Addis Ababa University

Addis Ababa Institute of Technology

School of Chemical and Bioengineering

Biochemical Engineering Stream

Optimization of Bio-paint production using Aspergillus niger under liquid state fermentation

By

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Oct, 2022

Addis Ababa, Ethiopia

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A thesis is submitted to the school of chemical and biochemical engineering of Addis Ababa University in partial fulfillment of the requirements of the degree of Master of Science in biochemical engineering.

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Thesis Advisor:

Dr. Solomon Kiros

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DECLARATION

I hereby declare that the thesis I am submitting for the M.Sc. degree at the University of Addis Ababa, titled " Optimization of Bio-paint production using Aspergillus niger under liquid state fermentation," is my original work and has not been previously submitted for a degree at this or any other university. All sources of materials used for this thesis have also been properly acknowledged.

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ABSTRACT

Synthetic dyes are hazardous to living organisms and the environment in general. This has progressively led to an increased demand for natural colorants. The fungus kingdom has been extensively researched as a source of bioactive chemicals with many industrial applications. However, in Ethiopia, such studies are lacking. In this work, the synthesis of natural pigments by Aspergillus niger spp. in a submerged fermentation system was investigated. The species were obtained from the biodiversity institute and grown on PDA agar for 5-7 days at a temperature of 30 °C. Even if many factors influence pigment production from microbes, in this study, only pH (3–8), maltose concentration (0.024–0.072 g), and fermentation time (7–28 days) were examined. For pigment production, cultivation of Aspergillus niger spp. was carried out in potato dextrose broth supplemented with a carbon source (maltose) for a day and pH adjusted in shaking conditions (centrifugal force of 150 rpm). Natural paint ingredients were used with fungal dyes to produce bio-paint. To establish optimal cultural conditions, Design Expert version 13 was used. To determine the optimal setting, a Box-Behnken design under response surface methodology was used. A second-order polynomial equation was fitted to the data using multiple regression analysis. A UV-Vis absorbance measurement was employed to quantify the pigment concentrations. Thin layer chromatography and FT-IR spectroscopy were employed to molecularly characterize the pigment. In this study, the maximum pigment concentration of 357.425 a.u. was obtained for the optimized values of pH, maltose concentration, and fermentation time of 7.678, 0.045 g, and 27.953 days, respectively. The bio-paint was found to have values of pH, density, viscosity and drying time of 6.8 $\pm$ 0.57, 1.3272 $\pm$ 0.04mg/ml, 55 mPas $\pm$ 1.34 and 30 min $\pm$ 1.63 min, respectively, and demonstrated comparable properties with synthetic paints. When applied to a wall, the bio-paint showed remarkable color and coating performance. Fungal pigments could, therefore, be utilized in expanding bio-colored industries in Ethiopia.

Key words: Pigment; Fungi; Aspergillus niger; Submerged fermentation; Paint; RSM; Optimization

ACKNOWLEDGEMENTS

I want to start by expressing my gratitude to the Almighty God for giving me the courage and strength necessary to complete this study. Next, I would like to thank my advisor, Dr. Solomon Kiros, for his continuous follow-up, advice, and constructive comments. I also want to thank Dr. Mesfin Redi, Dr. Mulat Abegaz, Dr. Shimelse Kebede, Ms. Hana Asrat and Mr. Hagos Tikue from the bottom of my heart for their insightful advice and kind support.

The Bahir Dar Institute of Technology deserves a special mention for providing the funding that made this research project possible.

I must thank the Ethiopian Biodiversity Institute for providing me with the microorganisms I needed for my experiment.

In order to finish my thesis, I truly want to thank all of my friends for their support and individual contributions. In every way, they've been really helpful. Not least of all, it is a huge honor for my dear relatives who worked hard to give me the chance to pursue an education from the start and moral support throughout my studies.

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ACRONYM

BBD	Box-Behnken Design
C/N	Carbon to Nitrogen ratio
EBI	Ethiopian Biodiversity Institute
FT-IR	Fourier transform- infrared ray
GC-MS	Gas chromatography
GRAS	Generally recognized as safe
LC-MS	Liquid chromatography-Mass spectrometer
MSG	Monosodium glutamate
NMR	Nuclear magnetic resonance
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RSM	Response surface methodology
SCBE	School of Chemical and Biochemical Engineering
SmF	Submerged fermentation
SOP	Second-order polynomial
SSF	Solid state fermentation
TLC	Thin layer chromatography
VOC	Volatile organic compounds

1. Introduction

1.1 Background

Pigments are coloring agents, and many archeologists have evidence that early humans utilized paint for esthetic purposes. This practice has existed since the Paleolithic era (Prabhu et al., 2017). All over the world pigments are used for coloring food, dyeing cloth, painting the furniture in our homes, houses, cars, road markings, and underground storage (Prabhu et al., 2017; Sastry, 2016). Pigments are classified as natural and synthetic pigments. Among the natural colorants, plant materials were used since ancient times. Whereas, the synthetic colorant gained importance and priority in the market due to presence of established production technology, relatively low-cost production, low level of unwanted flavors imparted to food, superior coloring properties, and use of small amounts for coloring. During the production and utilization of many synthetic pigments, they had never been tested for their toxicity or other adverse effects on health and the environment (Prabhu et al., 2017).

However, in recent decades, because of the development of scientific, technological, and analytical capacity, the detrimental effect of synthetic colors on human health and the environment has been identified, including properties such as poor degradation and longer persistence in the environment, and the potential to cause cancers/allergies, etc. (Akilandeswari & Pradeep, 2017; Lagashetti et al., 2019a). Such challenges have created for increased demand for natural, organic, and eco-friendly pigments to be used as colorants, color intensifiers, additives, antioxidants, etc., for many industries including textile, pharmaceutical, cosmetic, painting, food, and beverage industries (Kechi et al., 2013; Lagashetti et al., 2019a).

In addition, currently, waste from the paint industry mainly contains toxic heavy metals due to widespread usage of synthetic pigments which are potentially hazardous to human health and the environment. To mitigate the adverse effects of synthetic pigments, several decontamination methods were applied that are not only financially very expensive but also mostly result in incomplete metal removal. Further, they require high energy and a large volume of reagents and produce other secondary waste (Malakootian & Hossaini, 2008).

Natural pigments are widely extracted from plants, animals, and microorganisms, and are often recognized as bio-colors due to their biological origin and easy degradation. Pigments from those natural resources are considered and believed to be safe since they are non-toxic, eco-friendly, and biodegradable. Natural sources are, therefore, potentially better alternative sources as compared to synthetic pigments. Among the organic pigment sources, microbial sources are also potentially good alternatives compared to plant, animal, and synthetic pigments (Akilandeswari & Pradeep, 2017).

The usage of plants and animals for natural pigment production has many disadvantages such as the non-stability against light and heat, adversely affected by pH and low solubility of pigments, coupled with dependency on the season, and the loss of certain species for large-scale production (Noor et al., 2018; Prabhu et al., 2017). This in turn leads to deforestation and loss of biodiversity. Whereas, microbial pigment production is considered more advantageous due to their higher growth rate in short period of time, the fact that they are unaffected by seasonal changes, and the high stability of produced pigments, ease of handling, biodegradability, and higher compatibility with the environment (Nigam & Luke, 2016; Noor et al., 2018; Panesar et al., 2014). Furthermore, they are available for the most of the year (Aftab et al., 2021).

A literature survey showed that among the microorganisms, fungi are mostly preferred for industrial-scale production of natural pigments because bacteria have pathogenic nature as well as associated toxicity (Lagashetti et al., 2019a). Currently, fungi have emerged among the prominent, eco-friendly sources of natural pigments. Easy processing, fast growth in cheap media, and weather-independent growth make them an excellent alternative to all natural sources (Rajendran & Gunasekaran, 2015a).

Various fungi species are known to produce a variety of pigments as secondary metabolites such as carotenoids, melanins, azaphilones, flavins, phenazines, quinones, monascin, violacein, indigo, etc. with different chemical classes (Lagashetti et al., 2019a). Among the fungi species, the genus Aspergillus, such as Aspergillus niger is recommended due to its ability to synthesize many pigments such as Flavioline (orange-red), N-naptho–γ–pyrones (yellow), aspergillin (black), azanigerones A–F, asperenone (yellow), melanin (dark brown-black) which plays a role in stable bio-coloring production (Lagashetti et al., 2019a).

However, the production of pigments, its yield or color shade, from different fungi species is severely affected by various parameters such as growth media constituents (carbon source, nitrogen source, an amino acid source, salts, etc.), temperature, pH, time and light, etc. (Vinithkumar et al., 2019). Therefore, the present study was undertaking an optimization process to increase the pigment yield and color shade for cost-effective production and eco-friendly pigments. In addition, most studies done on pigment production from fungi focused on one factor at a time approaches. However, one-factor-at-a-time has limitations in doing many numbers of experiments, which is costly, time-consuming, less effective, and does not show the interaction among variables. Thus, the present study has implemented response surface modeling (RSM) which is a powerful statistical tool and technique that is efficiently used in studying multiple independent variables and their interactive effects, used for the optimization of several biochemical and biotechnological processes (Kaur et al., 2019).

Though there are several publications on natural pigments from fungi and bacteria, to our best knowledge, no work has been done on the optimization of pigment production from Aspergillus niger and paint production in worldwide. Although Ethiopia is rich in biodiversity which can have economic, social and environmental importance, due to lack of studies, it is under-utilized. Consequently, Ethiopia is forced to import various color ingredients from abroad. Therefore, replacing the synthetic pigment through natural pigments with bio-paint production greatly reduces the cost required for waste treatment and improves human health and the environment. Park et al. (2020) and Sastry (2016) recommended that the possible utilization of microbial sources for potential pigment production in large scale for bio-paint purposes.

Therefore, the present thesis work was aimed to produce natural pigment from Aspergillus niger species under submerged fermentation and optimize the conditions for natural pigment production as well as evaluate the characteristics of natural colorants used in bio-paint. The current study is not intended to cover the steps involved in making paint and the chemical structure (NMR and HPLC analysis) of the pigment.

1.2 Statement of the problem

Recently, the demand and utilization of colors have dramatically increased worldwide. Nowadays, synthetic pigments are significantly applicable in many industries like paint, textile, and food industries. However, a country like Ethiopia has imported several synthetic pigments for various pigment using industries. The paint recipes currently in use produce volatile organic compounds (VOC) during drying after coating, and heavy metal wastes dispose to the environment during production which is hazardous and toxic to humans, other living things, and the environment and it lasts for a long time in the ecosystem and it needs expensive treatment.

For these reasons the use and demand for bio-colorants among customers become grown in developed countries, due to the nature of the pigments which are environmentally friendly and nontoxic and low cost production. Despite the presence of demand for bio-colorant, still there are gaps in scientific information on the optimized production of microbial pigment to obtain high yield and stable. In addition, scientific information on the quality of the pigment produced and stability of the bio-color in paint was not adequate. Thus, to utilize the microbial potential for bio-color production on large scale, require a clear understanding of the growth media, and growth conditions of microorganisms, which are critical point to gain and optimize maximum yield for large-scale production.

Currently, many researchers are exploring microbes for the production of microbial pigments for food supplements and textile dyes while limited studying has been done on paint application. Besides, performance of liquid fermentation for the production of pigment from fungi species, Aspergillus niger, and evaluation of the molecular structure and quality of the fungi pigment for application of paints using old paint recipes to produce alternative paint was not documented.

Therefore, the present research study was mainly focused to produce pigments from the cheapest, available, and environmentally friendly resource microbes by optimizing the different factors that affect pigment production (pH, time, and media constituent) by performing different experimental runs by using design expert, which could help to monitor the production yield. In addition, UV/Vis spectrophotometer, TLC and FT-IR were applied for studying the molecular structure of fungal pigment.

1.3 Objectives

1.3.1 General objective

- The general objective of this study was to optimize Bio-paint production and characterization of the products using Aspergillus niger under liquid state fermentation

1.3.2 Specific objective

The specific objectives of this study are;

- ✓ To assess the growth condition of Aspergillus niger
- ✓ To investigate and optimize the selected parameters for the pigment production
- ✓ To characterize the physicochemical characteristics of the produced pigment
- ✓ To evaluate and validate bio-paint production from casein, paint recipes, and pigment

1.4 Research Questions

The research questions raised which were used to answer the listed objectives were:

- ✓ Does the species able to produce a pigment?
- ✓ Does the selected fermentation condition (pH, fermentation time, and maltose concentration) have an effect on pigment concentration and which factor is significant?
- ✓ Does the used statistical method used to optimize the pigment yield?
- ✓ What are the functional groups exist in the pigment compound?
- ✓ Which type of solvents used to purify a pigment under TLC analysis?
- ✓ Does the produced pigment can apply on coating application?

1.5 Scope of the study

This thesis project consisted of studying the potential of Aspergillus niger found in Ethiopia for bio-pigment production. Optimization of physical (pH and time) and chemical (maltose concentration) parameters that greatly affect the pigment yield were performed. Application of the pigment on coating application was evaluated. The pigment produced under optimized conditions was characterized by thin layer chromatography, and UV/Vis and FT-IR spectroscopic techniques.

