil and T. Ravi

ABSTRACT

Development of pest control measures using entomopathogenic fungi has received increased attention in various parts of the world to control the wide range of economically important pests associated with various crops. A lot of research has been carried out on the exploitation of entomopathogenic fungi for the pharmacological and environmental application.In this study, the effect of nutrient factors of sabouraud maltose yeast extract broth (SMYB) – a major medium used for entomopathogenic fungi cultivation and the process parameters such as pH and temperature on biomass production of *Beauveria bassiana, Nomuraea rileyi, Metarhizium anisopliae* and *Penicillium rubrum* was studied by response surface methodology (RSM) and the inocula derived from the fungi was formulated as consortium in jiggery water. Central composition design (CCD) was used for optimizing the parameters. Temperature (X_1), pH (X_2), Maltose (X_3), Peptone (X_4) was chosen as independent variables. The maximum percentage of biomass yield is observed at 34.7º C temperature, pH 7.15, peptone 4.52 g/mL and 0.62 g/mL. Biomass thus obtained was formulated in jiggery water and the formulated consortium to be was use for various purposes in the near future.

Key words; Entomopathogenic fungi, consortium, biomass, optimization, RSM

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INTRODUCTION

A microbial inoculant containing many kinds of naturally occurring beneficial microbes called Effective Microorganisms (EM) has been used widely in nature and organic farming consisting of lactic acid bacteria, photosynthetic bacteria, Yeast, Fermenting fungi and actinomycetes. Studies have shown that EM may have a number of applications, including agriculture, livestock, gardening landscaping, composting, bioremediation, cleaning septic tanks, algal control and household uses .The application of EM will improve soil and irrigation water. It can be used in seed treatment. It can be used to make organic sprays for the enhancement of photosynthesis and control of insects, pests and diseases (Hanekom *et al.*, 1999, Javaid *et al.*, 2008; Sekaran *et al.*, 2007) *B. bassiana*, *N. rileyi*, *M. anisopliae, P. rubrum*as biological control agents for insect species have increased global attention during the last few decades. More than 750 species of fungi are known to be pathogenic to insects and they belong to the families of entomophogens and deuteromycetes (Alter and Vandenberg, 2000). The mycoinsecticide based on *B. bassiana* (Balsamo), Vaillemin, *P. fumosoroseus* (Wize) Brown and Smith, *N. rileyi* (F.) Samson, *M. anisopliae* have been used to control various insect pests (Asensio *et al.*, 2003; Brar *et al.*, 2004; Enkerli *et al.*, 2004; Sahayaraj and Namasivayam, 2011). Success of biocontrol programme using these fungal organisms is primarily based on production of adequate quantities of a good quality inoculum (Sahayaraj and Namasivayam, 2008). Development of simple and reliable production system follows the basic multiplication procedures of submerged liquid fermentation for the production of blastospores which are short lived and hydrophilic (Rombach, 1989) or solid state fermentation (Roussos *et al.,* 1993) for the production of aerial conidia. However, the most viable mass production technologies make use of a diphasic strategy in which the fungal inoculum is produced in liquid culture, which is further utilized for inoculating the solid substrate(s) for conidia production (Gopalakrishnan and Mohan, 2000). Response surface methodology (RSM) is an important statistical technique employed for multiple regression analysis by using quantitative experimental data obtained from properly designed experiments using central composite design (Sivasubramanian and Namasivayam, 2014). RSM can identify the various interactions among different parameters and it has been extensively applied for optimization of cultural medium conditions and other process parameters in bio processes. Optimization of the variables in a fermentation process can give information about the main effects of the variables and also the interaction between variables in varying level. The main advantage of using response surface methodology includes the reduction in the number of experiments saving time, chemicals and labour and also its rapid and reliable prediction of response makes it a lucrative option to explore (Bhanu Prakash *et al.,* 1999; Sivasubramanian and Namasivayam, 2014). The objective of the study is to develop viable inoculum of entomopathogenic fungi consortium by optimizing various nutrient factors and process parameters of sabouraud maltose yeast extract broth (SMYB) which suggests the possible utilization of response surface methodology (RSM) as a strategy for in vitro mass multiplication of entomopathogenic fungal consortium for various purposes in the near future.

