

**Media optimization for *Fusarium pallidorozeum*,
a novel biofertilizer**

Thesis

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By

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*Pantnagar
November, 2021*



(Aabha Bahuguna)
Authoress

CERTIFICATE-I

This is to certify that the thesis entitled “**Media optimization for *Fusarium pallidoroseum*, a novel biofertilizer**” was submitted in partial fulfillment of the requirements for the degree of **Master of Science** with major in **Botany**, of the College of Post Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of bonafide research carried out by **Ms. Aabha Bahuguna**, Id. No. **55504**, under my supervision, and no part of the thesis has been submitted for other degree or diploma.

The assistance and help received during the course of this investigation have been duly acknowledged.

Pantnagar
November, 2021



(A.K. Sharma)
Chairman
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CERTIFICATE-II

We, the undersigned, members of the Advisory Committee of **Ms. Aabha Bahuguna**, Id. No. **55504**, a candidate for the degree of **Master of Science** with a major in **Botany**, agrees that the thesis entitled “**Media optimization for *Fusarium pallidoroseum*, a novel biofertilizer**” may be submitted in partial fulfillment of the requirements for the degree.



(A.K. Sharma)
Chairman
Advisory committee



(D.S. Rawat)
Member



(Anita Sharma)
Member

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LIST OF ABBREVIATIONS

$^{\circ}\text{C}$	Degree Celsius
%	Percentage
g/l	Gram per litre
ml/l	Millilitre per litre
PBD	Plackett Burmann Design
RSM	Response Surface Methodology
CCD	Central Composite Design



Introduction



Food is the main source for humans and the development of a nation is directly proportional to the amount of food or nutrient available to the population. For many years, the theory of food security has been regarded at the worldwide, national, regional, state, household, as well as in individual levels. Global food supply may soon be insufficient to feed the whole world's population as a result of increased global population growth. The world's population, which is now approximately 7 billion people, is expected to grow to over 10 billion people in the next 50 years. Agricultural growth must be considerably improved during the next few decades to feed all of these people. Providing enough food for an ever-increasing global population, on the other hand, is a difficult undertaking that will need a range of techniques and approaches. As the world's population grows, so does the market for more food grains. Fertilizers are used to increase the food grains yield of the crops. A fertilizer can be any ingredient that is used to increase farm productivity. It improves soil fertility by introducing nutrients into the soil, which aids plant growth. Fertilizer can be classified into chemical fertilizers, organic fertilizers and Biofertilizers. Chemical fertilizers are defined as fertilizers made from raw chemicals in solid or liquid form and manufactured in factories to meet the nutritional needs of plants. Nitrogen, phosphorus, and potassium, collectively known as NPK, are typically present in these chemical fertilizers, along with other nutrients (Youssef *et al.*, 2014). Increased use of chemical fertilizers in agriculture makes the country's food self-sufficient, but also caused ecological imbalance and depletion of natural resources causing a serious threat to the living things (Saritha *et al.*, 2019). Chemical fertilizers reach water bodies through rainwater due to inadequate absorption by plants, causing eutrophication and affecting biological creatures, particularly growth-inhibiting microorganisms. Excessive use of them in agriculture is expensive and has a variety of negative impacts on soils, including loss of water holding capacity, soil fertility, and nutrient inequality (Youssef *et al.*, 2014). For this, any effective and long-term solutions for feeding the globe must incorporate sustainable and ecologically

acceptable biological alternatives, (**Glick, 2013**) capable of producing in large quantities and of high quality while not depleting natural resources. In this context, because of its vast potentiality in increasing crop productivity and food safety, using plant growth-promoting microorganisms is seen as a viable alternative in the agricultural industry to chemical fertilizers whereas organic fertilizers are naturally available ingredients sources that contain a moderate amount of plant essential nutrients sources.

A 'biofertilizer,' also known as a micro inoculant, is a substance possessing live microorganisms (**Arora et al., 2010**) that colonize the rhizosphere or inside of plants and stimulate plant development by increasing the availability of nutrients to the host plant when applied to seeds, plants, or soil (**Bardi and Malusa, 2012; Malusa and Vassilev, 2014**). Biofertilizers are both cost-effective and ecologically friendly, and their long-term utilisation helps to improve soil fertility significantly (**Mahdi et al., 2010; Singh et al., 2011**). It has been stated that the use of biofertilizers tends to increase crop yields by 10–40% by increasing the content of protein, essential amino acids, vitamins, and nitrogen fixation (**Bhardwaj et al., 2014**). Biofertilizers supply plants with the nutrients that are abundant in the soil and environment. Certain microorganisms such as plant growth-promoting bacteria, fungus, Cyanobacteria, and others have been shown to exhibit biofertilizer-like properties (**Mahanty et al., 2017**).

Fungi interact with plants as pathogens or benefactors and therefore may have an impact on crop production (**Varma et al., 1999**). The well-known fungal genera *Aspergillus*, *Fusarium*, *Penicillium*, *Piriformospora*, and *Trichoderma* are the most frequently reported PGPF. On comparing the results of different studies, it appears that plant-PGPF interactions can have positive effects on belowground and aboveground plant organs. The most commonly reported effects are significant improvement in germination, seedling vigor, biomass production, root hair development, photosynthetic efficiency, flowering, and yield (**Hossain et al., 2017**). Arbuscular mycorrhizal (AM) fungi are also widely distributed and can be found in a variety of agroclimatic regimes (**Akhtar and Panwar, 2011**). AM fungus develops

symbiotic relationships with the roots of over 80% of terrestrial plants (**Akhtar and Panwar 2011; Smith and Read, 2008**). Originally, they were classified as part of the phylum Zygomycota, order Glomales (**Redecker et al., 2000**), but they have now been reclassified as part of the phylum Glomeromycota (**Schusler et al., 2001**), which presently has around 200 species. The presence of arbuscular, a distinctive extraradical mycelium branching haustoria-like structure within the cortical cells, distinguishes AM fungus (**Smith and Read, 2008**). Arbuscles are primarily responsible for increasing the surface area of roots during nutrient transfer (**Akhtar et al., 2011**). The root depletion zone and root system are extended by AM fungus colonizing the plant roots and penetrating the surrounding soil (**Akhtar and Panwar, 2011**). Furthermore, due to enhanced nutrient absorption, synthesis of growth-promoting compounds, greater tolerance to drought, salt, and synergistic interactions with other rhizospheric microorganisms, AM fungi have boosted the development of the host plant (**Akhtar and Siddiqui, 2008**). Though AMFs are recognized as a distinctive biofertilizer, they are difficult to mass-multiply since they are biotrophs, and their growth under axenic conditions has proved impossible (**Srivastava et al, 2011**). The lack of axenic culture is a significant bottleneck for basic research and biotechnological applications. (**Singh et al., 2000**). With this context, a newly described axenically cultivable endophytic and saprophytic fungus *Fusarium pallidroseum*, which mimics the capabilities of AMF has been discovered. It plays a substantial favourable impact on plant growth promotion (**Srivastava et al., 2011**). The mass production of this fungus is still one of the major constraints for their large-scale use and commercialization. There is little information about the culture media used to maximize the production of spores. The most common commercial culture medium used to promote the growth of *Fusarium pallidroseum* are Potato Dextrose Agar, CLA, Armstrong, Czapek. For more spore production and higher biomass of fungus, optimization of media is essential. Optimum culture condition imparts suitable physiological and physical conditions to fungus. Optimization of media requires the selection of carbon, nitrogen, phosphorus, potassium, and trace elements. Traditional or statistical approaches can be used to modify nutritional needs. There are several

studies on the optimization of carbon and nitrogen sources utilizing the traditional medium optimization approach, which involves altering one independent variable while keeping the rest constant. For a high number of variables, this can be extremely time-consuming and costly. The traditional approach of optimization of a single component while keeping other parameters fixed does not reflect the combined effect of all the factors involved (**Monreal and Reese, 1969**). To establish optimum values, the approach necessitates a huge number of questionable trials. By using statistical experimental design to optimize all of the effective parameters, all of the constraints of a single factor optimization approach may be eliminated (**Gohel et al., 2006**).

A 'screening design' is an experimental design that may be used when a large number of potential causal factors must be investigated to find the most important ones that may influence one or more of the desired responses. This will cut down on the number of variables that need to be explored in future experiments. Screening might be used to eliminate irrelevant elements before investing time and money in a more thorough investigation. Several useful elements are included in the screening design: It aids in the quality control process by identifying the upper and lower control boundaries of a variable. A less expensive method of refining the process is to identify the impacting elements (**Vanaja and Shobha Rani, 2007**). The ultimate objective is to reduce the number of experiments while maximizing information. Another benefit is that the product's quality may be increased by using an organized method that preserves ideas and information in a clear and comprehensible manner (**Trocine and Malone, 2000**). Several useful elements are included in the screening design: It aids in the quality control process by identifying the upper and lower control boundaries of a variable. A less expensive method of refining the process is to identify the impacting elements. The ultimate objective is to reduce the number of experiments while maximizing information. Another benefit is that the product's quality may be increased by using an organized approach that maintains ideas and information in a clear and comprehensible manner. Because it is a mathematical expression, the results may be verified quickly and accurately. Statistical experimental designs such as Plackett-Burman Design and response surface methodology (RSM)

(Kennedy and Krouse, 1999) can collectively optimize all the affecting parameters to eliminate the limitations of a single-factor optimization process.

The **Plackett-Burman design (PBD)**, is a prominent type of screening design. It provides a fast and effective way to identify the important factors among a large number of variables, thereby, saving time and maintaining convincing information on each parameter. It was designed to optimize the quality control process so that sound decisions could be made about the implications of design parameters on the system state. Orthogonal arrays, proposed by Plackett and Burman (PB), are useful for screening because they offer unbiased estimates of the key effects in the smallest design possible. In an 'n + 1' run PB design, a variety of 'n' factors can be examined. The sample size is a multiple of four rather than a power of two (4k observations with $k = 1, 2, \dots, n$) which is a defining characteristic.

PB designs are used to analyze $n-1$ variables in n experiments, proposing experimental designs for more than seven factors, notably for $n * 4$ experiments, i.e., 8, 12, 16, 20, etc., which are ideal for researching up to 7, 11, 15, 19, etc., factors. The term "saturated design" refers to such patterns. The fundamental benefit of saturated designs is that they require the least amount of observations to estimate an effect for a given factor. PBD requires fewer experiments than the highly fractionated factorial designs that include the same number of factors.