1.6 Significance of the study

The outcome of this work would significantly contribute in production of pigments alternatives for colorant employing industries from natural sources that have the benefit of being safe to human health environmental protection. In particular, this research study is will contribute an alternative paint production technology, which will contribute to the development of science and technology in general and to development of paint industries in particular. Further it is an important research in initiating other researchers who want to do similar topic in the future.

2. Literature review

2.1 History of Pigments

Everything around us is colorful, it can be said that life on earth depends on pigments. The food we eat, the cloth we wear, the furniture in our homes, the color of the house, etc. have color effects. The use of pigments as coloring agents practiced since ancient times. The use of pigments in prehistoric times was further proven at the time of pigments and grinding equipment was found in a cave at Twin Rivers, near Lusaka, Zambia in the year between 350,000 and 400,000 years old. Different parts of the world use pigments for different purposes. In Europe, it was practiced during the Bronze Age. China was using plants, barks, and insects for dyeing. During the Indus Valley period (2500 BC), pigments occurred in India. Henna was used before 2500 BC and mummies were also used by Egyptians. The coloring of food emerged in Egypt for coloring of their candy and coloring soybean and the adzuki-bean cake was seen in Japan (Prabhu et al., 2017).

Pigments can absorb visible light in the visible range (400-700 nm) and modify the color of reflected or transmitted light. The word pigment comes from Latin and was first used to describe a color, but its meaning was eventually expanded to include colored compounds (Tirumale & Wani, 2018). The uses of colors are for coloring houses, cars, roads, storage vessels, clothes, and food as a food additive, protecting the surface, and aesthetic and adding value. These coloring agents are called pigments.

In ancient times there was the use of natural colorants as discussed above from plants and animals. The first synthetic color, mauvine, was developed by Sir William Henry Perkin in 1856 and this development started a revolution in the history of synthetic colorants. Since then, the synthetic color industrial revolution has rapidly proceeded and the use of natural pigments become declining (Prabhu et al., 2017). Despite its hazardous effect on health and the environment synthetic pigments that took place of natural pigments in the middle of the 19^{th} century still lead the market. But through time the awareness of human safety and environmental conservation creates enthusiasm for natural sources of pigment (Noor et al., 2018). Synthetic pigments pose a serious influence on earth such as poor degradation, longer persistence, and cable to cause cancers and allergies in humans. These causes inspire the demand for natural, organic, and eco-friendly

pigments (Lagashetti et al., 2019a). Although the synthesis of these tiny molecular weight chemicals is not essential for regular microbe growth, they may provide the organism with various advantages (Pradeep & Pradeep, 2013).

Pigments are used extensively in industrial and biotechnological applications. Pigments and dyes are used for a variety of industrial products such as food and beverage, textile, leather, cosmetics, pharmaceuticals, paper, dye-sensitized solar cells, etc. for aesthetic and to increase the taste, quality, and durability. But they pose a serious impact on the health of well-being and the environment. They are health hazards due to their carcinogenic activity carmoisine or mutagenic activity, allergy, and hyperactivity associated with synthetic pigments. Therefore the legislature stopped it from using. Moreover, the effluents from various colored industries are accumulated and contaminate the environment. Different strategies were taken to solve even if they are expensive and inefficient (Padhan et al., 2021a). Not only these but also synthetic pigments cause behavioral problems in children (Gupta et al., 2011).

Today's special issues are the pollution of soil and water bodies due to pigmented industries such as the textile industry, etc., which utilizes a lot of dyestuff and effluents from the production process. They are also mutagenic, carcinogenic, allergenic, and cytotoxic, posing threat to all living things (Chadni et al., 2017a).

Currently, nanostructures for waste treatment are preferable due to the cost-effective and eco-friendly way of treating the waste by advanced oxidation processes such as photo-catalysis, sonolysis, and Fenton reactions. Several nanoparticles, composites, Nano-crystals, and sorbents have been used for the decontamination of wastes in colored industries. But they require large infrastructure, huge investment, skilled labor, and Nano-toxicity. For these reasons, the best possible alternative way to preserve health and the environment is to turn the face towards natural pigments (Padhan et al., 2021b).

2.2 Natural pigment

Natural pigments are natural colorants mostly extracted from plants and animals like annato, grapes, beet, paparika, female insects (Coccus cacti), and microorganisms like Monascus, Rhodotorula, Bacillus, Achromobacter, Yarrowia, Phaffia, etc... As discussed previously addition of color to processed foods is an old practice but microbial pigment production is a recent phenomenon (Joshi & Bhushan, 2014). Natural dyes, in addition to their non-toxic, non-polluting, and less hazardous properties, have anti-fungal, anti-oxidant, and anti-microbial properties, making them superior to artificial dyes (Sharma et al., 2012).

Even though natural pigments are produced from various sources, microbial sources take the first destination due to their natural character and safety to use, medicinal properties; nutrients like vitamins, production is independent of season and geographical conditions, and controllable and predictable yield (Joshi & Bhushan, 2014). Of course, colors come from plants. However, due to their low productivity, low eco-efficiency, seasonal supply, the disadvantage of variability, and loss of plant species have turned their attention to microbes (Gupta et al., 2011). The capability of fast-growing, the potential of standardized commercial microbes makes them more suitable for dyeing than plants and animals (Sharma et al., 2012).

Plant extract pigments are limited by seasonal changes, resulting in low output and increased costs, but bio-pigment production from microbes yielded a greater yield due to the ease with which process parameters could be controlled (Sehrawat, 2017).

A microbial pigment β-carotene which shifts its color towards orange-red gains a more attractive and unique property in microbial pigment but is absent in plant-derived pigments. In addition to this some microbial pigment is produce from industrial and agricultural residue which helps to reduce environmental and water pollution (Joshi & Bhushan, 2014). The application of natural pigments in foods, cosmetics, and pharmaceuticals become growing recently (Rajendran & Gunasekaran, 2015b). Some filamentous fungus can create pigments, which are gaining popularity as a promising fermentation-derived natural pigment alternative (K & Prabha, 2018).

The generation of secondary metabolites normally begins late in the microbe's development cycle, often when it enters the stationary or resting phase. Even if some fungal strains sporulate, they are deficient in producing secondary metabolites (Calvo et al., 2002).

It is crucial to distinguish various pigment extraction methods and choose among them because of the effect of extraction solvents and the condition on pigment quality, final composition, and efficiency of the process (Pailliè-jiménez et al., 2020). There are various intracellular pigment extraction methods are involves these are organic solvents, solid-phase extraction, homogenization, freeze-thaw method, ultra-sonication, inorganic acids, soxhlet method, and others. Among those using organic solvent for the extraction of pigment is the easiest and most cost-effective method (Padhan et al., 2021b). A report showed effect of using different solvents for the extraction of pigment from Bacillus subtilis PD5. Red and yellow pigments were extracted by using ethyl acetate and methanol and hexane organic solvents respectively (Trivedi et al., 2017).

2.2.1 Pigment-producing microorganisms

A vast number of microorganisms are used for the production of pigments for various applications such as bacteria, molds, yeasts, and algae. A suitable species for the production of pigments must satisfy the following criteria: Capability to use a wide range of carbon and nitrogen sources; Should have a tolerance to pH, temperature, and mineral concentration and possess moderate growth conditions; Reasonable color yield; Should be non-toxic and non-pathogenic; and Must be easily separable from the cell mass (Joshi & Bhushan, 2014). Pigment-producing microbes can produce different (vast) colored pigments and can be easily cultured to give a high yield. Pigments that are produced by microorganisms are; carotenoids, melanins, flavins, quinones, and more specifically monascins, violacein, phycocyanin, or indigo (Rajendran & Gunasekaran, 2015b).

Microorganisms create a wide range of colors through fermentation, with better pigment yields and less waste than plants and animals (Tirumale & Wani, 2018).

Microorganisms producing pigments as secondary metabolites can ever be found in different environmental sources (Kaur et al., 2019). It estimates that the use of microbial pigment in health, pharmaceutical, cosmetics, food, and feed is expected to rapidly increase by $2.0 billion by 2022 (Pailliè-jiménez et al., 2020). Among microbe fungi and microalgae are potent water-soluble bio

pigment producers but due to the lowest harvest algal culture and lowest harvest basidiomycetous fungi culture, filamentous fungi are preferable and feasible for large-scale production to exploit them for bio pigment production (Kalra et al., 2020). The high cost and limited productivity of microbial pigments produced by yeast, bacteria, and microalgae, however, renders them insignificant. Because of the low cost of fermentation and simple downstream processing that make ascomycetes and basidiomycetes industrially relevant, they are the focus of this investigation (Aftab et al., 2021).

It is self-evident that microbes producing secondary metabolites are evolved over hundreds of millions of years because microbes employed them as chemical signals for communication, habitat defense, and to prevent the growth of competitors (Chadni et al., 2017a).

Microbial pigments are synthesized by microbes under certain optimum conditions for a variety of industrial purposes. Even though the production of natural food colorants increases dramatically, microbial pigments gain the first hierarchy among other natural colorant-producing sources due to the cheaper production cost of pigments from microbes. The new existing fields of research need to explore more microbial pigment production from microbes (Trivedi et al., 2017). Microbial pigments can be applied to textiles without frustrating their toxic issue but for food colorants, safety criteria must not be ignored (Venil et al., 2021).

The various advantages of microbial pigments were reported by Sharma et al. (2012). It was concluded as the potential of Trichoderma sp. for textile dyeing, it has been founded to be antifungal property in addition to non-toxic to human skin. Moreover, for maximization of pigmentation for commercially mass scale production, it is important to optimize the fermentation in an inexpensive and environmentally friendly manner with numerous species found abundantly in soil, air, and other habitats (Sharma et al., 2012).

A microbe fungi can create a wide range of secondary metabolites, which are normally depending on the stage of development as well as environmental conditions such as nutrient concentrations, light, and temperature (Pradeep & Pradeep, 2013). Microbes will certainly be used more widely in the future for basic and practical research on pigments (Abdullatif et al., 2021).

2.2.1.1 Bacteria pigments

Bacteria are known to produce various pigments for industrial purposes. They are found everywhere in many ecological niches. Among different bacterial groups, actinobacteria are more likely to produce pigments than other groups (Prabhu et al., 2017). Most carotenoids are synthesized from bacteria which is a good source of pigments. Streptomyces chrestomyceticus subsp. rubescens has been employed to produce lycopene while zeaxanthin and lutein production from Flavobacterium sp. is gaining importance (Joshi & Bhushan, 2014).

A bacteria Bacillus subtilis produces a pigment on sporulation. The liquid medium for optimum pigment formation contained: Spizizen's salt, glucose 27.7; L-tryptophan 0.25; L-tyrosine 0.25 L-histidine 0.055; and MnS04 0.67 Mm. All the nutrients inoculate with the microorganism after sterilization in the autoclave (Joshi & Bhushan, 2014).

2.2.1.2 Fungi pigments

Fungi are found in many sources like; soil, sediment, waste, food waste, and spoiled food. Most soil fungi are saprobes; they decompose organic matter and contribute to nutrient cycling. They are producing secondary metabolites in their growth phase. Essential several bioactive chemical compounds from fungi are currently used for pharmaceuticals. Many fungi genetic resources are traditionally found on soils (Petit et al., 2009). Fungi, unlike bacteria and yeast, can survive at very low water activity because their hyphal mechanism of growth gives them an advantage over other microorganisms (Sehrawat, 2017).

Fungi are fascinating organisms that produce a diverse range of natural products known as secondary metabolites regularly. These natural products are used in many sectors including the medical, industrial, and agricultural sectors (Calvo et al., 2002). Amongst the natural products, colors are created by fungi with varied structures and biological activity (Tirumale & Wani, 2018). According to Sehrawat (2017), fungus-produced colorants are more stable and durable and can be employed in a variety of applications. The filamentous fungus are the most metabolically active in the synthesis of stable and commercially valuable compounds among the powerful producers of microbial pigments. Together with antibiotics, they also release extracellular enzymes and secondary metabolites such as pigments, organic acids, and food additives (Aftab et al., 2021).

Pigments from fungi microbes have a higher advantage over other microbes due to their property to be scaled up in fermenter and give high yield and also the extraction is easy either intracellular or extracellular. They are a good source of microbes in replacing synthetic colorants and natural colorants derived from plant materials (Rajendran & Gunasekaran, 2015b). Both the terrestrial and marine fungi are reported as the source of pigments and marine fungi is an excellent producer of a variety of unique pigments for therapeutic and industrial applications.

In addition number of yeasts and filamentous fungi acquired from Antarctic regions have been reported to produce a variety of colors (Lagashetti et al., 2019a). A fungus provides a wide variety of colors such as carotenoids, melanins, and polyketides, namely flavins, phenazines, quinones, monascins, violacein, and indigo. Not only used for coloring but also they have essential properties like anticancer, antioxidant, antimicrobial, anti-inflammatory, and immune-suppressor. Many industrial sectors health care, food, cosmetics, and textile industries use color. Therefore fungal pigments were applied there (Pailliè-jiménez et al., 2020). Many fungal pigments are stable at a wide pH and temperature range; these make fungal microbes interesting. Agricultural and industrial wastes are important as a source of the substrate in media forming to cut off the cost of media. Corn steep liquor instead of yeast extract as a nitrogen source and grape waste is used for pigmentation for Monascus ruber and Monascus purpureus fungal microbes (Prabhu et al., 2017).