MATERIALS AND METHODS

Entomopathogenic fungal strains

B.bassiana, M.anisopliae, N.rileyi, P.rubrum used in the present study were isolated from agriculture field soil as reported in our previous work (Namasivayam and Bharani, 2014). Pure culture was maintained on agar slant as monosporic culture (Fig. 1).

Optimization of parameters for inoculam development

Inocula preparation

Fungal inoculam was prepared from seven day old SMYA slant culture by scrapping off with a sterilized glass rod. A homogenous conidial suspension was prepared in sterile distilled water by adding a few drops of the wetting agent Tween 80 (0.01%). The conidial concentration of the suspension was determined using an improved Neubauerhaae mocytometer (Germany). It is used as the source of inocula.

Experimental design and optimization studies

Central composition design (CCD) was used for optimizing the parameters. Sabouraud maltose yeast extract broth (SMYB), the most commonly used medium for the cultivation of fungi used in the study. Experimental analysis was carried out in 250 mL Ermenlayer flasks. To 100 mL of sterile double distilled water were maltose and peptone added and used as medium for the growth of fungi. The flasks were maintained under static condition at different temperatures and pH (Table 1, 2). Experimental design was carried out using Design expert software (Stat Ease, 9.0 trial version). This software was used towards the construction of a quadratic model. Four independent variables such as temperature, pH, Maltose (carbon source) and peptone (nitrogen source) were studied. Three levels such as -1, 0, +1 were analyzed for each independent variable. The variable factors in coded and actual values are given in table 1.The second order polynomial function was also used for regression analysis to study the interaction effects. The regression equation contains four linear terms (X_1, X_2, X_3, X_4), four quadratic terms (X_1^2, X_2^2, X_3^2, X_4^2), and six cross interaction terms (X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_3X_4, X_3X_4) plus 1 block term.

Fungal consortium development

Biomass thus obtained under optimum condition was collected from the respective treatment and used for consortium development. Development of fungal consortium involved addition of 20 L of distilled water and 2 kg of Jaggery (pure cane sugar) to 1 gm of fungal inoculum. The mixture was poured into a clean airtight plastic container under an anaerobic condition. The container was kept in dark place at ambient temperature for 8-10-days. Gas was released at regular intervals until the fermentation was complete. During the period of activation, a white layer of fungal growth was formed on top of the culture media. The formation of white layer was due to the rapid growth that formed dense white mycelia, Collected biomass was characterized by field emission scanning electron microscopy (FESEM) performed by SUPRA 55-CARL ZEISS, Germany.

Statistical analysis

The statistical significance was evaluated by performing F-test and ANOVA using the design expert software 9.0. Temperature (X_1), pH (X_2), Maltose (X_3), Peptone (X_4) were chosen as independent variables. The model was found to be statistically valid with a lower probability value (P_{model}<0.0001) and the lack of fit value was found to be not significant (P= 0.1901), which also indicates the equation is adequate for the prediction of percentage of biomass yield under all conditions. The low coefficient of variation (CV= 0.74%), suggested that the model is reliable and precise.