The experimenter can use the projective property of the PB design to follow up on an initial design with runs that allow for efficient separation of main effects and interaction effects, PB designs are Resolution III designs that have the advantage of requiring the fewest number of runs, but they do not allow for the estimation of factor interactions; nevertheless, they can identify the major primary factors that make up the possible significant interactions.

Response Surface Methodology (RSM) is a mathematical and statistical approach for optimizing an operational condition that is impacted by several independent factors to obtain the highest yield through the most cost-effective procedure (Fan *et al.*, 2015). Based on the results of PBD, a few variables are

selected that have been found to have the largest influences on responses like spore count of fungus. The selected factors are then chosen for submerged flask culture through RSM. A set of experiments is conducted and the effects of the three factors under investigation, as well as their interactions, on the dependent variable, spore count per ml were computed in both linear and quadratic form. Analysis of variance was used to determine their significance (ANOVA).

Keeping in mind that *Fusarium pallidoroseum*, a plant growth-promoting fungus acts as a biofertilizer, the present work was carried with the following objectives:

1. Optimization of submerged fermentation media for a large number of spores through Plackett Burmann Design and Response Surface Methodology
2. Evaluation of highest spore-producing cultivation media composition



*Review
of
Literature*



Soil micro-organisms play a significant role in regulating the dynamics of decomposition of organic matters and the availability of plant nutrients such as N, P and S. It is well-recognized microbial inoculants are core component of integrated nutrient management, which leads to more sustainable agriculture. Furthermore, microbial inoculants can be employed as a cost-effective way to boost crop output by lowering fertilizers dosages and harvesting more nutrients from the soil. Biofertilizer is defined as a substance which contains living micro-organisms and is known to help with expansion of the root system and better seed germination. A healthy rhizosphere characterizes a healthy plant that carries several beneficial microorganisms. The major feature of biofertilizers that differentiate them from chemical and organic fertilisers is that they are cultures of specific bacteria and fungi that do not directly deliver nutrients to crops. In comparison to chemical fertiliser, biofertilizer manufacturing technology is quite simple, and installation costs are very inexpensive (**Chen, 2006**). Since biofertilizers contain living cells, therefore their effectiveness depends on the surrounding environment. Hence, the potentialities are bound to be inconsistent (**Mohammadi and Sohrabi, 2012**). A number of biofertilizers like N₂ fixers: *Azospirillum* sp., *Azotobacter* sp., *Rhizobia*; P solubilizers/mobilizers: *Pseudomonas fluorescens*, *Aspergillus* sp., AMF are being used at a commercial scale. Amongst these, AMF have gained most attention because of their varied characteristics: non-specificity towards crops except in members of the family Brassicaceae and Chenopodiaceae where they are not responsive; uptake of the limiting nutrient in soils *e.g.*, phosphorus; enhanced disease resistance in crops; capacity to withstand drought conditions and similar other functions (**Ferrol, 2002**). Nearly all soils also contain AMF, which associate with approximately 80% of all plant roots (**Smith and Read, 2008; Brundrett and Tedersoo, 2018**). AMFs are endowed with the ability to boost plant growth and yield while also increasing P absorption has been extensively demonstrated (**Miransari, 2010; Jansa et al., 2011; Smith et al., 2011; Smith and Smith, 2011; Nadeem et al., 2014; Brundrett and Tedersoo, 2018; Etesami, 2020**).

Despite the fact that AMFs are considered unique biofertilizers, they are difficult to mass multiply because they are biotrophs. Due to the obligate nature of these biotrophs, species level identification of AM fungal species could not be done at an early stage of development, and their propagation under axenic conditions was not possible. Though, AMF are widespread (**Giovannetti, 2006**), their biotrophic nature has proved the biggest bottleneck in application in agriculture (**Srivastava et al., 2011**). A newly described axenically cultivable endophytic and saprophytic fungus *Fusarium pallidroseum*, which mimics the capabilities of AMF has been discovered. It plays a substantial favourable impact on plant growth promotion (**Srivastava et al., 2011**).

About fungus: *Fusarium pallidroseum*

Fusarium semitectum Berkeley and Ravenel [syn: *Fusarium pallidroseum* (Cooke) Sacc.; syn: *Fusarium incarnatum* (Roberge) Sacc.] is a widely distributed and prevalent species in the tropics, subtropics, and Mediterranean areas, and is frequently linked to a variety of plant diseases (**Leslie and Summerell, 2006**). The formation of polyblastic conidiogenous cells is a notable feature of this area (**Booth, 1971**). *F. semitectum* comes in two varieties: vars. *semitectum* and *majus* (**Gerlach and Nirenberg, 1982**). *F. semitectum* var. *semitectum* generated 1–5 septate conidia, while *F. semitectum* var. *majus* produced 1–7 septate conidia. Sporodochia is absent in *F. semitectum* var. *semitectum*, whereas var. *majus* generated sporodochia, is another feature that distinguishes these types (**Booth, 1971; Joffe, 1974**).

Morphological characterization

All single-spored isolates of *Fusarium* that were successfully isolated were identified morphologically based on the microscopic and macroscopic characteristics.

Microscopic characteristics

Microscopic traits included conidiophore structure, conidia form, and the presence or absence of chlamydo spores and sporodochia (**Leslie and Summerell, 2006**). Each isolate was cultured onto Potato Dextrose Agar to enhance the formation of chlamydo spores and incubated at $25 \pm 2^\circ\text{C}$ for 2 weeks.

Macroscopic characteristics

The macroscopic characteristics such as colony appearances (texture and colour of aerial mycelium), pigmentations and growth rates were examined. In five replicates for each isolate, a 6 mm diameter mycelial disc was transferred and inoculated centrally onto PDA plates (90 mm diameter) and incubated at 25 °C. After two weeks of incubation, the colony morphology and pigmentation were evaluated, and the growth rate was measured every day until the colony was fully formed (6 - 9 days). The pigmentation study was carried out using the Methuen handbook of colour chart (**Kornerup and Wancher, 1978**).

Macroconidia

Sporodochia: Orange sporodochia produced on carnation leaves in CLA by some strains.

General morphology: Relatively slender with a curved dorsal surface and a straighter ventral surface. Apical Cell morphology: Curved and tapering to a point. Basal Cell morphology: Foot shaped. Number of septa: 3- to 5- septate. Abundance: May be difficult to find in some cultures.

Microconidia are pyriform to obovate and usually 1-septate and most common in older cultures. Mesoconidia are fusoid and 3- to 5- septate.

Aerial mycelium presentation: Individual spores per phialide, but often two spores per polyphialide to give a “rabbit ear” appearance.

Conidiogenous cells: Monophialides and polyphialides.

Abundance: Mesoconidia are abundant in the aerial mycelia. Microconidia are scarce and often are difficult to find.

Chlamydospores

Abundance/Speed of formation: Present, but not common. The absence of chlamydospores is not a reliable diagnostic character.

Location: Found in the hyphae both singly and in chains, and singly within conidia.

Appearance: Globose and smooth. Initially hyaline, but may become a light yellow color with age.

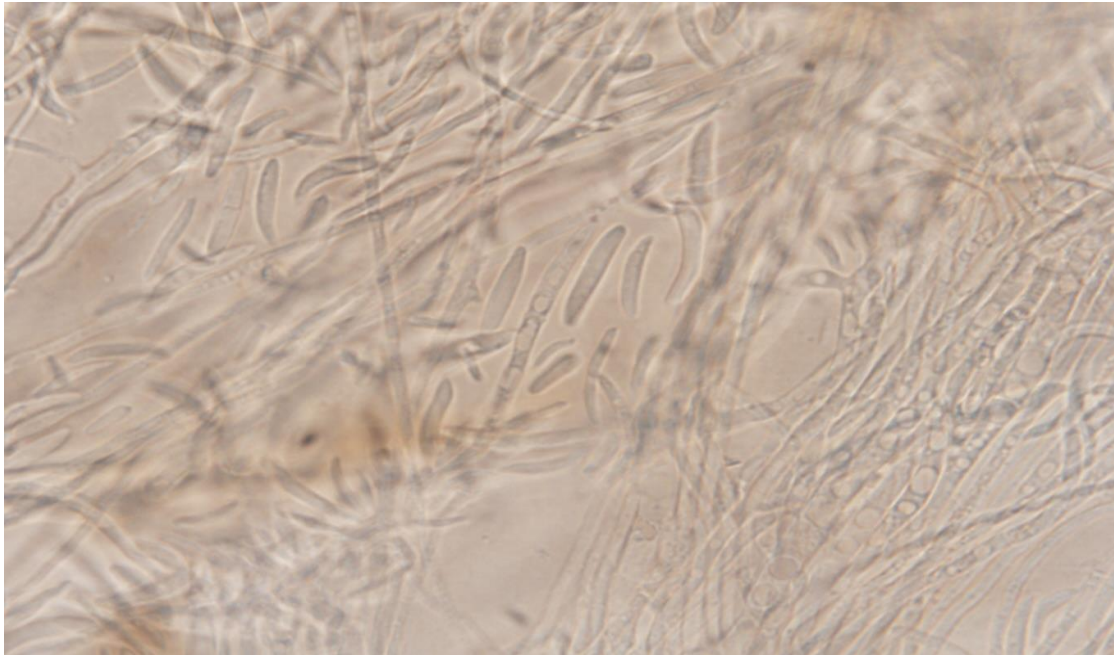


Plate 2.1 Mesoconidia of *Fusarium pallidoroseum*

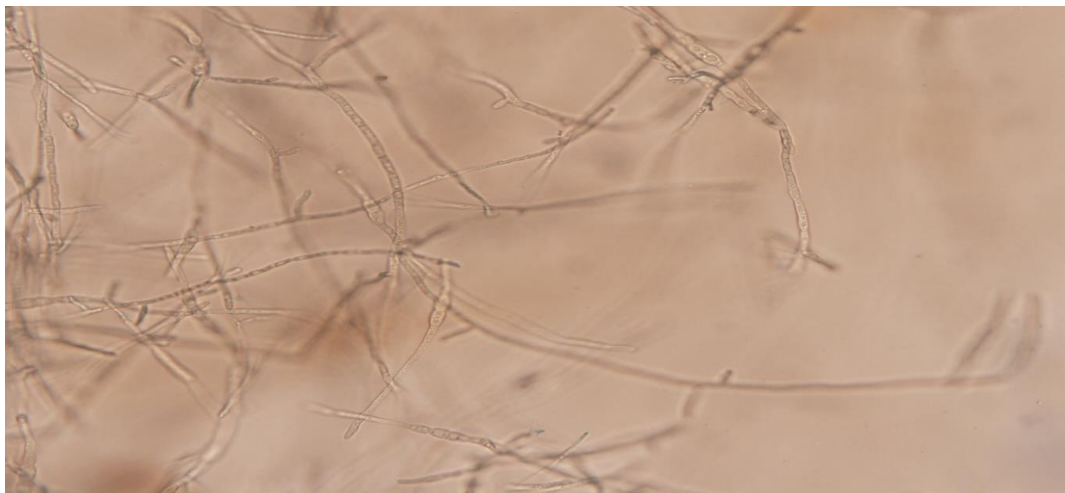


Plate 2.2 Chlamydospore of *Fusarium pallidoroseum*

Fusarium pallidoroseum have also been used as a potential bioherbicide for Parthenium management (Jeyalakshmi *et al.*, 2001)

Culex quinquefasciatus is one of the most annoying vectors and important man-biting mosquitoes. It works as the vector of lymphatic filariasis and Japanese encephalitis in India. They were killed within 4 days of exposure to *Fusarium pallidoroseum* at a concentration of 1.11×10^{10} conidia per m^2 (Mohanty *et al.*, 2008).