Fouillaud reported that because of their enormous adaptability and ability to synthesize a variety of different secondary metabolites, filamentous fungi are found all over the world. Food and beverages, animal feeds, pharmaceuticals, cosmetics, textile, leather, pulp and paper industries, bio-fuel generation, and environmental bioremediation are all places where pigments and enzymes are used (Fouillaud et al., 2017).

Monascus pigments are traditionally used for coloring foods and it’s extracted from M.purpureus grown on steamed rice by solid-state fermentation (Joshi & Bhushan, 2014). Penicillium species that belong to the genus family were reported as potent producers of pigment. Such as; the first commercial red colorant is arpink red color and a yellow pigment chrysogenum is from Penicillium chrysogenum. While many species among the member of the Aspergillus genus has the potential to synthesize pigments but reported that Aspergillus niger species produce a wide variety of

pigments, such as aspergillin, asperenone, azaphilones (azanigerones A–F), and melanin (Kalra et al., 2020).

To increase the color intensity of Aspergillus sp. a carbon source dextrin and maltose produce high yield whereas factors like nitrogen sources, pH, and temperature affect the pigment production under submerged conditions (Joshi & Bhushan, 2014). A. oryzae produces an orange-red pigment belonging to the anthraquinone group. The starch medium used includes: $MgS0_4.7H_20$ solution, 1 ml (24.658 g/100 ml); $CaCl_2$ 1 ml (14.79 g/100 ml); $FeCl_3$, 0.5 ml (16.221g/100ml); $ZnS0_4$, 1 ml (28.756 g/100ml); $NH_4H_2P0_4$, 0.173; sodium citrate, 0.1176 and carbon source 100 g/l (Joshi & Bhushan, 2014).

Currently, the utilization of filamentous fungi for food colorants is being grown to promote safe and healthier food additives. They are richly found in terrestrial systems. Researchers studied yellow pigment from Aspergillus niger species at pH 4.5 for 9 days incubation and use ethanol for the extraction of pigment for food colorant acceptable and they found that the pigment synthesized from Aspergillus niger cannot cause any toxicity during their toxic analysis on mice for 28 days and no abnormal activity was observed. The sensory evaluation (color/appearance, flavor, texture, and taste) was conducted by panelists. They evaluated the sensory scores of the colored cookies (8.48 ± 0.35) and the colored lemon juice (8.08 ± 0.30) as more acceptable than the colorless cookies (7.92 ± 0.19) and lemon juice (7.55 ± 0.23). They concluded that fungal pigments are an influencer for the future of the food industry (Toma et al., 2021).

Another investigator declared that various fungal strains of Aspergillus niger have shown different properties in producing pigment under the same media constitute and methanol and acetone are the best solvents for extraction of yellow pigment from Aspergillus niger species. They found optimal pH of 2.5 to 3 for 5 days incubation and ether, dextrose, maltose, yeast extract, corn steep liquor, corn oil, and $NaNO_3$ were found that enhance the pigmentation whereas Soybean oil, lard, and a kerosene-hydro carbon mixture in lesser degree and Malt extract, urea, and distiller's soluble not beneficial. Different strains of Aspergillus niger spp. have different potentials for pigment production and also some strains are unable to produce pigments within the same environmental condition (James et al., 1961).

Therefore it can conclude that from those two studies, both of them have the same objective in exploring yellow pigment from Aspergillus niger but acquire different results so for this thesis work the pigment potential of Ethiopian founded Aspergillus niger is studied since the climate, topographical, and the biodiversity of Ethiopian vary from other worlds and also within Ethiopia from different regions, places, terrestrial or aquatic, temperate or cold zone gain the same species with different characters along with their adaptation.

The United States Food and Drug Administration (USFDA) and the united nations World Food Organization (WFO) have designated Aspergillus oryzae and Aspergillus niger as GRAS (Generally Regarded As Safe) industrial microorganisms (Fisheries et al., 2015).

2.2.1.3 Yeasts pigment

Several yeast species are good sources of pigments such as Rhodotorula, Yarrowia lipolytica, Cryptococcus sp., and Phaffia rhodozyma. A red pigment Astaxanthin is mainly found in animals but rarely found in microorganisms like P. rhodozyma. The cultural condition plays a great role in producing a variety of carotenoids from P. rhodozyma. To grow Rhodotorula it needs too complex and requires several chemicals but it is expensive so it's recommended to use the waste from the apple juice processing industry Apple pomace. For Yarrowia lipolytica lactic acid promotes the development of browning activity of the yeast. During the exponential phase, the reddish-brown color develops and changes to deep brown during the stationary phase. The exponential phase occurred when the glucose level increased to 10 g/L from 0.5g/L and disappeared in the absence of Mn. And when the glucose amount (>10 g/l) delayed browning during the stationary phase (Joshi & Bhushan, 2014).

2.2.1.4 Algal pigments

Microalgae produce many pigments such as carotenoids, chlorophylls, and phycobiliproteins (PBPs). The biological functions of microalgae are described as anti-inflammatory, anti-angiogenic, neuro- and hepatic-protective, antiviral, anti-obesity, anti-diabetic, anticancer, and anti-osteoporotic. As well as they may help to regulate cardiovascular diseases, and cognitive function, protect from UV rays, enhance immune functions, present anti-aging properties, and prevent some blood-related disorders. They have high market demand in cosmetics, nutraceuticals,

pharmaceuticals, food colorants, textile dyes, painting, and feed additives for poultry (Pailliè-jiménez et al., 2020).

2.3 Synthesis of microbial pigment

Pigments, which are produced in the fermentation process, are categorized under secondary metabolites. Secondary metabolites (SMs) are described as chemical substances that are produced along certain biosynthetic pathways but are not required for the normal development and growth of the fungus in the laboratory. The fact that they are found in many species, though, suggests that they have a competitive advantage in nature given their persistence in evolution (Avalos & Limón, 2021). Whereas primary metabolites needed for appropriate growth, development, and reproduction that are produced by a wide variety of unrelated species (Keller, 2019).

Primary metabolites are largely polymerized by specific enzymes during the formation of secondary metabolites (often referred to as backbone or core enzymes). Additional enzymes, which can significantly alter the bioactivities of metabolites, are further customizing the metabolites formed by the backbone enzyme. The secondary metabolite produced is classified chemically by the backbone enzyme (Keller, 2019).

According to traditional definitions, secondary metabolites are not necessary for the development or survival of the providing organism (Bills & Gloer, 2016). Fungi have a variety of ways to release their secondary metabolites from cells, including as volatiles, excretions into the environment, incorporation into the cell's structural components, and cell-bound secondary metabolites (Bills & Gloer, 2016).

Chemically speaking, secondary metabolites are at one end of an array of metabolites and are defined as substances produced by pathways that use primary metabolites as building blocks to assemble more complex molecules, such as polyketides, terpenoids, non-ribosomal peptides, nuclear-encoded ribosomal peptides, and molecules of mixed biogenic origin produced by hybrid pathways. It's possible that secondary metabolite biosynthesis is closely controlled. Their expression is frequently induced by chemical or environmental triggers and linked to the morphogenesis and development of the providing organism (Bills & Gloer, 2016).

The four chemical families of polyketides (PKs), terpenoids, non-ribosomal peptides (NRPs), and hybrid non-ribosomal peptide/polyketides (NRP/PKs) make up the majority of the most well-known SMs. Acetyl-CoA stands out among the substrates from primary metabolism used in the synthesis of SMs since it is the precursor of polyketides and terpenoids. A certain kind of enzyme initiates each SM biosynthesis route, and it is finished by the action of a particular tailoring enzyme, adding additional modifications to the molecules (Avalos & Limón, 2021).

Growing strains under various nutrition and aeration conditions has been shown to significantly alter metabolite compositions and titers throughout the long history of fermentation technology. Secondary metabolite formation and the formation of biological molecules that can replicate themselves are likely related processes. One can easily envisage how basic amino acids interacted with the earliest macromolecular processes to generate new compounds; when these simple secondary metabolites had positive effects on macromolecular processes and ultimately on cell reproduction, they were preserved as a new cellular process (Bills & Gloer, 2016).

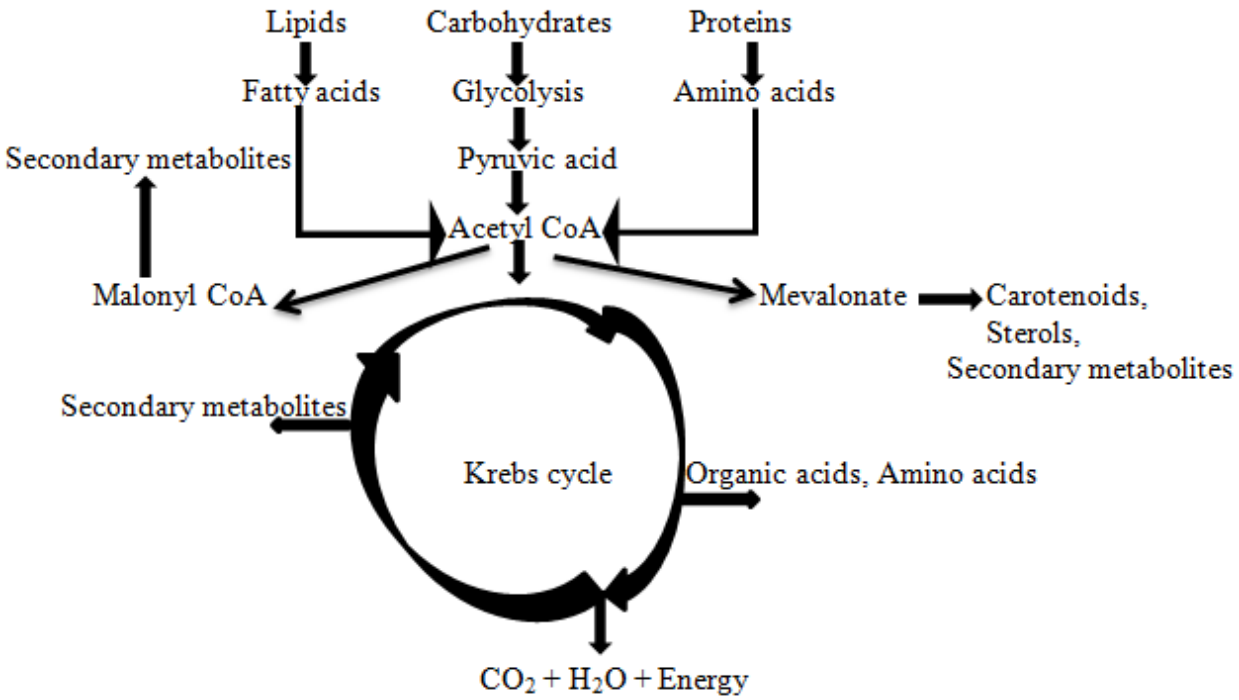


Figure 2. 1: The metabolic pathway of fungi shows how secondary metabolites produced (Moore et al., 2011)

2.4 Factors affecting Microbial pigment production

2.4.1 Temperature

The incubation temperature of microbes during fermentation affects pigment production. The growth and production of pigments from Monascus sp. and pseudomonas require 25-28^{0}C and 35-36^{0}C respectively (Joshi & Bhushan, 2014).

2.4.2 pH

The pH of the medium in which microbes grow affects the growth and type of pigment. Different microorganisms have varying responses to the pH and a slight change in pH may change the shade of the color. Optimum pH for Monascus sp. and Rhodotorula is 5.5-6.5 and 4.0-4.5 respectively and neutral to slightly alkaline pH favors lycopene formation whereas acidic pH favors β carotene synthesis (Joshi & Bhushan, 2014). Different studies have shown that the optimum pH for pigment production varies with the fungal species and different strains in submerged fermentation (Lagashetti et al., 2019a).

Different studies reported that pigment production at acidic pH favors fungi microbes; it blocks the production of conidia and facilitates pigment production by enhancing the transport of certain media constituents and activating enzymes in the biosynthesis of pigments. Therefore the effect of pH on transporting substrates and influencing the activity of enzymes can be prevented by knowing their favoring condition at which pigment production increases (Pandey et al., 2018). Additionally, Mangrove Penicillium DLR-7 showed that it could produce a yellow pigment at pH 2, with a maximum absorption wavelength of 420 nm. Even yet, Penicillium didn't grow much at this pH since there were no spores. Low pH may change the transfer of nutrients and ions between the media and the microbial cell by affecting the solubility of salts and the ionic state of nutrients, as low pH limits conidia formation and promotes pigment production. Moreover, some Penicillium species growth and metabolite formation facilitated when the pH keeps at neutral and alkaline pH (7, 8, and 9) (Aftab et al., 2021).

The optimal pH for pigment production from fungi was pH of more acidic under submerged fermentation, which is reported by Cho et al. (2002). The pH of the fermentation broth slightly

decline from pH 6 to pH 4 in the exponential phase and recovered to pH 6. To enhance the pigment production from fungi, a fed-batch mode of operating system is suitable to overcome sugar concentration depletion (Cho et al., 2002).