RESULT AND DISCUSSION

There is a lot of scope for the microbial pesticides as an alternative to the toxic synthetic pesticides for insect pest management. However, their production in developed countries has concentrated on high technology methods involving complex equipments and sterile culture conditions (Clark,1997). These methods are inappropriate for the developing countries which require production system using cheap, locally available raw materials and equipments (Gopalakrishnan, 2001). Several attempts have been made throughout the world to mass produce the entomopathogenic fungi by using both solid and liquid media (Feng *et al.*, 1994). With this aim, the present study is undertaken to produce viable inocula of entomopathogenic fungal mixture by optimizing nutrient and process parameters of SMYB media adopting response surface methodology (RSM). Fungal strains *B. bassiana, M. anisopliae, N. rileyi, P. rubrum* were isolated from groundnut field soil (pH 7.2, organic matter 2.55%, nitrogen, 25.0ppm, potassium 99ppm, phosphorous) collected from Chengalpet, Kanchipuram District, Tamil Nadu by culture dependent methods as reported in our previous finding (Namasivayam and Bharani, 2014). Regression analysis was carried out considering full quadratic model equation on the responses to evaluate the adequacy of fit. The determination of coefficients (R^2 values) for the model equation (1) were R^2= 0.9972, adjusted R^2=0.9945, and predicted R^2=0.9859 for the responses. The adjusted R^2 and predicted R^2 values indicate the experimental values agree with the predicted values (Fig. 2). Therefore, the model is found to be appreciable and adequate in representing the response and the yield of biomass which will be useful for further analysis. The regression equation shows an optimal increase in biomass yield with respect to temperature, pH and gradual decrease in maltose and peptone. But decrease in temperature, pH, maltose and peptone was observed in square term of regression equation. Response surface methodology is specially used to evaluate the effect of process variables on biomass yield optimization.

Table 1. Coded levels for independent factors used in experimental design

Factor	Coded levels		
	-1	0	+1
Temperature (X_1),ºC	25	30	35
pH (X_2)	5.5	6.5	7.5
Maltose (X_3), g/100mL	3	4	5
Peptone (X_4),g/100mL	0.25	0.5	0.75

Table 2. Effect of pH, temperature and nutrient factors (Maltose, peptone) on the biomass yield (g)

Temperature (^{o} C) (X_1)	pH (X_2)	Maltose (X_3) (g/100mL)	Peptone (X_4) (g/100mL)	Biomass weight (g)
25	5.5	3	0.25	1.26±0.1
	6.5	4	0.5	1.81±0.1
	7.5	5	0.75	1.96±0.4
30	5.5	3	0.25	2.14±0.3
	6.5	4	0.5	2.21±0.2[a]
	7.5	5	0.75	2.33±0.4[a]
35	5.5	3	0.25	2.56±1.2[a]
	6.5	4	0.5	2.76±0.1[a]
	7.5	5	0.75	2.89±0.1[a]

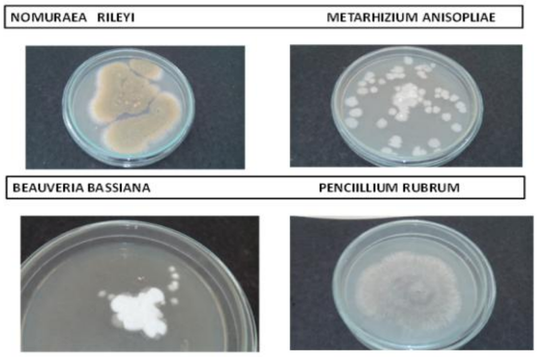

Fig. 1. Entomopathogenic fungi selected for consortium development

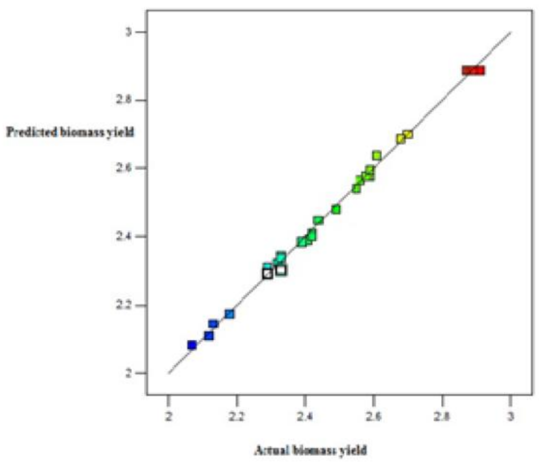

Fig. 2. Graphical comparison between actual and predicted percentage of biomass yield.