F. semitectum was reported to be effective against many sucking pests viz., sugarcane wooly aphid, *Ceratovacuna lanigera* (Aswini, 2007 and Nagaratha, 2004), on cowpea aphid, *Aphis craccivora* Koch (Rani, 2008). Field experiments conducted to estimate efficacy of *F. pallidoroseum* to manage *Aphis craccivora* using different spore formulations discovered that 82% mortality of mite, *Calepitrimerus azadirachtae* by *F. semitectum* at 2.3×10^9 spores per mL (Navik *et al.*, 2015).

A significantly enhanced proline level in plants infected by *F. pallidoroseum* also showed their role as plant growth promoter (Srivastava *et al.*, 2011). While, (Monga *et al.*, 2010) also reported *F. pallidoroseum* to cause 80-95% mortality of cotton mealybug, *Phenacoccus solenopsis*. The bioefficacy of *Fusarium pallidoroseum* against cowpea sucking pests was studied, and it was found to be a very effective entomopathogen against *Aphis craccivora* and *Riptortus pedestris*, with consistently higher mortality as exposure time increased. However, against *Beauveria bassiana* and *Metarhizium anisopliae* it was recorded moderately effective (Singh and Kaur, 2020; Tarekegn *et al.*, 2020). Spraying of spore formulation of *Fusarium pallidoroseum*, at 1×10^{10} spores⁻¹ml conc. was the most effective against *A. gossypii*, recorded 93.33% mortality of nymphs and 66.67% mortality of adults; could be used under IPM practice (Srivastava and Shukla, 2021).

Media optimization

The optimization of process variables, particularly physical and chemical parameters, is crucial in the development of any fermentation process because of the impact on economy and feasibility of the process (Liu and Tzeng, 1998). Furthermore, due to the complexity of the metabolic state in fungus, there is a clear requirement to optimize the essential process variables, depending on the substrate, for improved accumulation of desired product (Dhillon *et al.*, 2011).

For media optimization **Plackett Burmann Design** is applied, which is a prominent class of screening designs that may be used to analyse the impact of design parameters on the system state so that informed judgments can be made and through partial regression fitting of the experimental parts, Response Surface Methodology (RSM) is used, which is a thorough experimental design and mathematical modelling

(Wang *et al.*, 2011). The ability to reduce the number of experimental runs required to produce sufficient information for statistically acceptable results is its key advantage. RSM has been used in studies to improve a variety of processes, including fermentation conditions (Wang *et al.*, 2011).

It was created to improve the quality control process so that intelligent judgments could be made after studying the impacts of design parameters on the system state. Orthogonal arrays, developed by Plackett and Burman (PB), are valuable for screening because they offer unbiased estimates of the key effects in the smallest design feasible. In 'n + 1' run PB design, a variety of 'n' variables may be examined. A characteristic feature is that the sample size is a multiple of four rather than a power of two (4k observations with $k = 1, 2, \dots, n$). PB designs are used to investigate n-1 variables in n experiments proposing experimental designs for more than seven factors and especially for $n \times 4$ experiments, *i.e.*, 8, 12, 16, 20, etc., that are suitable for studying up to 7, 11, 15, 19, etc., factors, respectively. Such designs are known as saturated designs.

The main benefit of saturated designs is that they require the fewest number of observations to calculate an effect for a given factor. A PBD with 12 runs is used to analyse 11 components, whereas fractional factorial designs require 16 observations. PBD, on the other hand, necessitates fewer experiments than highly fractionated factorial designs with the same number of elements. The experimenter can use the projective characteristic of the PB design to follow up on an initial design with runs that allow for efficient separation of primary effects and interaction effects (Vanaja and Rani, 2007).

The aliasing pattern in PB design is substantially more sophisticated; each key effect is aliased with every two-way interaction that does not include that impact. It's tough to determine if there's a lack of fit, as first-order effects might be muddled by interaction effects. PB designs are Resolution III designs that have the advantage of having the fewest number of runs, but they do not allow for the estimate of factor interactions; nonetheless, they can identify the major primary factors that make up the probable significant interactions. Further, the analyst would be able to discover and

estimate the major interaction terms through a deeper examination of the important main components. As a result, using a Plackett-Burman design for screening is acceptable (**Trocine and Malone, 2000**).

Various steps involved in a screening design can be summarized as:

- Select the factors
- Define the levels for the factors
- Select the responses to be measured
- Generate a design matrix of PBD
- Randomize (block) and perform the experiments described in the experimental set up
- Replicate the design
- Develop a model
- Statistical or graphical analysis of the effects
- Interpret and conclude from the statistical analysis
- Recommend possible improvements and if necessary apply higher resolution energy.
- Build verification product (**Van Leeuwen *et al.*,1990**)

Fusarium sp. isolated from dairy runoff was used to optimize extracellular lipase production. Initially, the physicochemical factors which have a significant impact on enzyme synthesis were examined by modifying one factor at a time (OFAT). The ideal parameters for lipase production were discovered to be a mesophilic temperature of 40°C, an alkaline pH of 8, and a 72-hour incubation period. The disaccharide sucrose was the best carbon supply, palm oil was the best inducing lipid substrate, casein and (NH₄)₂SO₄ were the best organic and inorganic nitrogen suppliers, and the Ca²⁺ ion was the best trace element among the media components. The Box-Behnken design of Response Surface Methodology was used in the following phase of development to perform statistical optimization of medium

components (RSM). This methodology observed that the optimum concentrations of three significant components, namely palm oil, $(\text{NH}_4)_2\text{SO}_4$, and CaCO_3 , to be 5% (v/v), 5.5 g/L, and 0.1 g/L, respectively. A maximum lipase output of 73.3 U/ml was obtained using an RSM-guided design of trials, which is a 1.7-fold increase over the unoptimized medium. These findings indicate to the model's success in designing a technique for producing lipase, an enzyme with great industrial interest (**Kanmani et al., 2013**).

Lignin depolymerization is aided by aryl alcohol oxidase (AAO), an extracellular H_2O_2 -producing enzyme. Due to the low yield of enzyme-producing microorganisms and the high cost of fermentation media, cost-effective AAO synthesis has yet to be accomplished. The goal of this research is to create a low-cost medium for high-yield AAO production in submerged culture utilising a recombinant *Aspergillus nidulans* strain. The results show that corn steep liquor (CSL) is a good source of trace metals and vitamins (like pyridoxine) for *A. nidulans*, and that CSL can offer enough trace metals and vitamins (like pyridoxine). The major influencing factors in AAO production were determined using a two-level Plackett-Burman design. A RSM was used to further optimise the medium, yielding the best medium component concentrations (61.0 g/L maltose, 26.4 g/L CSL, and 13.8 g/L NaNO_3). With a protein content of 0.75 g/L, the highest AAO activity was 1021 g/L (**Liu et al., 2020**).

By protoplast mutation of UV light and diethyl sulphate, a mutant *Fusarium maire* K178 with high paclitaxel (taxol) synthesis was chosen (DES). Plackett–Burman design as well as response surface methods were used to optimise medium components. The Plackett–Burman design was used to screen key components in trace elements after comparing various nitrogen sources. The most essential components were found to be NaOAc, NH_4NO_3 , and MgSO_4 , which were further examined using response surface methods. NaOAc 2.02 g/l, NH_4NO_3 7.84 g/l, and MgSO_4 0.68 g/l were the best concentrations. After the strain improvement and optimization of the media the yield of taxol increased from 20 to 225.2 g/l (**Feng et al, 2006**).



*Materials
and
Methods*



The details of experimental plots, materials used and the techniques adopted during the course of investigation are described in this chapter

3.1 Material used:

3.1.1 Chemicals: All the chemicals used were AR and molecular grade purchased from different suppliers like Hi media, Sigma Aldrich, and other standard firms in India.

3.1.2 Glass-wares and Plastic-wares: All the glass-wares and plastic-wares used were manufactured by Borosil, Schott, Duran and Tarson.

3.1.3 Instruments: The following instruments were used for performing the experiments:

S.No.	Instrument	Manufacturer
1.	Electronic Weighing Balance	Afcoset
2.	Autoclave	Metrex
3.	Laminar Air Flow	Narang Scientific Works
4.	Rotator Shaker	Remi
5.	Microwave oven	Cookworks
6.	Hot Air Oven	Metrex
7.	Magnetic stirrer	Tarsons
8.	Vortex	Tarsons
9.	Refrigerator	LG
10.	Haemocytometer	Marienfeld
11.	Compound Microscope	Olympus DP71

3.2 Revival of fungal culture: *Fusarium pallidoroseum* used in this study were provided by the Rhizospheric Biology Lab, Department of Biological Sciences, Pantnagar. Fungal isolates were maintained in Potato Dextrose Agar (PDA) throughout the investigation.

3.3 Optimization of media by Plackett Burmann Design and Response Surface Methodology

3.3.1 Plackett-Burman Design: A prominent type of screening design to optimize the fermentor media is used. They have the advantage of having the fewest number of runs and allowing for the identification of the major primary factors that make up the possible significant interactions. The following factors were chosen for media optimization: K_2HPO_4 , $MgSO_4$, $CaCl_2$, Sucrose, Ferric Ammonium citrate, EDTA, Trace, Na_2HPO_4 and Peptone. Among all of these factors important factors influencing the spore count of *Fusarium pallidoroseum* was identified. The responses were analysed using the Design-expert v10 software. Following equation was used for mathematical modelling:

$$Y = \beta_0 + \sum \beta_i x_i$$

where , Y- Predicted response

β_0 -model intercept

β_i - linear coefficient

x_i - level of an independent variable

For each of the 13 combinations, 100 mL media was made in Erlenmeyer flasks (500 mL). Media was then autoclaved at 121°C under 15psi of pressure. 5 mL preactivated culture of *Fusarium pallidoroseum* was inoculated. The inoculation was carried out inside laminar air flow. It was then kept in incubation for 4 days at 27 ± 2 °C. The cultivation was carried out for 4 days in shaking incubator, 150 RPM, at 27 ± 2 °C. On 4th day spore concentration was determined using Haemocytometer using Compound Microscope.