Different reports from the literature indicated that more acidic condition is preferred to produce promising fungal secondary metabolites products and many strains of Aspergillus niger could not provide yellow pigment at high pH (James et al., 1961)

2.4.3 Media constituents

For the synthesis of bio-pigment, either submerged or solid-state fermentation is used, and yield is significantly affected by growth media, agitation, and pH (Sehrawat, 2017). The mycelial growth of pigment-producing microorganisms is affected by the source of carbon like glucose, fructose, maltose, lactose; galactose, etc. glucose and its oligosaccharides are better sources of carbon for growth and pigment production. Starch and dextrin is the best source of carbon for Monascus sp. whereas glucose and maltose are moderate and poor on fructose. In M. purpureus, fermentation with maltose and glucose as carbon sources gave very dark liver pigment, whereas sucrose produced a light and uneven red pigment. Cellobiose is the best source of carbon for pigment production from Phaffia rhodozyma. While D-mannitol is used for pigmentation, glucose supports both growth and pigmentation. A carbon source also affects the shade of the pigment (Joshi & Bhushan, 2014). Maximum pigment production was achieved when the carbon source is fructose and lactose for M. purpureus and M. ruber respectively (Lagashetti et al., 2019a).

As a source of energy for microorganisms and a key factor in both growth and the generation of primary and secondary metabolites, carbon is by far the most crucial component of the medium. The pace of carbon source metabolization frequently affects the generation of primary or secondary metabolites as well as biomass production (Singh et al., 2017). As Pandey et al. (2018) studied the effect of supplementing a 2% carbon source on the production of orange pigment from Penicillium sp. (GBPI_P155), he got maltose increased pigmentation.

Menezes et al. (2020) reported that the optimal glucose concentration for pigment synthesis was 18g/L. Above this quantity of glucose, less productivity was seen, possibly because the

predominant metabolic pathway was the fermentative one, which produces more ethanol and inhibits the enzymes that breakdown the respiratory chain (Crabtree Effect).

Mukherjee & Singh (2011) discovered that the optimal concentration of glucose for the formation of red pigment was 18 g/L. above this concentration, there was a decrease in pigment production that may have been caused by respire-fermentative metabolism. this result was at odds with the positive correlation between pigment and carbon.

Thus it can be generalized as various glucose concentrations frequently have an impact on the pigment production rate and the synthesis of secondary metabolites is inhibited by an excessive glucose concentration. Additionally, it may make the growth slightly acidic, which is not good for pigment formation. Maximum growth and pigment generation were reported and advised to be kept under 20 g/L of glucose concentration for M. purpureus's. Growth rates, pigment synthesis, and significant ethanol production were increased by high glucose concentration (50g/L), possibly as a result of high glucose levels' induction of respiro-fermentative metabolism (Crabtree effect) in M. purpureus's aerobic SmF (Manan, 2017). Many fungus, Yeasts, and Ascomycetes produce ethanol under aerobic condition and maximum amount of sugar (Chen & Johns, 1994).

In liquid cultures, maltose, soluble starch, ethanol, and glucose have been suggested as being superior to alternative carbon sources for pigment production (Chen & Johns, 1994). Chen & Johns (1994) reported that, a carbon concentration in submerged culture must be regulated whether the substrate is glucose or maltose.

Bio-pigment synthesis decreased at high substrate concentrations, according to Sehrawat (2017), due to a decrease in oxygen availability, which could cause shear stress. Menezes et al. (2020) reported, when glucose levels are too high, the generation of secondary metabolites is inhibited thus preventing pigment production.

The effect of the nature of carbon source on production of antibiotics were investigated; and it was found that lactose was comparatively most suitable source of carbon for production of Penicillium whereas glucose was found to suppress the synthesis of Penicillium (Singh et al., 2017). Manan (2017) also reported that glucose and its oligo- and polysaccharides are superior to other carbon sources for both growth and pigment production. The formation of pigment is favored by

disaccharides and polysaccharides, whereas growth is favored by monosaccharaides in case of Penicillium species (Aftab et al., 2021).

Depending upon the type of microorganisms, pigment production is influenced by different nitrogen sources such as ammonium, peptone, sodium nitrate, glutamic acid, monosodium glutamate, 6-furturylaminopurine, and tryptophan (Lagashetti et al., 2019a). Ammonium chloride and peptone are the best for the production of Monascus pigment. In M. purpureus, 1.5% MSG medium produced an appealing red color, whereas other nitrogen sources produced faint or foggy red pigment. Ammonium phosphate proved for the growth of an organism Aspergillus sp. for the production of pigment. By incorporating tyrosine and histidine nitrogen sources in the medium the pigment production in Bacillus subtilis enhanced (Joshi & Bhushan, 2014).

The ratios of carbon to nitrogen (C/N) have been reported to be an important factor for pigment production in the culture medium. The high C/N ratio increased the synthesis of pigments in many fungi by limiting the source of nitrogen for protein synthesis or growth. It is reported that the ratio of C/N is 9:1 for N. crassa to increase β-carotene. Surface active agents known as surfactants play a role in transporting intracellular pigments to extracellular micelles, to prevent pigment degradation, lower the intracellular pigment concentration, and product inhibition. However, their effects are not fully understood due to their not consistency (Gmoser et al., 2017a).

Minerals also play an important role in pigmentation. Zn stopped the growth medium in liquid fermentation whereas vigorous growth and pigmentation were reported in solid-state fermentation. Zn sometimes acts as an inhibitor and stimulates the uptake of glucose in the medium for the synthesis of pigment. Mn increases the synthesis of pigment from Lactobacillus Plantarum and Streptococcus lactis. A salt NaCl has been reported as an enhancer for pigment production and inhibits citrinin production in M. purpureus (Joshi & Bhushan, 2014).

Since they are heterotrophic creatures, fungi need an external supply of nutrients. As a result, the media's composition affects growth and metabolite production. The production of metabolites also depends on metabolism; therefore simply using any nutrient is insufficient. As a result, it's crucial to choose the right nutrients by understanding the microorganism's nutritional needs as well as the metabolic route that would facilitate its metabolism. In this manner, the culture medium might be

provided with the necessary substrates to efficiently transform into the desired products with higher productivity in a short amount of time (Aftab et al., 2021).

Moreover controlling the media constituent not only affects the pigment yield but also influences toxin production. Therefore to boost the market demand for microbial pigments it is important to control the media besides understanding the metabolic pathway and selection of toxic producing strains (Prabhu et al., 2017).

Secondary metabolites productions were affected by the broth viscosity in submerged culture thus due to the increase in mycelial concentration. In the cultures of Penicillium chrysogenum and Aspergillus niger the transfer of oxygen dramatically decline, this is because of the increase mycelial concentration (Gibbs et al., 2000).

The concentration of biomass and the shape of fungus cells are the two main parameters affecting the rheology of fermentation fluids. The number of particles (in this example free or aggregated mycelia) in the fermentation fluid, and consequently the number of potential interactions between them, increases as the biomass concentrations increase, thus resulting in an increase in broth viscosity (Gibbs et al., 2000).

Since fungi synthesize secondary metabolites, which are often formed during the late growth phase (idiophase) of microorganisms, the increased biomass does not necessarily equate into an increase in pigment production (Pombeiro-Sponchiado et al., 2017).

2.4.4 Type of fermentation

The growing of microorganisms on moist solid surfaces in the absence of free-flowing water is known as solid-state fermentation (SSF). Besides growing on liquid media called submerged fermentation (SmF) (Fisheries et al., 2015). Though media composition, pH, temperature, and agitation influence pigment production, also fermentation type affects pigment production. The report showed that solid cultures yield 3 folds more pigment than a submerged type of fermentation. Even in similar conditions, the solid culture yields more pigment than liquid fermentation. In M.purpureus solid fermentation gave greater production of red and yellow pigments than submerged fermentation and also under similar conditions the solid culture gains

superior (Joshi & Bhushan, 2014). Submerged culture can produce more mycelial biomass in a short period and with fewer contamination risks (Pradeep & Pradeep, 2013).

A Monascus pigment produced by species of the genus Monascus under solid-state fermentation is an unpurified substance. Thus, the method is not appropriate for large-scale industrial production due to the low productivity, high labor costs, and control issues in the solid-state fermentation (Vendruscolo et al., 2014). In order to circumvent the space, scale-up, and process control issues associated with solid culture, submerged fermentation for the manufacture of Monascus pigments has been examined. Similarly, by minimizing the work required for solid-state fermentation, the use of submerged culture can increase the production of several secondary metabolites while lowering production costs (Vendruscolo et al., 2014).

Submerged fermentation (SmF) truly gives the space to develop pigments because of their unwavering commitment to cutting-edge control mechanisms and straightforward monitoring procedures during manufacturing (Venkatachalam et al., 2020)

2.4.5 Effect of agitation

Submerged fermentation is performed either in agitation or without agitation in an incubator shaker. The importance of agitation is to prevent sediment formation. In addition to preventing the sedimentation of the biomass and retaining suspended solids, the primary goal of an agitation system is to facilitate the transfer of oxygen from the gas phase to the culture medium and, subsequently, to the microbe (Cinbiz et al. 2010). According to Menezes et al. (2020) work, F. solani BRM054066 was found to produce more pigment when it was stirred at 200 rpm as opposed to 100 rpm or without stirring. Menezes et al. (2020) and Gunasekaran & Rajendran (2008) reported that the pigment intensity of Fusarium solani BRM054066 and Pencillium sp. were increased when fermentation performed in maximum agitation respectively.

2.4.6 Fermentation time

Pigment production under submerged fermentation from microbes is affected by an incubation time. Due to the number of cells growing smaller and going to die, pigment production seizes after a week (Djamaan, 2016). Pigments began to be produced at 48 hours, reached their peak at 140

hours, and then gradually increased until the fermentation process was complete (Venkatachalam et al., 2018).

Santos-Ebinuma et al. (2014) reports that different incubation times were studied for pigment production, due to the change in nutritional component concentration, which affects not only microbial growth but also its metabolism. During an incubation period there is a change in color hue from yellow to red. Thus the pigment yield starts and increases until stationary phase then decline, due to the pigment demolishing or changing the structure (Venkatachalam et al., 2020).

2.5 Stability of microbial pigments

The major disadvantage of microorganisms used as food colorants is their instability and being largely degradable. Since microbes are sensitive toward heat, light, acidity, air, and water activity. The pH and chemical modification to increase water solubility have an important role in pigment production and improve stability (Joshi & Bhushan, 2014).

2.6 Optimization for enhancement of bio color production

The traditional "one-factor-at-a-time" method of medium optimization entails modifying one component while keeping the others fixed at a particular level. The non-statistical "one-factor-at-a-time" approach is tedious and takes an excessive amount of time, and it also misses out on the interactions between the variables under investigation. By using statistical techniques, these shortcomings of a non-statistical optimization method could be resolved (Jinendiran et al., 2019).

Frequently utilized techniques that are helpful in fermentative process optimization include response surface analysis and orthogonal experimental design (Santos-Ebinuma et al., 2014). Response surface methodology (RSM) is used for designing the experiments, it analyses and modeling of the experiment in which the dependent variable is affected by several parameters and its objective is to show the effect of the parameter, the interactions among several factors, and to optimize the conditions under which best result obtained. The statistical analysis of the data and three-dimensional plotting is done using design expert software version 13 (Kaur et al., 2019). Process optimization for pigment synthesis can be accomplished using response surface methods

(RSM). This reduces the number of experimental trials required to evaluate numerous variables by solving the multivariate data acquired by solving the multivariate equations (Sen et al., 2018).

There are different parameters listed above that affect pigment production. An enhancement of parameters was reported among different researchers for different fungal strains. And some studies were on the assessment of pigment production from fungi on natural substrates (rice, corn, wheat, cassava, whole sorghum grain, dehulled sorghum grain, and sorghum bran) and different agro-industrial residues (feather meal, fish meal, cheese whey, grape waste, soybean protein, soybean meal, chicken feather, and rice husk, orange processing waste). Xylindein production was enhanced by adding test woods (Acer saccharum, Populus tremuloides, spalted P. tremuloides, and Ailanthus altissima) in agar-based media. The optimum fermentation of Aspergillus niger in corn cob solid medium was reported by a researcher at a temperature of 30^0C and incubation time of 6 weeks after cultivation (Djamaan, 2016).

2.7 Application areas of pigments

2.7.1 Painting

Paint is any liquid that has a mastic composition when applied on the substrate it turns to an opaque solid film. It has four components solvents, pigments, binders or resins, and additives. Commonly used for aesthetic, color, or to provide texture and protect an object such as house, car, road marking, and underground storage vessels. There is a great demand for commercial paints due to their various applications. They can be purchased or can be made in different colors. Currently, the market demand shows that non-toxic paints are highly commercial demand, scope, and preference than synthetic paint due to environmental and health concerns. Bio-paints are paints made from bio-based solutions, and they are belonging to the world of natural paints, eco paints, low VOC bio paints, or organic paints.