Fig. 3. (1) Interaction effect of Temperature(X_1) and pH(X_2), (2) Interaction effect of temperature (X_1), peptone (X_4), (3) Interaction effect of maltose (X_3) and peptone (X_4) and (4) Interaction effect of maltose (X_3) and peptone (X_4) on biomass yield

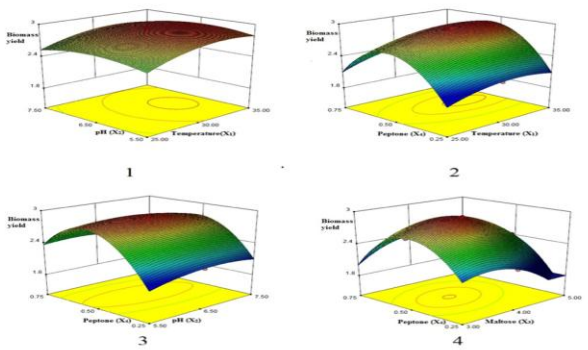

Fig. 4. Consortium in jaggery water

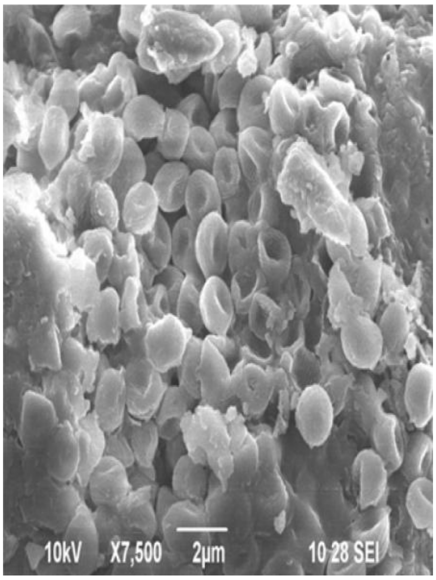

Fig. 5. Scanning electron microscopic image of fungal consortium

To evaluate and investigate the interaction effect of two factors on the percentage biomass yield, 3D surface plots have been used and analyzed. In a four parameter study, 3D surface plots play interactive role by varying any two parameters and keeping the other two variables as constant. Figure 3(1), shows the interaction effect between temperature (X_1) and pH (X_2) towards biomass yield and the maltose and peptone are kept fixed. Table 2 shows the effect of various parameters on the biomass yield of *B. bassiana* which reveals the effect yield was maximum at 35ºC,7.5pH 7.5, 5 and 0.75g of maltose and peptone run. A gradual decrease in biomass was recorded in decreased pH and temperature. Generally most of the fungi prefer to grow at acidic pH and lower temperature. In our present study, the tested entomopathogenic fungal strains exhibited best growth at high temperature and alkaline pH which might favor the viability of fungi even when exposed to high temperature pH (field condition) and the viability of the inocula due to the supply of major carbon (maltose) nitrogen (peptone) sources from the culture media at optimum level. The 3D surface shows maximum percentage biomass yield, at the temperature around 33.4ºC and pH around 4.68. Further increase in temperature and pH will decrease the yield of biomass. Figure 3(2), that shows the pH and maltose were kept constant respectively. The maximum percentage of biomass yield was observed at 34.7ºC and peptone at around 0.72 g/mL. Figure 3(3) represents the graph between pH and peptone and keeping the temperature and maltose constant. The maximum percentage of biomass yield is obtained when the pH is around 7.15 and peptone is at 0.66g/mL. Figure 3(4) represents the graph between maltose and peptone and keeping the temperature and pH constant the maximum yield was obtained by adding maltose around 4.52g/mL and peptone around 0.62 g/mL. Supply of nutrient factors like maltose and peptone mainly carbon and nitrogen sources at optimum concentration is used by the cells for various metabolic activities which in turn affect the viability of fungal infectious inoculum (Sharma, 2004).

Fungal biomass thus obtained under optimum metabolites productions which are known to cause undesirable odor and improves the viability (Sekaran *et al.,* 2007). Figure 5 shows electron microscopic image of constructed fungal consortium which reveals the presence of aggregates of fungal structure. The present study would suggest effective strategy for the mass production of viable inoculam by optimizing nutrient and process conditions using RSM which minimize the usage of more chemical components and improves viability.

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