Table 3.1: Experiment matrix for the Plackett-Burman design

RUN	K₂HPO₄ (mL)	MgSO₄ (mL)	CaCl₂ (mL)	Sucrose (g)	Fe(NH₄)₃ Citrate (mL)	EDTA (mL)	NaCl (g)	Trace (mL)	Na₂HPO₄ (mL)	Peptone (g)
1	2	0.5	0.5	10	2	0.5	2	2	0.5	4.5
2	2	0.5	2	30	2	0.5	0.5	0.5	2	1
3	2	0.5	2	30	0.5	2	2	2	0.5	1
4	0.5	2	2	10	2	2	2	0.5	0.5	1
5	1.25	1.25	1.25	20	1.25	1.25	1.25	1.25	1.25	2.75
6	0.5	2	2	30	0.5	0.5	0.5	2	0.5	4.5
7	0.5	2	0.5	30	2	0.5	2	2	2	1
8	2	2	0.5	10	0.5	2	0.5	2	2	1
9	2	2	2	10	0.5	0.5	2	0.5	2	4.5
10	0.5	0.5	0.5	30	0.5	2	2	0.5	2	4.5
11	0.5	0.5	2	10	2	2	0.5	2	2	4.5
12	0.5	0.5	0.5	10	0.5	0.5	0.5	0.5	0.5	1
13	2	2	0.5	30	2	2	0.5	0.5	0.5	4.5

For 1000 mL media.

3.3.2 RESPONSE SURFACE METHODOLOGY

Response Surface Methodology is a mathematical and statistical approach for optimising an operational condition that is impacted by a number of independent factors in order to obtain the highest yield through the most cost-effective procedure. The outcome is mostly determined by the primary components that have a significant impact on our interest response, as well as their combined impacts. The homogeneity of the error distribution and the appropriateness of the suggested model are checked using CCD residual plots. The least-squares regression model provides a credible explanation for each factor's effect as well as probable relationships between the independent and dependent variables. Based on the results of PBD, three variables are selected that have been found to have the largest influences on responses like spore count of fungus. The selected factors are then chosen for submerged fermentation through RSM. A set of experiments is conducted and the effects of the three factors under investigation, as well as their interactions, on the dependent variable, spore count per ml were computed in both linear and quadratic form. Changing the concentrations of the three specified factors while keeping the concentrations of the other factors constant for each culture, 20 sets of experiments were carried out. RSM was carried out in the same way as PBD, with 100 mL medium prepared in Erlenmeyer flasks. The media was then autoclaved at 121°C under 15 psi pressure. 5 mL of pre activated culture of *Fusarium pallidoroseum* is inoculated in 500 mL capacity Erlenmeyer flasks under laminar air flow. The cultivation was carried out for 4 days in shaking incubator, 150 RPM, at 27 ± 2 °C. On 4th day spore count per mL of *Fusarium pallidoroseum* were determined by using Haemocytometer under compound microscope.

The following quadratic equation describes the mathematical link between the response (Y) and the significant independent variables X1, X2, and X3:

$$\Psi = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where,

Y= response (Spore count per ml)

X₁, X₂, and X₃= significant independent variables

$\beta_1, \beta_2, \beta_3$ = linear regression coefficients

$\beta_{11}, \beta_{22}, \beta_{33}$ = quadratic regression coefficients

$\beta_{12}, \beta_{13}, \beta_{23}$ = interactive regression coefficients

β_0 = constant term

Table 3.2 : Experimental matrix for Response Surface Methodology

RUN	Peptone (g/L)	K ₂ HPO ₄ (mL/L)	Trace elements (mL/L)
1	4	0.9	2.5
2	4	0.9	2.5
3	6	1.5	4
4	4	0.9	2.5
5	0.636414	0.9	2.5
6	4	1.90908	2.5
7	4	0.9	-0.0226892
8	4	0.9	2.5
9	4	0.9	2.5
10	6	0.3	1
11	2	0.3	1
12	2	0.3	4
13	4	-0.109076	2.5
14	4	0.9	5.02269
15	2	1.5	4
16	6	0.3	4
17	4	0.9	2.5
18	2	1.5	1
19	7.36359	0.9	2.5
20	6	1.5	1

For 1000 mL

Responses i. e. spore count per ml of *Fusarium pallidoroseum* were analysed using the Design-expert v10 software. Three parameters that significantly and positively affected the spore count were chosen for the RSM experiment among the various factors.

3.4. Spore Count using Haemocytometer

1 mL of homogenous sample of fungi were taken from each of the Erlenmeyer flask and transferred to 9 mL of sterilized distilled water containing Tween 80 (0.01 %) solution. Counting of spores at dilution 10^{-3} were made after the serial dilution of the suspension using Neubauer Haemocytometer for determining the number of spore in 1 mL of the sample. Following is the formula for determining the number of spores :

$$\text{Spores}^{-1} \text{ mL} = n \times \text{Dilution factor} \times 10^4$$

where

n = the average cell count per square of the four corner squares counted.



*Results
and
Discussion*



This chapter discusses the findings of the experiments as well as the logical explanations for the outcomes. Tables and figures are used to present the observations and models, which are then statistically examined. According to the results obtained during the investigations relevant discussions have been made.

4.1 MEDIA OPTIMIZATION: Media optimization of *Fusarium pallidoroseum* was performed to develop a medium for higher production of spores in submerged culture using Plackett-Burmann design and Response Surface Methodology (Design-Expert version 10 software).

Plackett-Burman design is a mathematical and statistical tool for identifying and selecting the factors that affect a process, as well as determining their relative significance in optimizing the process.

4.1.1 Screening of significant factors for production of spore count per mL using Plackett–Burman design

The current study for 13 trials was performed to select the main factors that influence the production of higher number of spore count of *Fusarium pallidoroseum*. 10 variables representing 10 chemical factors namely K₂HPO₄, MgSO₄, CaCl₂, Sucrose, Ferric Ammonium Citrate, EDTA, NaCl, Trace, Na₂HPO₄ and Peptone, were investigated in a total of 13 experimental runs. Each factor was assigned with the highest and the lowest coded values as shown in (**Table 4.1.1**). Experiments were conducted as per the matrix and response was recorded as spore count ⁻¹ mL (as shown in **Table 4.2.1**). A first order polynomial model, as shown in Eq. (1), was used to analyse the results of PBDs, which are based on Hadamard matrices (**Goupy, 2005**).

$$Y = \beta_0 + \sum_{i=1}^{10} \beta_i X_i$$

where Y is the predicted response; β_0 is the model intercept; β_i is the linear regression coefficient; and X_i is the coded independent variable (concentration of each factor).

The data was fitted in the model using Design- Expert version 10 software at a significant level of $P < 0.05$. The significant factors were considered for further optimization using Response Surface Methodology.

Table 4.1.1: Factors with levels in PBD for screening of main factors in fungal spore production of *Fusarium pallidorozeum*

S. No.	Variables (media components)	Coded levels for variables	
		High (+1)	Low (-1)
1.	K_2HPO_4	2	0.5
2.	$MgSO_4$	2	0.5
3.	$CaCl_2$	2	0.5
4.	Sucrose	30	10
5.	Ferric Ammonium Citrate	2	0.5
6.	EDTA	2	0.5
7.	NaCl	2	0.5
8.	Trace elements	2	0.5
9.	Na_2HPO_4	2	0.5
10.	Peptone	4.5	1



Plate 4.1: PBD 13 experiments by using Erlenmeyer flask containing medium components as per matrix *Fusarium pallidorozeum*

Table 4.1.2 Predicted and experimental responses obtained from PBD responses influencing *Fusarium pallidoroseum* spore production

Run Order	Actual Value	Predicted Value¹	Residual	Leverage	Internally Studentized Residual	Externally Studentized Residual	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
12	17.74	18.38	-0.64	0.667	-1.576	-2.217	0.552	-3.135 ²	12
1	32.00	32.63	-0.63	0.667	-1.551	-2.128	0.535	-3.010 ²	7
4	10.50	10.98	-0.48	0.667	-1.196	-1.293	0.318	-1.828	2
6	28.30	28.42	-0.12	0.667	-0.289	-0.253	0.019	-0.357	10
9	25.30	25.33	-0.027	0.667	-0.066	-0.057	0.001	-0.081	9
3	35.00	34.98	0.017	0.667	0.041	0.036	0.000	0.051	3
13	22.00	21.98	0.017	0.667	0.041	0.036	0.000	0.051	1
11	34.00	33.88	0.12	0.667	0.289	0.253	0.019	0.357	5
8	26.30	26.17	0.13	0.667	0.314	0.275	0.022	0.389	8
7	13.30	12.82	0.48	0.667	1.196	1.293	0.318	1.828	4
2	30.30	29.81	0.49	0.667	1.221	1.335	0.331	1.888	11
10	20.00	19.36	0.64	0.667	1.576	2.217	0.552	3.135 ²	6
5	28.00	28.00	0.000	1.000 ³					13

Multiple regression statistical analysis and ANOVA (the analysis of variance) of the Plackett - Burman Design was performed is summarized in **Table 4.1.3** to assess relationship between independent variables and spore count⁻¹ml (dependent variable). ANOVA is used to determine which of the factors had a significant impact on the dependent variable of interest. F-value of model 38.87 proving our model to be statistically significant which is further consolidated by a p-value of 0.0005 (p-value < 0.05). K₂HPO₄, MgSO₄, CaCl₂, NaCl, trace elements and Peptone significantly impacted the process with p-value of 0.0004, 0.0006, 0.0022, 0.0098, 0.0006 and 0.0037 respectively.