The main difference between natural paints from conventional latex paints is; that natural paints are durable, breathable, prevent moisture problems, contribute to a positive room climate, use safer technology, and are less energy-intensive to produce and also used to improve indoor air quality and reduce urban smog and offer beneficial characteristics such as low odor, excellent durability,

and a washable finish. Bio-paints are produced by using natural colorants and natural paint recipes. Natural paint recipes have been used successfully throughout the world for thousands of years. These natural paint recipes are clay paint, milk paint, lime paint, unearthed plant glue paint, latex, recycled latex, and bio-acrylic paints. Among those reported natural paint recipes milk paint is the most durable form of ancient paint which is milk protein casein and crushed limestone form a tough coating that hardens over time like concrete (Sastry, 2016).

Pigments or dye molecule is a key ingredient of paint that provides color, opacity, and gloss, but not only provides color but also protects the surface from corrosion and weathering as well as help to hold the paint together. Paints are formed from either synthetic or natural pigments but the synthesis of synthetic pigments requires toxic reagents and is not environment friendly. As a result, natural pigments are preferable because of safety, toxicity, health, and environmental problems. Microbial pigments or dyes are good sources of colorants that replace synthetic pigments. In addition, they contribute to the remediation of the environment by evading inherent environmental disposal problems and offer significant opportunities as an ingredient of bio-paints, dyeing textiles, and other potential applications (Sastry, 2016).

Ancient knowledge and history of paint recipes were used for bio-paint production under some researchers' study. They used fungal colorants by isolating and extracting pigment from fungi species found on soil or plant. They combine fungal pigment with hydrated lime, borax, sea salt, chalk, clay, stone powder, linseed oil, and casein from milk to make bio-paint by applying the ancient history of paint. They found that acacia and linseed oil make the paint have good quality. They reported fungal colorants that are relatively more stable and robust such as anthraquinone and dyes with siderophore properties. They suggest that microbial biodiversity studies can give more hues and cost-effective durable natural paints can be prepared using selected stable fungal colorants with commonly available natural ingredients using scientific information based on the history of paints. But pH, Media in general, salt in specific, sampling source, and environmental conditions play a role in the production of fungal dyes so they must be explored well (Sastry, 2016).

The paint recipes currently in use produce VOC (volatile organic compounds) during drying after coating, and heavy metal wastes dispose to the environment during production. Wastes in paint

industries preliminary contain heavy metals including Cd, Cr, Pb, Cu, Ni, and Co, which harm human beings' health and pollute the environment when dispose of. Heavy metals are non-biodegradable and persistent toxic and recalcitrant and exist for a long time and cause their effect after a long time. The main source of heavy metal waste in paint industries is the pigments they use. They used different mechanisms to treat the waste either through chemical or bio-sorption process which incurs additional expenditure (Malakootian & Hossaini, 2008). But managing the waste from the source is the best way in reducing the waste as well as increasing the profit of the company by lessening the expenditure for wastewater treatment. So substituting synthetic pigments with bio-based pigments to manufacture paint is the best solution to manage the waste and the environment.

Here in Ethiopia, almost all Ethiopian pigment industries import synthetic pigments from abroad (UNIDO, 2019), which has significantly affected the biodiversity of the country. In order to mention, textiles, paint, leather, and the food sector etc., as a result to save the ecosystem from disturbance, it needs to find a solution to have an organic environment. For these reasons, it's important to study and use natural pigments. Currently in Ethiopia many researchers studied on the production of natural dyes for textiles from plants to mitigate the adverse effects results from usage of synthetic pigments (Kechi et al., 2013), but using of plants for natural dyes is non-economical, non-availability throughout the year etc.. as reported by Prabhu et al. (2017). For these reasons looking for other potential sources of natural dyes is important such as microbes, which was more works were done in developed countries. Especially for textiles, and food fabrics and very few works were done on painting. But no works were done on the potential pigment production of microbes in Ethiopia for all types of pigment using industries. As a result this thesis work aims to work in this field.

The prepared bio-paint was characterized by measuring its pH, specific gravity, and viscosity with a pH meter, density meter, and rotational viscometer respectively. The drying time and hiding power also tell us the quality of the paint.

Viscosity: It indicates how the paint is viscous. The viscometer is an instrument used for measuring viscosity.

Specific gravity: It indicates how the paint dissolves with water and it shows how the paint is denser relative to water.

pH: It indicates the acidity and basic property of the paint measured using a pH meter.

Hiding power: It indicates how the paint covers the object or the wall. It is simply measured by observing the wall of the surface by applying paint.

Drying: The drying capability of bio paint will evaluate by applying both synthetic paint and the produced bio paint on a wall and then the drying time will be measured and compared.

2.7.2 Food colorant

Currently, many fungal pigments are applied as food colorant as many works shows and the use of fungal pigments as food additives in different food products has been assessed and reported by many researchers. Food colorant fungal pigments such as Monascus pigments, arpink red from P. oxalicum, riboflavin from Ashbya gossypii, and β-carotene from B. trispora are on market (Lagashetti et al., 2019a).

2.7.3 Pharmaceutical

Many studies reported that fungal pigments have the potential as antimicrobial, antioxidant, anticancer, and as cytotoxic agents. All these studies proved the utilization of bioactive pigments as a food preservative and antibacterial ingredient in the food and pharmaceutical industries. Pigment extract reported that has antimicrobial against pathogenic bacteria, yeast and fungi are Monascus, Fusarium, Talaromyces, Trichoderma, Penicillium, and Aspergillus. A. alternata and Thermomyces spp. were reported for dyeing cotton and silk, etc. against pathogenic bacteria and used for medical applications to produce bandages, suture threads, face masks, etc... Anthraquinone pigment-producing microbes such as Aspergillus spp. are essential for herbicidal and phytotoxic activities due to their antibacterial and antifungal properties (Toma et al., 2021).

2.7.4 Cosmetic industry

Nowadays Monascus and Monascus-like pigments have already entered the market for application in cosmetics such as skin conditioning and skincare products, lipsticks, etc. Pigments, like melanin, carotenoids, lycopene, etc., have been reported for their application in cosmetics, sunscreens, sun lotions, sun-blocks, face creams, anti-aging facials, etc.

2.7.5 Textile industry

Some fungal pigments belong to the genera Monascus, Fusarium, Aspergillus, Penicillium, Talaromyces, Trichoderma, Alternaria, Curvularia, Chlorociboria, Scytalidium, Cordyceps, Acrostalagmus, Bisporomyces, Cunninghamella, Thermomyces, and Phymatotrichum have been studied for their potential application in dyeing silk, cotton, and wool. Fungal pigments have an immense advantage over synthetic pigments for textile dyeing because of their good color stability, colorfastness properties, and dye uptake potential. In addition, fungal pigments do not have adverse effects on fabric and are non-toxic to human skin (Lagashetti et al., 2019a).

2.8 Characterization or analysis of fungal pigment

There are various analytical instruments used to determine the chemical structure of compounds which constituents in the microbial pigment. Molecular weights of molecules, the types of bonds that engage in molecular conformation, the polarity of the structures, and their functional groups, etc. are analyzed by different analytical methodologies stated below. However, the drawback in determining the chemical structure is the lack of industry reference criteria that enable adequate characterization of the compounds (Valenzuela-gloria et al., 2021). Those techniques used to identify and characterize microbial pigments are UV-VIS spectrophotometry, FT-IR, TLC, HPLC, and NMR (Rana et al., 2021).

2.8.1 Absorption spectra of pigments (UV/Vis spectrophotometer)

A spectrophotometer is a device used for analyzing the components in pigment extract from UV to visible range by measuring the maximum absorption of pigment extract (Rajendran & Gunasekaran, 2015b) and also the characteristic lambda max of the pigment measured (Pandey et

al., 2018). The UV-Vis spectrophotometer for the orange fluorescent pigment from Bacillus Endophyticus (AVP-9(Kf527823) shows that maximum peak at 493 nm which detects the presence of carotenoid pigments from the species (Ram et al., 2017). These devices are used to measure pigment production indirectly by measuring the absorbance of the extract (Pradeep & Pradeep, 2013).

2.8.2 Fourier transforms infrared spectroscopy (FTIR) analysis

FTIR spectrometer was used to analyze the structure of fungal pigment and to measure the infrared spectra of extract solution at each wavenumber. The FT-IR analysis of thermomyces species was reported as the infrared spectra showed the presence of hydrogen bond OH groups (3300-3400 cm^{-1}) and carbonyl function with carbonyl stretching vibration frequency of the pigments were between 1635-1639 cm^{-1}. The stretching frequency is close to phenol and quinone (Rajendran & Gunasekaran, 2015b).

2.8.3 Liquid chromatography-Mass spectrophotometer (LC-MS)

This method of pigment analysis is used to identify possible compounds that are constituents in the pigment. It is followed after TLC separation (Pandey et al., 2018).

2.9 Industrial application and safety consideration of Aspergillus niger

Aspergillus niger is a filamentous fungus that thrives on organic substances and grows aerobically. It can be found in nature in soil and litter, compost, and decaying plant matter. The species can grow in a wide range of temperatures and pH from 6 to 47^0C with a relatively high optimum temperature of 35-37^0C and 1.4 to 9.8 respectively. For decades, Aspergillus niger has been studied and used in industry. When its potential to make citric acid was industrially exploited in 1919, it became of practical importance. Since the 1960s Aspergillus niger was found to be a source of a range of enzymes that are used in fruit processing, baking, and the starch and food industries. Experts from the FAO and WHO have analyzed and approved enzyme preparations from A. niger, as well as the organism itself (Frisvad, 2002).

Aspergillus niger can be classified as “generally recognized as safe” (GRAS) if nonpathogenic and nontoxic strains are utilized in manufacture, as well as current good manufacturing practices evolved in production. Aspergillus niger is generally thought to be a harmless organism. This is reflected in lists of organizations in charge of workplace health and safety. It’s reported that a species is non-pathogenic and in the inhalation research, it had no significant effect on the animals. These microbes have never been recognized as the main causative agent of any human disease. In a manufacturing environment, the risk of allergic sensitization to inhaled spores can be controlled by reducing worker exposure to spore dust (Frisvad, 2002). Nielsen et al. (2009) also agree with the above explanations that Aspergillus niger can be a safe industrial production organism.

3. Methodology

The research was conducted in the laboratory of the school of chemical and bio engineering at the Addis Ababa Institute of Technology.

3.1 Frame work of the Experiment

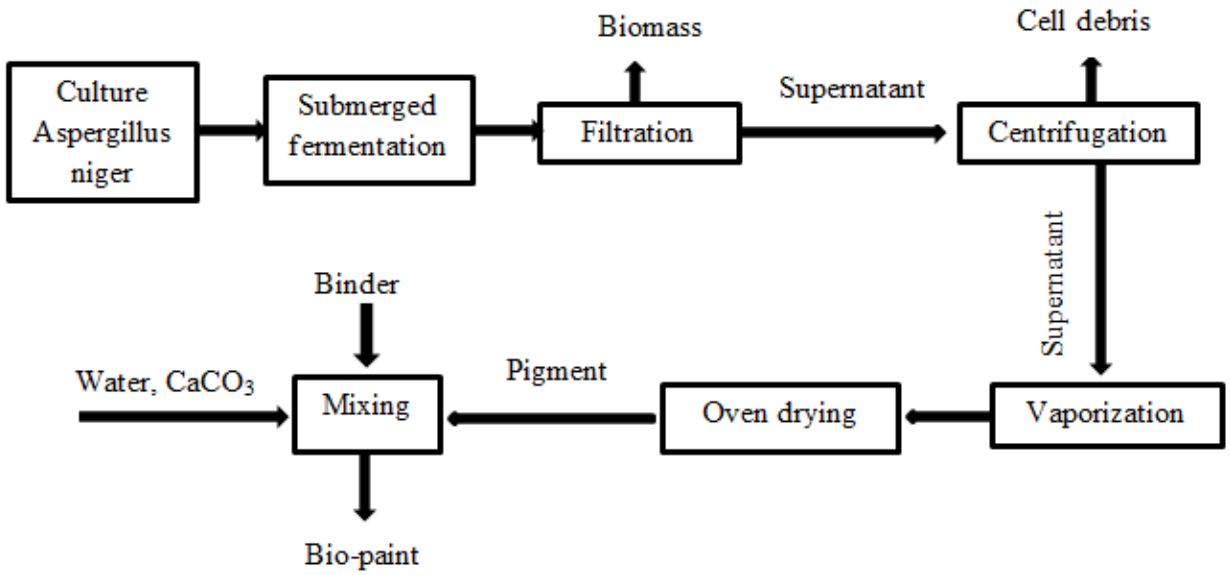


Figure 3. 1: Process flow chart of pigment and paint production

3.2 Materials and Methods

3.2.1 Materials, Chemicals, and Equipment

Materials and Chemicals

Raw materials and chemicals used during the present series of experimental studies include Aspergillus niger (was obtained from Ethiopia Institute of Biodiversity, (EIB)) , whereas other chemicals listed in the table with their functions during the experimental work were obtained from Biochemical Engineering laboratory, SCBE, AAiT and bought from the local market and prepared to the required concentrations.