Table 4.1.3: ANOVA analysis and Regression statistics of variables for spore production using *Fusarium pallidoroseum*

Source	Sum of Squares	df	Mean Square	F value	p-value
MODEL	700.34	7	100.05	38.87	0.0005*
A-K₂HPO₄	184.55	1	184.55	71.69	0.0004**
B-MgSO₄	156.53	1	156.53	60.81	0.0006**
C-CaCl₂	85.65	1	85.65	33.27	0.0022*
D-Ferric ammonium citrate	9.26	1	9.26	3.60	0.1164
E-NaCl	42.34	1	42.34	16.45	0.0098*
F-Trace metals	154.51	1	154.51	60.02	0.0006**
G-Peptone	67.50	1	67.50	26.22	0.0037*
Residual	12.87	5	2.57		
Cor Total	713.21	12			

** highly significant, * significant

Std. Dev.	1.60	R-Squared	0.9820
Mean	24.83	Adj R-Squared	0.9567
C.V. %	6.46	Pred R-Squared	0.9494
PRESS	36.06	Adeq Precision	19.068

*Significant values

4.1.1.a Pareto chart of Effects

The Pareto chart illustrates the order of significance of the variables affecting spore count of *Fusarium pallidoroseum*.

The ANOVA effect estimates are arranged in this graph from highest absolute value to lowest absolute value. A typical Pareto chart is a bar graph with different-length columns that represent the absolute value of the estimated effects divided by the standard error. The bars are arranged in order of effective value, i.e. t-value, with the highest effects on top and each bar representing a variable. The independent variables are grouped above and below the horizontal line denoting the t-value limit in declining order of their impact on spore count. A high t-test score and a low probability suggested that the factor had a significant impact on the process. The factors with positive effects are shown by orange, while the factors with negative effects are represented by blue (as shown in Figure 4.1.1). The large values of positive or negative factors indicate a high significance of those factors on spore count of *Fusarium pallidoroseum*. While the near-zero effect indicates that the factor has little to no effect on spore count. **Figure 4.1.1** represents the factors (K_2HPO_4 , Trace elements, $CaCl_2$, Peptone) influencing positively the production of spore count of *Fusarium pallidoroseum*. Among all of the positively affecting factors K_2HPO_4 , Trace elements and Peptone were selected as main factors for production of higher spore count in submerged media components.

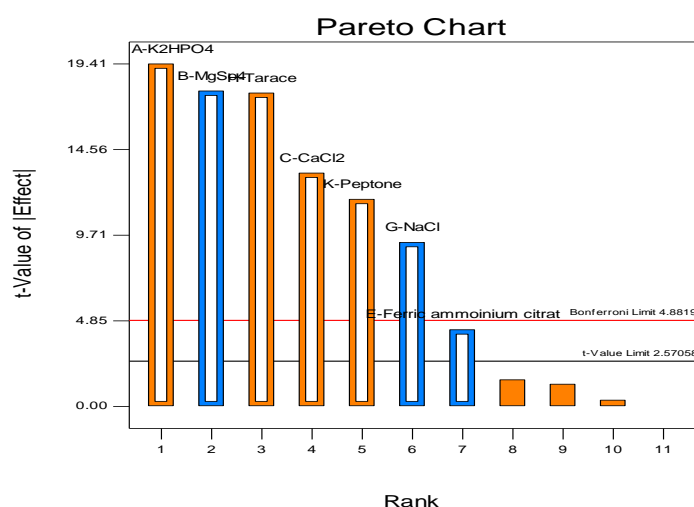


Fig. 4.1.1: Pareto chart showing the significant variables i.e., K_2HPO_4 , Trace metals, $CaCl_2$, and Peptone influencing the production of *Fusarium pallidoroseum* spore.

Regression Analysis

The R^2 or "determination coefficient" is used to verify the model's ability to represent the data and the variability of the response influenced by the independent variables. A regression model with R^2 -values greater than 0.9 is considered to have a high correlation. The regression model in this study has R^2 value of **0.9820** (Table 4.1.3) which suggests that 98.20 % of the variation in the spore count can be validated by the independent variables and only 1.8% of the total changes are due to reasons other than the independent variables. As a result, the regression model provides a perfect explanation for the link between spore count⁻¹ml and the independent variables.

The high precision of the experiment is indicated by the value of C.V. (6.46). The PRESS (Predicted Residual Error Sum of Square) value for the model is 36.06, with standard deviation and mean values of 1.60 and 24.83, respectively.

Table 4.1.4: Coefficient of the factors affecting Spore production of *Fusarium pallidoroseum*

Term	Coefficient
Intercept	+14.74369
K ₂ HPO ₄ (A)	+5.22889
MgSO ₄ (B)	-4.81556
CaCl ₂ (C)	+3.56222
Ferric Ammonium citrate (D)	-1.17111
NaCl (E)	-2.50444
Trace (F)	+4.78444
Peptone (G)	+1.35524

Regression equation in terms of the independent variables is shown as:

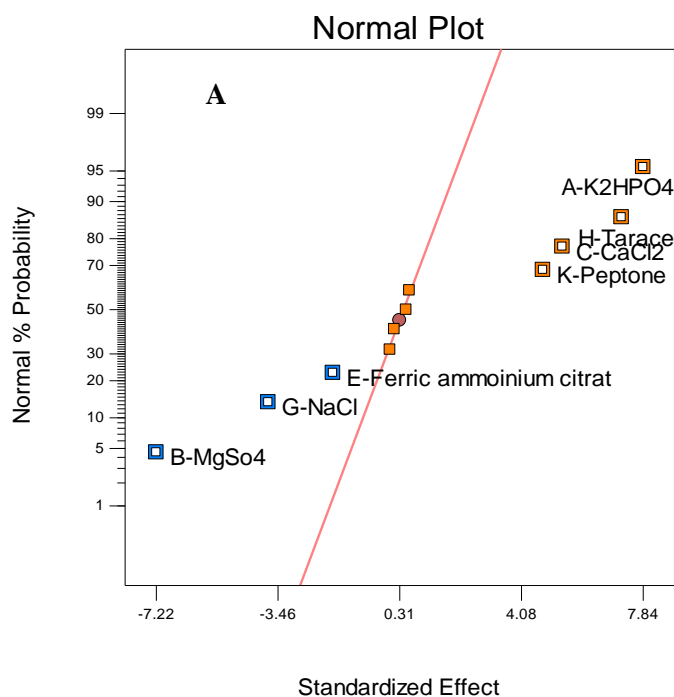
Response (spore production Spore Count⁻¹ mL): **+14.74369 + 5.22889A - 4.81556B + 3.56222C - 1.17111D - 2.50444E + 4.78444F + 1.35524G**

4.1.1.b Normal probability Plot

The normal probability plot (NPP) is a chart showing whether the residuals are distributed normally. It tells us if our model is good enough to explain our results and experiments. The distribution of residual values is examined before accepting a certain "model" that comprises of a certain number of effects. The data are plotted against a theoretical normal distribution. The plotted point in the normal probability plot of the studentized residuals **Fig 4.1.2 (A)** is near to a straight line, indicating that the model has been well fitted with the experimental results.. These are calculated as the difference between the predicted and observed values. The actual residual values are represented along the horizontal X-axis, while the expected normal values for the relevant values are plotted along the vertical Y-axis after they have been rank-ordered. If all of the values fall on a straight line, the residuals are said to have a normal distribution. A graphical representation is shown in the **FIG 4.1.2 (B)** implying that the model is adequate to determine the effect of media composition for spore production.

Design-Expert® Software
R2

Shapiro-Wilk test
W-value = 0.905
p-value = 0.458
A: K₂HPO₄
B: MgSO₄
C: CaCl₂
D: Sucrose
E: Ferric ammonium citrat
F: EDTA
G: NaCl
H: Tarace
J: Na₂HPO₄
K: Peptone
L: MSG
■ Positive Effects
■ Negative Effects



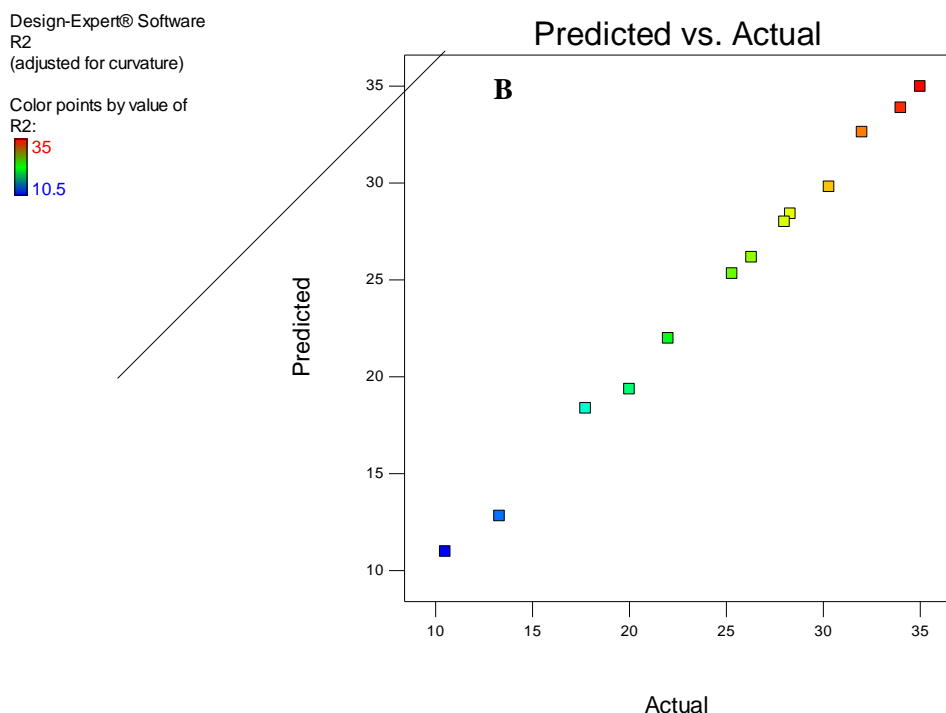


Fig 4.1.2: (A) Graph showing normal probability plot of the studentized response. (B) correlation between the experiment and Predicted values of spore production by *Fusarium pallidoroseum*

4.2 Optimization of significant culture parameters with Response Surface Methodology based on the central composite design (CCD)

To determine the optimum combined effect of the most significant independent variables, the **Response Surface Methodology (RSM)** was applied according to central composite design (CCD). The central composite design was used to determine probable interactions between the factors that were significant and positively affecting spore count, as well as to determine optimal levels of these variables to produce a higher spore count. The composite complex design matrix is presented in **Table 4.2.1**. The central objective of the response surface is to find the optimum values of the variables as quickly as possible so that the response can be maximized. Peptone, Trace and K_2HPO_4 , factors were chosen for RSM. CCD was created for these three parameters, yielding a matrix of 20 experiments (**as shown in Tale 4.2.2**).

Table 4.2.1: Levels and code of variables chosen for central composite design

S.No.	Variables (media components)	Coded levels for variables	
		High	Low
1	Peptone	0.636414	7.36359
2	K ₂ HPO ₄	-0.109076	1.90908
3	Trace metals	-0.0226892	5.02269

**Plate 4.2.: 20 RSM experiment performed in Erlenmeyer flask for spore production**

Table 4.2.2: Experimental matrix for CCD using designed expert software package for medium components with actual and predicted responses (spore production)

RUN	Coded levels of selected variables			Spore production (Spore count ⁻¹ mL)		Residual
	A:Peptone	B:K ₂ HPO ₄	C:Trace metals	Actual value	Predicted value	
1	4	0.9	2.5	9.50	10.13	-0.63
2	4	0.9	2.5	8.00	8.40	-0.40
3	6	1.5	4	8.20	8.51	-0.31
4	4	0.9	2.5	5.20	5.49	-0.29
5	0.636414	0.9	2.5	10.50	10.77	-0.27
6	4	1.90908	2.5	8.25	8.54	-0.29
7	4	0.9	-0.0226892	12.80	13.06	-0.26
8	4	0.9	2.5	10.25	10.41	-0.16
9	4	0.9	2.5	10.25	10.13	0.12
10	6	0.3	1	10.25	10.13	0.12
11	2	0.3	1	10.25	10.13	0.12
12	2	0.3	4	10.25	10.13	0.12
13	4	-0.109076	2.5	10.25	10.13	0.12
14	4	0.9	5.02269	10.00	9.84	0.16
15	2	1.5	4	15.30	15.03	0.27
16	6	0.3	4	9.25	8.99	0.26
17	4	0.9	2.5	5.50	5.20	0.30
18	2	1.5	1	10.30	10.02	0.28
19	7.36359	0.9	2.5	11.00	10.70	0.30
20	6	1.5	1	6.50	6.08	0.42

Table. 4.2.3 Regression statistics and analysis of variance (ANOVA) for response surface models

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	99.29	9	11.03	66.83	< 0.0001	significant
A-Peptide	13.39	1	13.39	81.14	< 0.0001	
B-K ₂ HPO ₄	4.69	1	4.69	28.42	0.0003	
C-Trace metals	6.50	1	6.50	39.39	< 0.0001	
AB	2.20	1	2.20	13.36	0.0044	
AC	3.51	1	3.51	21.27	0.0010	
BC	1.28	1	1.28	7.75	0.0193	
A ²	19.10	1	19.10	115.68	< 0.0001	
B ²	27.67	1	27.67	167.63	< 0.0001	
C ²	14.95	1	14.95	90.57	< 0.0001	
Residual	1.65	10	0.17			
Lack of Fit	1.18	5	0.24	2.52	0.1665	not significant
Pure Error	0.47	5	0.094			
Cor Total	100.94	19				

Std. Dev.	0.41		R-Squared	0.9836
Mean	9.59		Adj R-Squared	0.9689
C.V. %	4.24		Pred R-Squared	0.9031
PRESS	9.78		Adeq Precision	34.204
-2 Log Likelihood	6.87		BIC	36.82
			AICc	51.31

Each significant variable was assigned a "central coded value" and examined at various axial coded levels. In each experiment, the response value was spore count mL^{-1} . The highest spore count mL^{-1} is obtained in run number 15 with the value of 15.30 where peptone, K_2HPO_4 , and Trace metal values were 2, 1.5 and 4 respectively (as shown in table 4.2.2). The CCD experimental data from the 20 experiments were statistically evaluated to find the association between variables with the most significant positive effects and higher number of spore count using "design expert software".

The summary of fit for the CCD is presented in **Table 4.2.3**. The model F-value of 66.83 and P-value of < 0.0001 implicates that the model is significant. The model has coefficient of determination (R^2) value of 0.9836, which suggests that 98.36 percent of the differences in spore count per ml can be describe by the independent variables utilized, while 1.64 percent of the variability cannot be explained by independent variables. This suggests that the regression model used in this study explains the relationship between spore count mL^{-1} and the independent variables quite well. Furthermore, an Adjusted R^2 value of **0.9689** proves the model's accuracy in calculating expected spore count mL^{-1} . The highest accuracy of the experiment is indicated by the figure of C.V. (**4.24**). Furthermore, the PRESS value was 9.78, while the standard deviation and mean were 0.41 and 9.59, respectively (as shown in table 4.2.3).

The coefficients of linear, quadratic and interaction terms were estimated and listed in Table 4.2.4. The statistical significance of each variable for spore count⁻¹ mL were considered as significant with $P < 0.05$, further indicating the linear coefficients of (A) Peptone (A), (B) K_2HPO_4 and (C) trace metals are found to be significant. According to **Table 4.7**, (A) Peptone, (B) K_2HPO_4 and (C) trace metals were observed to be significant with P value of < 0.0001 , < 0.0001 , 0.0003 respectively.

The interaction between AB, AC and BC along with their quadratic effect A^2 , B^2 and C^2 are found to be significant as shown in (**Table 4.7**), suggesting their contribution in affecting the spore count. The P value of the model was less than 0.0001, which indicated that the model was reliable to predict spore count per ml. The

lack of fit, which is used to test the failure of the model to fit the experimental data, had a P value of 0.1665. The non-significant lack of fit demonstrated that the model fits the experimental data and can predict spore count.

The equation of regression can be obtained in coded terms:

$$\text{Response (Spore count ml}^{-1}\text{)} = -2.98365 + 3.74319A + -3.09030B + 4.00666C - 0.43750AB - 0.22083AC - 0.44444BC - 0.28777A^2 + 3.84904B^2 - 0.45267C^2$$

Where A is Peptone, B is K_2HPO_4 and C is trace elements

The model with the highest adjusted and predicted R^2 and the lowest standard deviation was chosen using the model summary statistics. With a very low P-value (0.0001), the quadratic model is a high-significant and suitable model adequate for the CCD for spore production. The model also revealed a high lack of fit F-value = 2.52 (insignificant lack of fit, P-value = 0.1665). The quadratic model has the highest coefficient of determination $R^2 = (0.9836)$ and adjusted $R^2 = (0.9689)$, which illustrates the good fit. In addition, the quadratic model has the minimum standard deviation (0.41).

The regression model's 3D response surface plots were designed to show the impacts of the independent variables and the interaction of each independent variable on the response variable. In accordance with the current findings **Figure 4.2.2** shows three-dimensional response surface plots that can be used to visualise the relationship between spore count per ml and variable interactions, as well as to discover the best submerged medium components for the production of spores. By plotting spore count per ml against two factors and leaving the third variable at its zero levels, three-dimensional plots were constructed for pairs of the three significant variables.

The three-dimensional response surfaces were plotted by the response (spore count per mL) with two factors (**Fig 4.2.2 (A)** Peptone and K_2HPO_4 , **Fig. 4.2.2 (B)** K_2HPO_4 and trace metal, and **Fig. 4.2.2 (C)** Peptone and trace metals while holding the other factor at 0 level.

Figure 4.2.2 (A) depicts the simultaneous effects of Peptone and K_2HPO_4 on spore count per ml while level of trace metal were kept to a minimum. The highest

spore count of 12U/mL was reached when the concentration of peptone and K₂HPO₄ was 4 ml/L and 0.9 ml/L respectively.

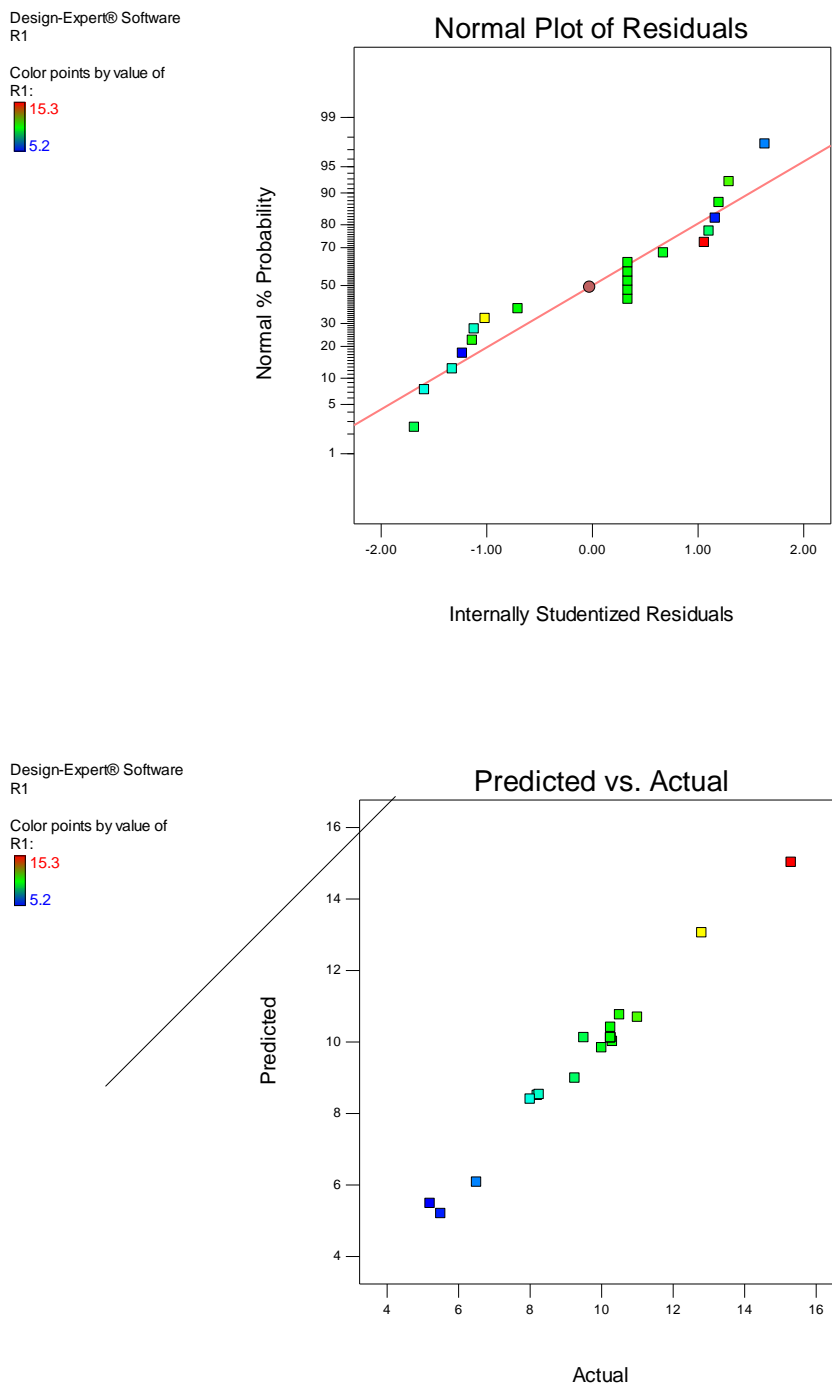


Fig 4.2.1 Plots of standardized residuals vs normal % probability for spore production (A). Actual and predicted value of spore production by *Fusarium pallidoroseum*