Table 3. 1: Types of chemicals used in the experiment with their function

Chemicals	Used	Chemicals	Used	Chemicals	Used
Aspergillus niger	For pigment production	Chloroform, hexane, toluene, Methanol, Ethyl acetate	Solvents used during TLC analysis	Maltose, Starch soluble, glucose	As carbon source
Distilled water	For solution preparation, and to clean	Alcohol (ethanol 99%)	To remove contaminants	NaOH (3 M), HCl (3 M)	To adjust the pH of media
PDA	To culture the species	$CaCO_3$, raw milk, vinegar	For paint ingredients	PDB	To prepare liquid media

Equipment used

Equipments that were used during the experiments include Freezer (BEKO DN139110; Energy class A), Autoclave (model LX-B100L (digital)), Cabinet or Hood (cabinet model: FlowFast V 15 P), Incubator (Model 100-800, Germany), Incubator shaker (New Brunswick Scientific Edison, New Jersey, USA, Model EXCELLA E24R), Vacuum filter, centrifuge, rotary evaporator, Electronic analytical balance (FA2004, Number 201803172), Erlenmeyer flasks (50 ml, 100 ml, 250 ml, 500 ml, 1000 ml), Measuring cylinders, test tubes, Petri dishes, pH meter (Model 3505, UK), Stove, Oven (202-0A), Beaker, Microscope (Microscope DM-25 binocular with LCD screen), Hemocytometer, striker loop, Spreader, Density meter, Viscometer, UV-Vis spectrophotometer (Lambda 950-UV-Vis-NIR, PerkinElmer, UK), Fourier Transform-Infrared Spectrometer (Perkin Elmer, spectrum 65 Spectrophotometer, USA).

Table 3. 2: Materials and equipments used during experimental work and their functions

Materials	Used	Equipments	Used
Erlenmeyer flask, measuring cylinder, spatula, petridish, striker, test tubes, spreader	To prepare media and culture the species	Freezer	To preserve
		Microscope, Hemocytometer	Used to count the spores of Aspergillus niger
		pH	To determine the acidity and basicity of media, and paint
Stove	Heater	Autoclave	Sterilizer
Vacuum filter	To separate biomass	Cabinet hood	To inoculate
Centrifuge	To remove cell debris	Incubator/ Incubator shaker	To grow the species, for fermentation
Rotary evaporator	To concentrate the pigment	Density meter	To measure density and specific gravity of paint
Oven	To dry semi sold pigment	FT-IR	Functional group determination
TLC	To purify pigment	Viscometer	To determine viscosity of paint

3.2.2 Methods

Microbial selection

In this study the fungal species Aspergillus niger among filamentous fungus was selected and obtained from Ethiopian Institute of Biodiversity. Thus, due to the pathogenic effect of bacteria and the lowest harvest culture from algal culture and basidiomycetous fungi (Lagashetti et al., 2019a). In addition the easy processing and fast growth of the fungi microbe makes the fungi species preferable source of natural pigment (Calvo et al., 2002; Gunasekaran & Rajendran, 2008; Sehrawat, 2017). A filamentous fungi Aspergillus niger is recommended due to its ability to synthesize many pigments and the species recognized as safe industrial microorganisms (Aftab et al., 2021; Lagashetti et al., 2019a; Nielsen et al., 2009). That's why this thesis work was aimed to

evaluate the pigment producing potential of Aspergillus niger in Ethiopia for wall paint application.

Cultivation of Aspergillus niger

After the fungal species Aspergillus niger species was obtained from EIB, the species was stored in a freezer (BEKO DN139110; Energy class A) at temperature below -18°C under the condition stated by Humber (1997). The laboratory apparatus including Petri dish, Tips, Erlenmeyer flask, and the Spreader were washed successively with distilled water, and then sterilized and prepared to culture the species.

The fungus was cultured using a PDA. The PDA agar solution was prepared according to the manufacturer recommendation. Accordingly, 3.9 g PDA powder was weighed using an analytical balance in an Erlenmeyer flask and filled to 100 ml with distilled water. The solution was boiled until the solid PDA was completely dissolved. The prepared solution and materials were sterilized for 15 min at 121^{o}C using vertical steam sterilizer (model LX-B100L (digital)) according to Anchana (2014).

After the sterilization was complete, the prepared solution and the materials were brought immediately in the biosafety cabinet (FlowFast V 15 P) in order to avoid contamination. Alcohol was used to clean up the cabinet table and avoid any contamination as well as Ultraviolet light was applied to sterilize the inside of the cabinet and hood environment. Then, the PDA solution in the Erlenmeyer flask was poured on to the sterilized petri dish and left for 20 min until the agar solution in the petri dish was solidified.

Then, 0.1 ml of Aspergillus niger species was pipette out from the vile, which was taken out of the freezer before 20 min to release out the ice formed, and subsequently poured into the petri dish and the species were smoothly smeared along the petri dish by the spreader. One petri dish was left alone as a control to check out whether contaminations might have happened. Finally, according to the procedure in the literature (Pradeep & Pradeep, 2013; Toma et al., 2021), the culture was incubated at 30^{o}C for seven days. Then the growth of culture was observed on the next days.

After seven days, the petri dish with matured fungi was wrapped with paraffin and kept at 4 degrees Celsius in the freezer for further experiment. Same experimental procedures were repeated to multiply the species by sub culturing the seven days old cultures. To subculture, a striker was used to take a spore (8 $\times 10^5$ spores) using Hemocytometer to count the spores from the previous old culture and mixed with sterilized 8 ml distilled water in a test tube. Then the prepared culture in the test tube was stored in the refrigerator at 4^{o}C for further experiment according to Alam et al. (2011).

Production and extraction of natural pigment

A submerged type procedure of fermentation was employed for the production of pigment from Aspergillus niger species, according to the method proposed by Anchana (2014) with some modifications. This method has been widely used for the production of secondary metabolites, especially for filamentous fungi. In addition, this type of fermentation method has a potential benefit in producing more mycelial biomass in a smaller area in a shorter amount of time with less chances of contamination (Pradeep & Pradeep, 2013). Submerged fermentation (SmF) seems to be a useful and economical fermentation approach for the production of fungal pigments (Venkatachalam et al., 2020).

Accordingly, a liquid medium was prepared using potato dextrose broth (PDB). 2.4 g of PDB was weighed and poured in to 100 ml of distilled water in to an Erlenmeyer flask. The pH of the solution was adjusted to 3 and 8 using 3M HCl and 3M NaOH. The medium was supplemented with maltose (1%-3%). Then, the prepared media and pipette tips were sterilized at 121°C for 15 min using an autoclave (Anchana, 2014).

In addition to alcohol (99% ethanol), UV light was applied to the hood to prevent contamination. Then, based on the procedure recommended by the researchers (Alam et al., 2011; Beiri et al., 2021; Morales-Oyervides et al., 2017; Toma et al., 2021), 2 ml of old Aspergillus niger species with a spore suspension (1×10^5 spores/ml) counted using Hemocytometer was inoculated in to the liquid media and covered with gauze and incubated in incubator shaker (NEW BRUNSWICK SCIENTIFIC Edison, New Jersoy, USA, Model EXCELLA E24R), at 30°C and 150 rpm, for 7-28 days. According to the experimental design (BBED) shown in Table 3.3, seventeen (17) series

of submerged fermentations were conducted. The medium pH, maltose concentration, and fermentation time could be fixed using the BBD matrix (Table 3.4). Throughout the whole culture time, the flasks were incubated in a controlled orbital shaker under fixed conditions.

After fermentation time was over, the flasks were taken out of the incubator. The culture broth containing extracellular pigments was then vacuum filtered using Whatman filter paper No. 2 in order to separate it from the mycelia (Fouillaud et al., 2017). Further, the cell culture was centrifuged at 6000rpm and at 4^{o}C for 15 min to remove left over cell debris (Morales-oyervides et al., 2017a).

Finally, the extracted extracellular pigment was concentrated using a rotary evaporator at 65°C and 30 rpm to remove cell debris and obtain a semisolid pigment (Rajendran & Gunasekaran, 2015b). The concentrated pigment was then applied to glass Petri dishes and dried for three days at 60^{o}C using an oven (Ahmad et al., 2012). The pigment was kept under refrigeration at 4^{o}C for further analysis (Morales-oyervides et al., 2017a).

For this study, only the extracellular pigment was considered. The concentration of the extracted extracellular pigments were quantified by measuring the absorbance over the wavelength 200-800 nm using UV/Vis spectrophotometer (Morales-oyervides et al., 2017b).

3.3 Experimental Design and Statistical Analysis

In this study, the effect of process parameters on pigment synthesis was investigated and optimized using the Box-Behnken experimental design (BBED) and response surface modeling (RSM) using three factors (pH, fermentation time, and maltose concentration) at three levels.

The process parameters were: pH (A), fermentation time (B), and maltose concentration (C). The ranges of the process parameters were defined based on literature recommended values (Hiroshi Tanaka, Pie-Lang Wang, 2014; James et al., 1961; Pandey et al., 2018). Each independent variable was given a three-level coding system: -1 (low), 0 (center point), and +1 (high) which were: pH of the liquid media (A; 3-8), fermentation time (B; 7-28 days), and mass of maltose (C; 1-3%).

Table 3. 3: Coded and actual values of the variables for the three factor Box–Behnken experimental design

Symbol	Variables	Units	Levels		
			-1 (low)	0 (medium)	1 (high)
A	pH	-----	3	5.5	8
B	Fermentation time	Days	7	17.5	28
C	Maltose concentration	g	0.024	0.048	0.072

There were 17 trial runs in total, and five of them had center points (table 3.4). The experimental data were statistically examined in this study, and the experimental design was designed using the Design-Expert® Software (Version 13, Stat-Ease Inc.).

The obtained experimental results need to be satisfactorily accommodated by the empirical second-order polynomial model (Equation (3.1)). The second-order polynomial equation provides an explanation of the relationship between factors and outcome. The following equation is given in mathematical form:

$$y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \varepsilon \quad \text{Eq}\ldots\ldots\ldots\ldots 3.1$$

The coefficients of the polynomial are represented by β_0 (constant), β_1, β_2, β_3 (linear effects); β_{12}, β_{13}, β_{23}, (interaction effects); and β_{11}, β_{22}, β_{33}, (quadratic effects) and is ε is random error.

Design-Expert® version 13, statistical software, was used for the regression analysis and determination of the coefficients. Analysis of variance (ANOVA) was used to assess the suitability of the model equations. The F-test, p value, coefficient of determination (R^2), prediction coefficients of determination (Pred R^2), and adjusted coefficients of determination (adj-R^2), coefficient of variation (CV) were used to describe the quality of fit and their statistical significance. ANOVA was used to test whether there were any differences in the activity and concentration of the samples, and a P value of less than 0.05 was regarded as statistically

significant (Vendruscolo et al., 2014). In addition, the validity of the models was analyzed by comparing the experimental and predicted values on the graph.

Table 3. 4: The suggested Box-Behnken Design (BBD) and response of pigment concentration

	Factor 1	Factor 2	Factor 3	Response 1
Std	A:pH	B:Fermentation time Days	C:Maltose concentration g	Pigment concentration a.u
1	3	7	0.048	9.5
2	8	7	0.048	55.4
3	3	28	0.048	113.3
4	8	28	0.048	345.03
5	3	17.5	0.024	24.11
6	8	17.5	0.024	193.05
7	3	17.5	0.072	19.09
8	8	17.5	0.072	180.54
9	5.5	7	0.024	25.61
10	5.5	28	0.024	251.72
11	5.5	7	0.072	27.07
12	5.5	28	0.072	205.46
13	5.5	17.5	0.048	274.23
14	5.5	17.5	0.048	259.1
15	5.5	17.5	0.048	262.18
16	5.5	17.5	0.048	260.06
17	5.5	17.5	0.048	264.42

3.4 Optimization of Pigment Production and Validation

In this research, the extracellular pigment parameters were studied. Although different physical (temperature, pH, time, agitation, aeration, etc.) and chemical (media constituent) parameters affect color production, only three factors (pH, time, media constituent) were studied in this project; and the other factors adjusted to optimized values recommended in the literature (Toma et al., 2021). This is due to the fact that, the capability of pigment production was highly affected by

fermentation time, pH, and media. For instance, pH and fermentation time are crucial factors that affect the pigment synthesis, and different results were provided by different researchers, and recommended for further studies (Anchana, 2014; Rajendran & Gunasekaran, 2015a).

In addition, numerous studies have demonstrated that, among other environmental parameters, the medium's pH and carbon source have a significant impact on the growth and production of metabolites in submerged cultures (Venkatachalam et al., 2020).

A fungus's structure, morphology, physiology and ability to synthesize metabolites are all influenced by pH, which is a major environmental factor. Fungi cannot grow and metabolites cannot be produced above a particular pH range. When pigments are synthesized as secondary metabolites, the enzyme activities that are engaged in those metabolic pathways are controlled by pH (Aftab et al., 2021).

Accordingly, the pH of the liquid cultivated media was studied, which is a critical factor for pigment production. Hence, the present study was investigated the effect of pH on Aspergillus niger identified from an Ethiopia source which was owing to a considerably different ecology.