Table. 4.2.4 Experimental and predicted values of spore production by *Fusarium pallidorozeum*

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residual	Externally Studentized Residual	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
17	9.50	10.13	-0.63	0.166	-1.686	-1.890	0.057	-0.844	18
14	8.00	8.40	-0.40	0.607	-1.590	-1.746	0.391	-2.171 ¹	14
18	8.20	8.51	-0.31	0.670	-1.326	-1.386	0.357	-1.974	3
11	5.20	5.49	-0.29	0.670	-1.231	-1.268	0.307	-1.806	1
20	10.50	10.77	-0.27	0.670	-1.137	-1.155	0.262	-1.646	4
19	8.25	8.54	-0.29	0.607	-1.120	-1.136	0.194	-1.413	10
13	12.80	13.06	-0.26	0.607	-1.017	-1.018	0.160	-1.266	11
15	10.25	10.41	-0.16	0.670	-0.705	-0.686	0.101	-0.977	7
1	10.25	10.13	0.12	0.166	0.336	0.321	0.002	0.143	15
2	10.25	10.13	0.12	0.166	0.336	0.321	0.002	0.143	19
4	10.25	10.13	0.12	0.166	0.336	0.321	0.002	0.143	16
8	10.25	10.13	0.12	0.166	0.336	0.321	0.002	0.143	17
9	10.25	10.13	0.12	0.166	0.336	0.321	0.002	0.143	20
10	10.00	9.84	0.16	0.670	0.672	0.652	0.092	0.929	2
6	15.30	15.03	0.27	0.607	1.059	1.067	0.174	1.327	12
12	9.25	8.99	0.26	0.670	1.103	1.117	0.247	1.591	5
5	5.50	5.20	0.30	0.607	1.163	1.186	0.209	1.475	9
3	10.30	10.02	0.28	0.670	1.198	1.228	0.291	1.749	8
16	11.00	10.70	0.30	0.670	1.293	1.344	0.339	1.915	6
7	6.50	6.08	0.42	0.607	1.633	1.810	0.413	2.250 ¹	13

Design-Expert® Software

Factor Coding: Actual

R1

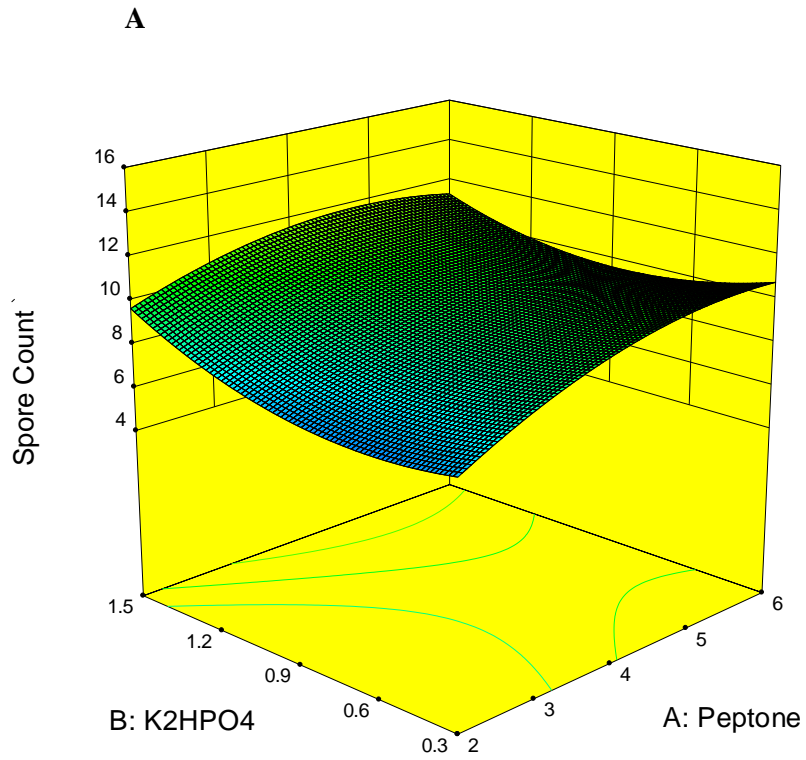


X1 = A: Peptone

X2 = B: K2HPO

Actual Factor

C: Trace metals = 1.68919



Design-Expert® Software

Factor Coding: Actual

R1

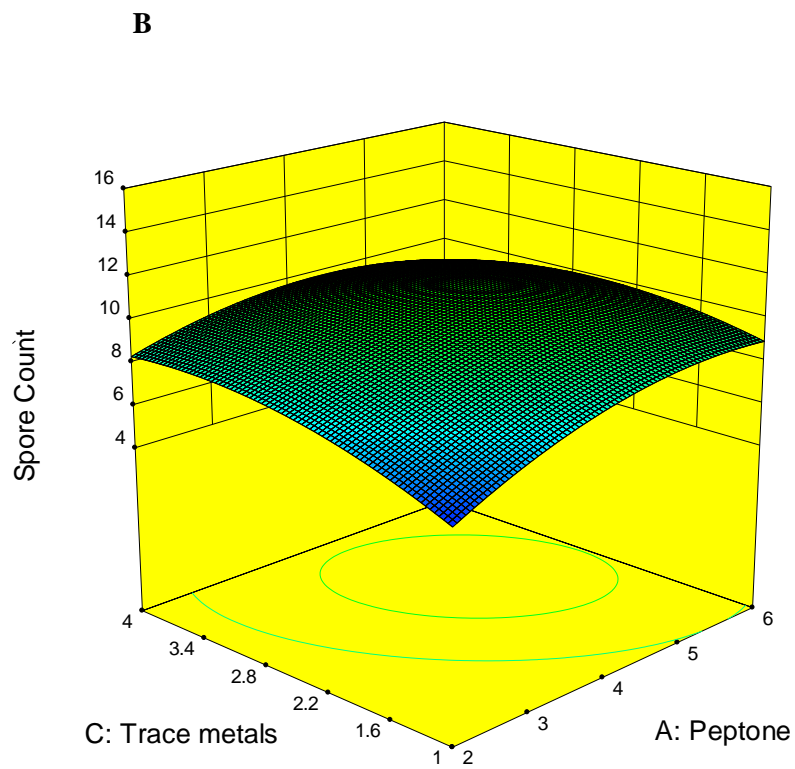


X1 = A: Peptone

X2 = C: Trace metals

Actual Factor

B: K2HPO = 0.9



Design-Expert® Software
Factor Coding: Actual
R1



X1 = B: K2HPO
X2 = C: Trace metals

Actual Factor
A: Peptone = 4

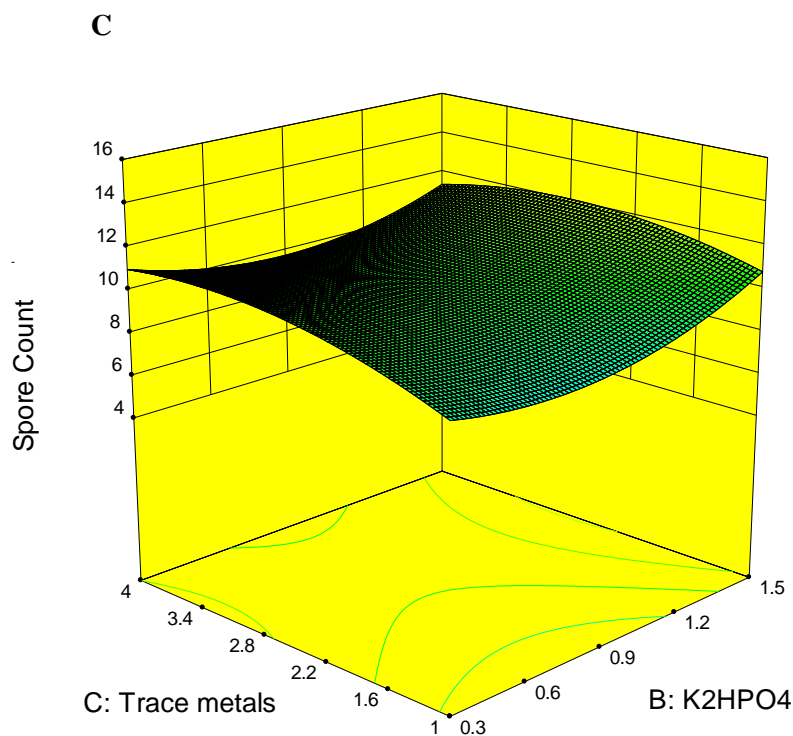


Figure 4.2.2 3D Response surface plots showing interaction between factors and their combined effect on spore count (A) Peptone and K_2HPO_4 , (B) Trace metals and Peptone, (C) Trace metals and K_2HPO_4

The 3D surface and contour plots in **Figure 4.2.2 (B)** illustrates spore count per ml as a function of K_2HPO_4 and Trace elements while Peptone was fixed at a central point. **Figure 4.2.2 (B)** indicates that higher spore count of 11 U/mL was found when concentration of K_2HPO_4 was 0.9 mL/L and trace metals was 2.5 mL/L. Further spore count gets decreases with increasing value of K_2HPO_4 and decreasing value of trace elements i.e. when spore count was 6.50 U/mL then the concentration of K_2HPO_4 and trace elements was 1.5 mL/L and 1 mL/L respectively. **Figure 4.2.2 (C)** represents the response surface contour plots. The response surface represents spore count per mL as a function of concentration of the peptone and trace, with K_2HPO_4 being at an optimal level. From the study of plot, higher spore count per ml of 9.50 U/mL was found when concentration of peptone was 4g/L and trace elements was 2.5 mL/L.



*Summary
and
Conclusions*



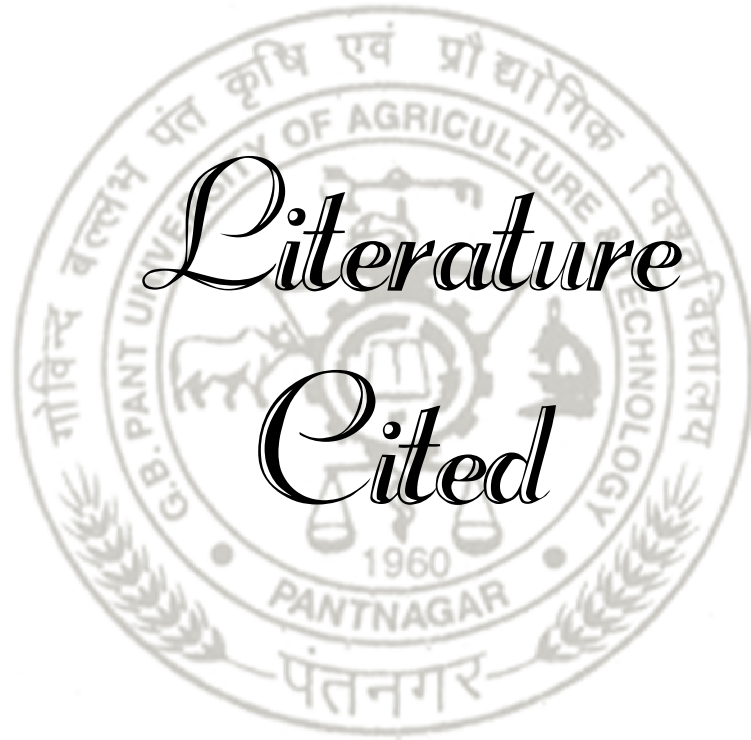
The continuous increasing population in India leads huge pressure on agriculture lands to produce more food. Food is the main source for humans and development of a nation is directly proportional to the amount of food or nutrient available to the population. As a result of increasing global population growth, global food supply may soon be insufficient to feed the whole world's population. For this, any effective and long-term solution for feeding the globe must incorporate sustainable and ecologically acceptable biological alternatives, capable of producing in large quantities and of high quality. Therefore, using microorganism as biofertilizer is seen as viable alternative in the agriculture industry to chemical fertilizers. With this context, a newly described axenically cultivable endophytic and saprophytic fungus *Fusarium pallidroseum*, which mimics the capabilities of Arbuscular Mycorrhizal Fungus has been discovered. There is little information about the cultural media used to maximize the production of spores. For more spore production of fungus optimization of media is essential. Optimum culture condition imparts suitable physiological and physical conditions to fungus. To solve this problem, our current study is based on following features:

- 1. Plackett Burmann Screening Design**, a mathematical and statistical tool for identifying and selecting the factors that affect a process, as well as determining their relative significance in optimizing the process. 13 trials were performed for 10 variables representing 10 chemical factors namely K_2HPO_4 , $MgSO_4$, $CaCl_2$, Sucrose, Ferric Ammonium Citrate, EDTA, NaCl, Trace, Na_2HPO_4 and Peptone for identifying important media components affecting spore count of *Fusarium pallidroseum* and their impact in optimizing the process. A screening run is one that isolates the most important input factors for the response. Based on the results of the screening experiments, it leads to the exclusion of those elements regarded to be of small significance to the intended output i.e. spore count per mL. PBD therefore simply screens the

design space to detect large main effects. PBD data has been used to develop optimal media for high spore counts in cultures, along with factors that could positively or negatively affect the spore count⁻¹ mL. Peptone, K₂HPO₄, Trace metals were found to be the most significant factors which were further investigated using response surface methodology.

2. After the factors (**Peptone, K₂HPO₄, Trace metals**) selected from PBD, they are further optimized using **Response Surface Methodology (RSM)**. This method is a combination of statistical techniques that use design of experiments to develop models, evaluate the effect of variables, and estimate the best medium components for production of higher number of spores. The optimization resulted in the production of higher number of spores containing submerged medium components 0.0592 (g/L) K₂HPO₄, 0.0531 (g/L) MgSO₄, 0.113 (g/L) CaCl₂, 25.6 (g/L) Sucrose, 0.03 (g/L) Ferric Ammonium citrate, 0.002 (g/L) EDTA, 0.063 (g/L) NaCl, 2 (ml/L) Trace metals, 0.434 (g/L) Na₂HPO₄, and 6 (g/L) Peptone.

In conclusion, the overall study shows that PBD and RSM are effective methods for determining and increasing spore production for *Fusarium pallidorozeum*. And we have developed a new medium formulation for the production *Fusarium pallidorozeum*. Spore in submerged cultivation process in a cultivation period of 72 hours with fungal active spore 7×10^6 CFU/ mL was found. It has an advantage over solid state cultivation as production time of spores is short period of 3 days.



*Literature
Cited*



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2.	B.Sc.	D.A.V PG College, Dehradun	2019	7.29
3.	Intermediate	D.S.B International Public School, Rishikesh	2014	84.6 %
4.	High School	M.D.S, Uttarkashi	2012	80.2 %

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Publication: Nil

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List of papers presented in conference/seminar during degree programme: 0

Software Skills: MS Word, MS Excel, MS Power Point.

Professional Skills: Critical thinking and problem solving.

Professional Affiliations (Membership, etc.): Nil

Awards/Honours/Achievements: Nil

Place: Pantnagar

Date: November, 2021

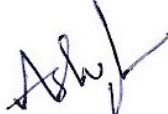

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No. of pages : 39 Advisor : Dr. A.K. Sharma

ABSTRACT

Food is the main source for humans and development of nation is directly proportional to the amount of food or nutrients available to the population. The growing population and limited cultivation land demand the application of fertilizer to meet the human needs. Because of its vast potentiality in increasing crop productivity and food safety, the application of microorganisms as biofertilizer is seen as potential alternative to chemical fertilizer. Though Arbuscular Mycorrhizal Fungi (AMF) are recognized as a distinctive biofertilizer yet they are difficult to mass multiply since they are biotrophs, and their growth under axenic conditions has proved to be impossible. Therefore, a newly described axenically cultivable endophytic and saprophytic fungus *Fusarium pallidorozeum*, a novel biofertilizer which has a substantial favourable impact on plant growth promotion has been discovered. There is a little information about the culture media used for the production of spores. Therefore, our current study was carried out to optimize submerged fermentation medium for *Fusarium pallidorozeum* and evaluating highest spore producing cultivation media composition by Plackett Burmann Design (PBD) and Response Surface Methodology (RSM). The PBD selected 6 significant medium composition out of the 10 screened. Among which three significant factors Peptone (P value of < 0.0001 and F = 81.14), K₂HPO₄ (P value of < 0.0003 and F value = 28.42) and trace metals (P value of < 0.0001 and F value of 39.39) were chosen for RSM to produce higher spore production. Therefore, an optimized cultivation media 0.0592 (g/L) K₂HPO₄, 0.0531 (g/L) MgSO₄, 0.113 (g/L) CaCl₂, 25.6 (g/L) Sucrose, 0.03 (g/L) Ferric Ammonium citrate, 0.002 (g/L) EDTA, 0.063 (g/L) NaCl, 2 (ml/L) Trace metals, 0.434 (g/L) Na₂HPO₄, and 6 (g/L) Peptone was obtained through PBD and RSM.

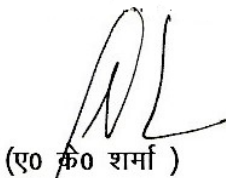

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शोध शीर्षक	: “फ्युसेरियम पैलीडोरोसिअम, एक नवीनतम जैव उर्वरक के लिए मीडिया अनुकूलन”		
पृष्ठ संख्या	: 39	सलाहकार	: डॉ. ए.के. शर्मा

सारांश

भोजन मनुष्य के लिए मुख्य स्रोत है और राष्ट्र का विकास जनसंख्या के लिए उपलब्ध भोजन या पोषक तत्वों की मात्रा के सीधे आनुपातिक है। बढ़ती जनसंख्या और सीमित कृषि भूमि के लिए मानव आवश्यकताओं को पूरा करने के लिए उर्वरकों के प्रयोग की आवश्यकता होती है। फसल उत्पादकता और खाद्य सुरक्षा बढ़ाने में इसकी विशाल क्षमता के कारण, जैव उर्वरक के रूप में सूक्ष्मजीवों के अनुप्रयोग को रासायनिक उर्वरक के संभावित विकल्प के रूप में देखा जाता है। यद्यपि अर्बुस्कुलर माइकोरिजल कवक (एएमएफ) को एक विशिष्ट जैव उर्वरक के रूप में पहचाना जाता है, फिर भी उनका प्रचुर मात्रा में उत्पादन करना मुश्किल है क्योंकि वे बायोट्रॉफ़ हैं, और एकसैनिक परिस्थितियों में उनकी वृद्धि असंभव साबित हुई है। इसलिए एक नव वर्णित एकसैनिक रूप से खेती योग्य एंडोफाइटिक और सैप्रोफाइटिक कवक फ्युसेरियम पैलीडोरोसिअम, एक जैव उर्वरक, जिसका पौधों के विकास को बढ़ावा देने पर पर्याप्त अनुकूल प्रभाव पड़ता है, की खोज की गई है। बीजाणुओं के उत्पादन के लिए प्रयुक्त कल्चर मीडिया के बारे में बहुत कम जानकारी है। इसलिए हमारा वर्तमान अध्ययन फ्युसेरियम पैलीडोरोसिअम के लिए जलमग्न किण्वन माध्यम को अनुकूलित करने और प्लैकेट बर्मन डिजाइन (पीबीडी) और रिस्पॉस सरफेस मेथडोलॉजी (आरएसएम) द्वारा उच्चतम बीजाणु उत्पादक खेती मीडिया संरचना का मूल्यांकन करने के लिए किया गया था। पीबीडी ने 10 स्क्रीनिंग में से 6 महत्वपूर्ण मध्यम संरचना का चयन किया। जिनमें से तीन महत्वपूर्ण कारक पेप्टोन (<0.0001 का P मान और F = 81.14), K₂HPO₄ (<0.0003 का P मान और F मान = 28.42) और ट्रेस धातु (<0.0001 का P मान और 39.39 का F मान) को अधिक बीजाणु उत्पादन करने हेतु RSM के लिए चुना गया था। इसलिए, एक अनुकूलित खेती मीडिया 0.0592 (g/L) K₂HPO₄, 0.0531 (g/L) MgSO₄, 0.113 (g/L) CaCl₂, 25.6 (g/L) सुक्रोज, 0.03 (g/L) फेरिक अमोनियम सिट्रेट, 0.002 (g/L) ईडीटीए, 0.063 (g/L) NaCl, 2 (g/L) ट्रेस धातुएं, 0.434 (g/L) Na₂HPO₄, और 6 (g/L) पेप्टोन पीबीडी और आरएसएम के माध्यम से प्राप्त किया गया था।


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