Many researchers suggest that replacing a carbon source with maltose may lead to an increase in pigment production (Chen & Johns, 1994; James et al., 1961; Pandey et al., 2018). In addition, Chen & Johns (1994) reported that maltose, glucose, and soluble starch are the best sources of carbon relative to others for pigment production. However, the effect of types of carbon source with its concentration on pigment production using Aspergillus niger species was not studied either. Thus, supplementing the carbon sources with different concentrations of maltose was investigated in this study. That’s why it is due emphasis was given in this study.

Different results were reported for the optimum incubation time including three weeks (Hiroshi Tanaka, Pie-Lang Wang, 2014), nine days (Toma et al., 2021), and 5-7 days (James et al., 1961). This indicated that there was no well-defined incubation time to produce a high yield. Thus, the incubation times suggested in the present study were considered to determine the incubation time. Every microbe needs an ideal period of time to grow and generate pigments at the highest possible level, thus the incubation period is a crucial consideration in the optimization process. Each microbe has a unique incubation period, which differs amongst species (Aftab et al., 2021).

In this optimization study, the incubation temperature for the fermentation was adjusted to 28 ± 2°C as recommended by Anchana, 2014; Rajendran & Gunasekaran, 2015a; and Toma et al., 2021.

Many researchers proved that an acidic condition (pH=3.5, 4, 5, and 6) was favored for pigment production from filamentous fungi (Cho et al., 2002; James et al., 1961; Joshi & Bhushan, 2014; Lagashetti et al., 2019b; Toma et al., 2021). And also Aftab et al. (2021) reported that neutral and slightly alkaline pH was good for some filamentous fungi metaboloic activity. Based on this reports this thesis was used to study the pH effect on pigment production on the pH between 3 and 8 since the effect of pH highly depends on the species, and different strain types with their different ecological environment. The pH range was taken to include the acidic (3-7), slightly basic (8) and the neutral condition (7).

A literature survey showed that 2 % suplementation of maltose enhance pigment production from filamentous fungi (Pandey et al., 2018). So this thesis work was aimed to investigate the effect of maltose concentration, i.e., the effects of maltose concentration below and above 2 %, from the previously studied 2 % was studied.

Various optimum fermentation time were recorded to enhance pigment yield. They found pigment production with in one week, two week, three week, and four weeks due to the species type, and environmental conditions etc (Aftab et al., 2021; Hiroshi Tanaka, Pie-Lang Wang, 2014; James et al., 1961; Toma et al., 2021). As a result it can concluded that there is no well defined optimum fermentation time to give high yield. For these reasons the effect of fermentation time between one week and four weeks were studied in this thesis work.

Therefore, optimization of pigment production from Aspergillus niger species was conducted through serial experimental runs as per the experimental design generated by design expert software version 13. Accordingly, supplementing maltose supplement (1%-3%), pH (3-8), and fermentation time (7-28 days) of the medium were considered. After fermentation, the filtrate was used for the determination of concentration based on absorbance measurements.

The effects of process variables on pigment concentration were optimized using a numerical optimization method to enhance the pigment yield under the set objectives. The experiment was done three times under the predicted optimal condition and the average was taken. The model validity was checked by comparing the results found in the experiment with the predicted values.

3.5 Effect of carbon source

After optimization was done, further, the effect of the nature of carbon on pigment production was studied by using the optimum condition. This is done by supplementing the carbon source; starch soluble and glucose were used instead of maltose under submerged fermentation with a 150 rpm shaker. To extract the pigment, all the procedures as described above were applied. In addition, fermentation without supplementing carbon sources in the media was studied. The experiment was carried out three times.

3.6 Characterization of the produced pigment

3.6.1 Purification of pigment using TLC

Thin Layer Chromatography (TLC) was used to analyze the purity and polarity of the compounds of the pigment using different solvents of varying polarity. In pre-coated thin layer chromatography, the extracellular pigment was eluted using different solvents including methanol, ethanol, ethyl acetate, chloroform, toluene and hexane as a mobile phase. The sample was placed on the plate at a height of 1 cm from the bottom, and the plate was then placed in one of the solvent-filled beakers and covered with aluminum foil.

The TLC plates were seen under UV light after the solvent font reached the specified line. The distances traveled by the solvent and the pigment compound were used to calculate the retention factor (R_f).

$$Rf = \frac{Distance\ traveled\ by\ compound}{Distance\ traveled\ by\ the\ solvent} \qquad \text{Eq}\ldots\ldots\ldots\ldots\ldots\ldots\ 3.2$$

3.6.2 FT-IR Analysis of the pigment

The FT-IR spectrophotometer was used to analyze the chemical structure of fungal pigment and to measure the infrared spectra of the extract solution at 4 cm^{-1} resolution and the wavenumber range between 400– 4000cm^{-1}. From the spectrum of the sample obtained, the functional groups which resulted in absorption of light were determined.

3.7 Evaluate and Validate Bio-paint production

3.7.1 Preparation of casein from skimmed milk

Casein was prepared from skimmed milk according to the procedure proposed by Sastry (2016). 1 L of fresh raw milk was bought from the market and the skimmed milk was prepared, and then 100 ml of vinegar was added to it and mixed well and stayed overnight in a warm place to get curd solution. On the following day, the liquid whey was allowed to drain and milk solids containing casein were obtained by filtration using a sieve.

3.7.2 Preparation of bio paint using bio-paint ingredients

According to Sastry (2016), bio-paint ingredients including natural pigment (coloring agent), raw milk (for binder preparation), water (solvent) and filler ($CaCO_3$) were purchased from the local market and the pigment was synthesized from the fungus species. First, the extracellular pigment obtained from the broth was mixed well with the casein protein. Then other ingredients were added successfully with the ratio of 48:40:10 pigment and filler, water and binder respectively. Afterwards, viscosity, density and pH were measured through a rotational viscometer, density meter and pH meter, respectively. The drying time of bio-paint was measured and compared with synthetic paint. The measurement was carried out three times and the average was taken. There are numerous techniques to test paint. In order to achieve accurate results, various industries use a variety of paint testing techniques (Panda H., 2022). The proportions for mixing ingredients to prepare paint, and the procedure for measuring some properties of paint were conducted in accordance with the procedures currently being used by Ethiopian paint factories (BEETAR paints factory, Bahir Dar Ethiopia).

4. Results and Discussions

4.1 Growth condition and its pigment production potential

The fungal species Aspergillus niger shows efficient growth on the PDA agar plate. The growth appearance of Aspergillus niger spp. was initially whitish, which changed rapidly and became a black mold after three days. Similar observations have been reported in the literature (Toma et al., 2021). The species was found to be growing well at a temperature of 30^{o}C. There was no microbial growth in the control group, indicating that the experiment was done carefully.

This study suggests that an Ethiopian localized Aspergillus niger spp. has a potential for the production of pigments of reddish brown or purple red color under submerged fermentation (Fig. 4.1 c&d). The color of the broth darkened in the course of fermentation, implying that the species has a potential to generate pigment as reported by Fouillaud et al. (2017). In contrary to Calvo et al. (2002) in which he claimed that some fungal strains are deficient in producing secondary metabolites, this study confirms the ability of Ethiopian Aspergillus niger species to have potential for producing pigment. Such differences might occur due to the differences in microbial strain, sources and optimization parameters.

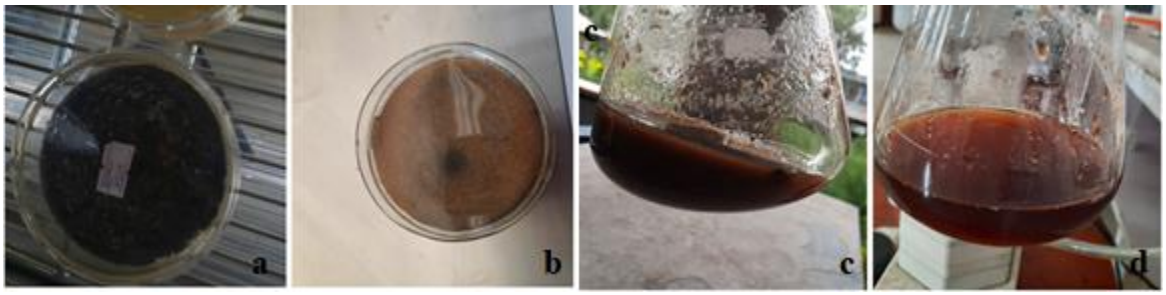


Figure 4. 1: a - Obverse face on PDA; b - Reverse face on PDA; c - Culture in PDB supplemented with maltose as carbon source; d - Extracellular pigment (filtrate from culture broth)

The Ethiopian fungal species is found to produce an orange to dark orange colored (reddish brown or purple red) pigment depending on the pH value, maltose concentration and fermentation time, but Gnanamani, (2019); James et al. (1961); and Toma et al. (2021) reported that Aspergillus niger

has a potential to produce yellow pigment under acidic condition. This indicates that the pigment poteintial of a microbe under the same species may provide different metabolites. Thus due to the strain types, and the environmental conditions controlled the growth and metabolite production (Gmoser et al., 2018).

This shows that pigments produced by the microbes depend highly on the species type, strain and isolates coupled with carbon sources and concentration and fermentation time and others (Aftab et al., 2021). The present study findings were in line with the report presented by Fouillaud et al. (2017). These findings clearly show that isolates from the same species synthesize and secrete various pigments, and so behave differently when it comes to producing colored compounds by optimizing the parameters required for pigment production. As a result, the current study confirmed that Ethiopia has a potential pigment producing Aspergillus niger, which is suitable for Ethiopian ecological conditions and to utilize them on a large scale to replace the synthetic pigments, and there is still room for research on the potential of pigment production and color of pigments under various conditions Aspergillus niger, since Ethiopia has a wide variety of ecological environment which may affect the type species.

As shown in Figure 4.2, the color appearance of the analyzed samples gradually changes in the course of pigment production (from Fig. 4.2 “a” to “d”). In order to measure the color depth, the absorbance of each sample, prepared under different pH values, maltose concentration and fermentation time were measured.

Figure 4. 2: Submerged fermentation of Aspergillus niger species for color production

All the spectra of the samples showed a wavelength of maximum absorbance at around 447 nm. The results of the current study agreed with the report by Fouillaud et al. (2017). The spectra were fitted with a Gaussian function and then the integral areas of absorption were determined, which is demonstrated in Figure 4.3. The values of the integral absorption are used to optimize the extent of pigment production.

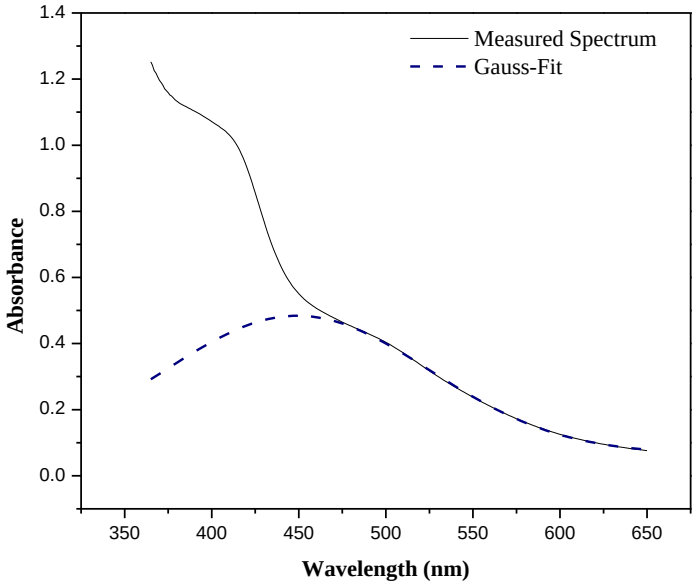


Figure 4. 3: Visible spectrum and gauss fit of three fold diluted pigment (pH=5.5, Fermentation time=17.5 days, Maltose concentration=0.048g, Integral area=87.4 a.u., wave length range of integration= 350-650 nm)

4.2 Box-Behnken Experimental Design Analysis

Through the use of an experimental design, the statistical techniques chosen examined the effects of changing the operating factors and their interactions with one another on a system or a process (Venkatachalam et al., 2020). In order to explore and optimize the impact of the process variables on the yield of pigment for Aspergillus niger, this study used Design-Expert® Software (v13) and the Box-Behnken experimental design with three factors at three levels. Thus, by varying the pH values, maltose concentration and fermentation time 3-8, 1%-3% and 7-28 days, respectively, 17

samples were prepared and evaluated using the BBD model to optimize the process parameter values in order to maximize the pigment yield.

4.3 Second-Order Polynomial Modeling

The experimental data was checked for model adequacy to see if the fitted model would produce inaccurate or deceptive results. The experimental data were fitted using four-degree polynomial models, including linear, interactive (2FI), cubic, and quadratic models (Venkatachalam et al., 2020). In this study, two distinct tests, the sequential model sum of squares and the model summary statistics, were run to assess the suitability of the various models for representing the responses. The adequate model summary output showed that the quadratic model was statistically highly significant because of its greater R^2 and lower p value ($p<0.0001$), compared to the other models (Table 4.1). As a result, the quadratic model was chosen in this study to examine how process variables affect a response (pigment production in Aspergillus niger).

Table 4. 1: Fit Summary

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	Remarks
Linear	0.0063	< 0.0001	0.5089	0.3998	
2FI	0.7483	< 0.0001	0.4317	0.1523	
Quadratic	**< 0.0001**	**0.1025**	**0.9934**	**0.9642**	**Suggested**
Cubic	0.1025		0.9972		**Aliased**

4.4 Determination of Second-Order Polynomial Equations

The finding of the experiment, which was based on the Box-Behnken experimental design, was fitted to the input variables using an empirical relationship defined by a second-order polynomial equation with interaction terms. Based on the responses, the software recommended that a quadratic model simulating the process be used to better describe the pigment production process as shown in equation 4.1.

Concentration of pigment= -789.10711+138.61037×pH+19.48569×fermentation time + (14270.73264×maltose concentration) + (1.76981×pH × fermentation time) – (31.20833×pH×maltose concentration) – (47.34127×fermentation time × maltose concentration) – (12.51664pH²) – (0.498517×fermentation time²) – (1.41617×10^{5}×maltose concentration²) …….equation 4.1

4.5 Statistical Analysis

ANOVA was used to determine the significance of the developed models, with the results displayed in Table 4.3. The ANOVA results showed that the developed models accurately represented the real relationship between the independent variables and responses within the specified range. The significance of each variable was determined using analysis of variance and Fisher's statistical test (F-test). The high F- values, 269.86 for the pigment yield, showed that the constructed regression models could represent the majority of the response variation.

The probability values (p) for the reddish brown colorant are displayed in Table 4.3. Which variables were important to the process can be inferred from the p value. Independent variables with p values less than 0.05 are considered significant in this study because a confidence level of 95% was used. P-values less than 0.05 indicated that the developed model and the terms were statistically significant. The accompanying p-values were used to evaluate whether F is large enough to signal statistical significance (Venkatachalam et al., 2020). In this investigation, the response's p-values were less than 0.0001. It demonstrated the accuracy and precision of the created models.

To determine if the constructed models were adequate and accurate, the determination coefficient (R^2), adjusted R^2 ($AdjR^2$), predicted R^2 ($PreR^2$), and coefficient of variation (CV %) were determined. The proportion of overall variation in the responses that the models had predicted was shown by the R^2. The quadratic model was successfully fitted to the experimental data due to the values of R^2 (0.9971). The values of $AdjR^2$ (0.9934) in this investigation were also high and very close to the R^2 values, indicating a better model prediction. $AdjR^2$ will typically never be more than R^2 or equal to it. $PreR^2$ measures how well a model can estimate a response value. In this case, the $PreR^2$ (0.9642) and $AdjR^2$ are reasonably in accordance. Furthermore, the model's fit

statistics, showed that the difference between the predicted R^2 (0.9642) value and the adjusted R^2 (0.9934) value, was less than 0.2, implying that the predicted and actual values were collinear.

The CV%, which measures how far the experimental points deviate from the second-order polynomial (SOP) models' predictions, was discovered to be 5.70 for a pigment concentration. The extremely high degree of precision and good reproducibility of the experimental data was clearly demonstrated by the low values of CV%. A good estimate of the system response over the tested ranges could be provided by the developed model, according to the high R^2 value and low CV% value. Venkatachalam et al. (2020) reported that high values of R^2 and low CV% represented the models significant

The model's lack of fit has a very low F value, indicating that the lack of fit was non-significant. The ANOVA analysis revealed that the quadratic model is suited to fit the experimental data due to the non-significance of the lack of fit as shown in table 4.3. The model's adequate precision, or signal to noise ratio, was found to be 49.3, which is significantly higher than 4, indicating that the model is reasonably reliable for use as a navigation tool in the design space (Table 4.2).

Table 4. 2: Fit Summary Statistics

Std. Dev.	9.29	R^2	0.9971
Mean	162.93	Adjusted R^2	0.9934
C.V. %	5.70	Predicted R^2	0.9642
		Adeq Precision	49.3401

Table 4. 3: Analysis of variance (ANOVA) Box-Behnken quadratic model for pigment concentration

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.095E+05	9	23281.10	269.86	< 0.0001	Significant
A-pH	46211.04	1	46211.04	535.65	< 0.0001	
B-Fermentation time	79586.54	1	79586.54	922.51	< 0.0001	
C-Maltose concentration	485.63	1	485.63	5.63	0.0494	
AB	8633.20	1	8633.20	100.07	< 0.0001	
AC	14.03	1	14.03	0.1626	0.6988	
BC	569.30	1	569.30	6.60	0.0371	
A^2	25767.48	1	25767.48	298.68	< 0.0001	
B^2	12719.02	1	12719.02	147.43	< 0.0001	
C^2	28016.46	1	28016.46	324.75	< 0.0001	
Residual	603.90	7	86.27			
Lack of Fit	456.23	3	152.08	4.12	0.1025	not significant
Pure Error	147.68	4	36.92			
Cor Total	2.101E+05	16				

4.6 Effect of Process Variables on Pigment Yield

In the present work, the fungus growth in PDB results in the generation of secondary metabolites, which are observed through the production of a colored broth during the fermentation process. The effects of each variable and their interaction on pigment production were studied. Fermentation time and pH play a key role in the formation of pigments as a combined active mechanism that is likely influenced by the genetic and metabolic regulation of defensive mechanisms. This has an impact on the level of pigment concentration and the high productivity of pigments (Afshari et al., 2015).

4.6.1 Combined Effects of fermentation time, pH, and maltose concentration on reddish brown pigment concentration

The mutual interaction between the considered process parameters was revealed by the RSM analysis, and the results are depicted as surface plots in Figures 4.4, 4.9 and 4.10. The interaction between pH and maltose concentration, on the other hand, was found to be statistically non-significant where its p-value greater than 0.05. By altering the levels of any two independent variables while maintaining the level of the third variable at its fixed middle value, the influence of independent variables on pigment concentration was examined. By taking into account all potential combinations, three response surface plots were produced.

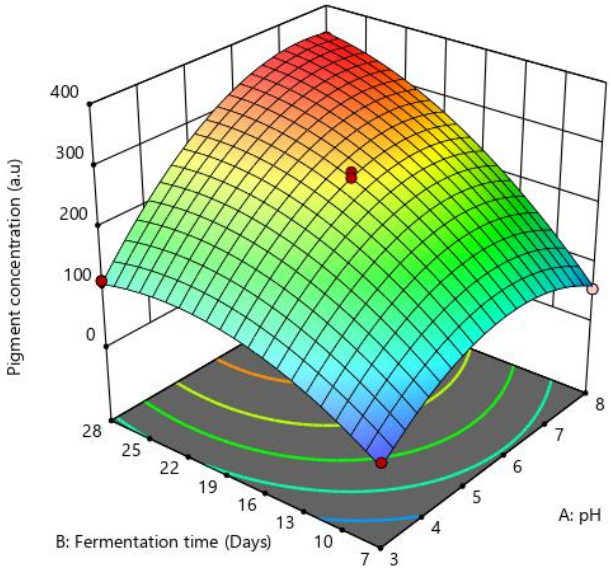
The interaction effect of pH and fermentation time

Figure 4.4 shows the effect of interaction between pH and fermentation time on pigment concentration. It was shown that both elements had a negative effect on the synthesis of the pigment at their lower concentrations. The synthesis of pigment gradually increased as pH and fermentation time increased. After pH 7.5 and fermentation time 27 days, the pigment concentration does not change much, which becomes constant. The pigment concentration during the fermentation process is better facilitated at a relatively low medium pH value and extended fermentation time with a maximum pigment concentration of 113.3 a.u. than high pH with a minimum fermentation time, which resulted in a lower pigment concentration of 55.4 a.u. Fermentation time has an effect on the pigment concentration when the pH increases to 8. This shows their interaction effect on the response.

Surprisingly, after twenty-seven days, the rate of pigment formation slightly decreases in a given range, which is attributed to cell autolysis due to the formation of toxic metabolites that lethally attack the microbes and also due to the reduction in nutrients. This stage of microbial growth is the decline phase. Increasing the fermentation time after pigment formation is a waste of time and also demolishing of pigments happens.

Even though fungi may grow in a wide range of pH (pH 1.4-9.8), pigment production is facilitated by a specific enzyme that best operates at a specific pH value (Frisvad, 2002). In this study, the result showed that raising the pH from 5.5 to 7.5 increased the pigment intensity.

Due to that, at this pH the enzymes in the species have favored conditions to synthesize the pigment. Beiri et al. (2021) reported that pH affects the functional activity of microbial enzymes and also more desirable products are produced only when the environment is more favorable for the production of products. As a result of changes in pH, the intensity and hue of the pigment is varied (Gmoser et al., 2017a). Here in the figure below the interaction effect of pH and fermentation time were depicted at maltose concentration 0.048 g.



C-Maltose concentration-0.048 g

Figure 4. 4: Three-dimensional response plot showing interaction effects of pH and fermentation time on pigment production

Figure 4. 5: The effect of fermentation time on pigment production

As can be observed from the experiment, the submerged fermentation process progressed with increasing pH and fermentation time in a given range. The color appearance developed from pH 5.5 to 8 to reddish brown whereas a light reddish brown color appeared at high acidic pH, when supplemented with maltose for 28 days (Fig. 4.6). This shows that the pH of the media significantly affects the color of the resulting pigment. In addition, the fermentation time also affects the pigment production process, in which the fungal production first increases and decreases at longer stay due to the possible formation of toxic metabolites. Similar observations were reported (Fouillaud et al., 2017).

a, b,

Figure 4. 6: A light reddish brown color at 28 days under acidic condition supplemented with maltose (a), an orange color at 28 days under acidic condition with PDB only (b),

In this study it was observed that, as other researchers studied, the pH significantly affected the shade of the pigment. The higher pigment intensities were observed from pH 5.5 to 7.5. This finding implies that more acidic pH was not favorable to the formation of intense pigments in the selected isolated fungi, leading to lower pigment production, whereas moderate acidic, neutral and moderate alkaline pH were more preferable. This finding opposes the results found by Gmoser, who interprets that high acidic pH is preferable for pigment production by fungal species, specially filamentous fungi (Gmoser et al., 2017b)

Figure 4. 7: The effect of pH on pigment intensity

The interaction effect of maltose concentration and fermentation time

The PDB in liquid media was supplemented with (1-3%) maltose. As indicated in table 4.3, there is a mutual interaction between maltose concentration and fermentation time, where its P value is less than 0.05, which is significant. From the results, it was concluded that increasing fermentation time and maltose concentration from 7-27 days and 0.024-0.048 g respectively enhanced pigment synthesis and then decreased it. As indicated in figure 4.8, increasing fermentation time enhances pigment production from 0.024g-0.048g maltose concentration. In any case, secondary metabolite formation often occurs after fungal growth has halted, as a result of food constraints combined with an abundance of carbon. This allows them to be manipulated in their formation (Fouillaud et al., 2017). When maltose is supplemented with the liquid media, the pigmentation is increased over the liquid media which constitutes only PDB. This is known by measuring based on visual observation of pigment color in flasks and the related UV-visible spectra.

From the above stated findings, it can be concluded that supplementation of carbon source facilitates and increases pigment formation, i.e. pigmentation increased when the carbon source was increased from 1% to 3% (Fig 4.8). These results corroborate with the results of some literatures (Chadni et al., 2017a; Pandey et al., 2018). Similar findings are also reported (Beiri et al., 2021; Chadni et al., 2017b; Menezes et al., 2020).

Figure 4. 8: The effect of supplementing carbon source (maltose) on media for pigment synthesis

As observed from Figure 4.8, the pigment intensity is higher for the sample supplemented with 0.072g of maltose than that supplemented with 0.024g of maltose.

In contrary, the pigment concentration decreases when above 0.048 g of maltose concentration supplemented to the fermentation process. Even if many researchers have reported that supplementing carbon source to the liquid media enhances pigment formation, the amount that is to supplement is also an effect on pigment formation. In this work it's concluded that supplementing carbon source up to 0.048 g to the media enhances pigment formation and lowers pigment production when much of sugar supplemented to it. This is because of a decrease in oxygen transfer due to the viscosity of the broth increases and the formation of primary metabolites. Similar reports were reported by Gibbs et al. (2000) where broth viscosity negatively influences oxygen transfer in submerged culture.

Thus, that means that the biomass growth enhances when maltose concentration is high. As a result the broth viscosity increases, which lessens the flow of oxygen to the bottom of the cell. Only the cells found on the top of the fermentation flask facilitate the fermentative product. Thus, fermented products kill the growing microorganisms. These primary metabolites may have adverse effects on the growth of fungal species. Those primary metabolites inhibit the work of enzymes which produce energy for the cell in the respiratory chain. These findings are consistent with those of

researchers findings (Manan, 2017; Menezes et al., 2020; Mukherjee & Singh, 2011; Sehrawat, 2017).

Pigment concentration of a fermentation process with low maltose concentration and low fermentation time (25.61 a.u.) has lower concentration than a fermentation process with high maltose concentration and lower fermentation time (27.07 a.u.). And also, a fermentation process with a high maltose concentration and maximum fermentation time (205.46 a.u.) has lower pigment concentration than a fermentation process with maximum fermentation time and lower maltose concentration (251.72 a.u.). The interaction effect between fermentation time and maltose concentration on pigment yield at fixed pH 5.5 was shown in figure 4.9.

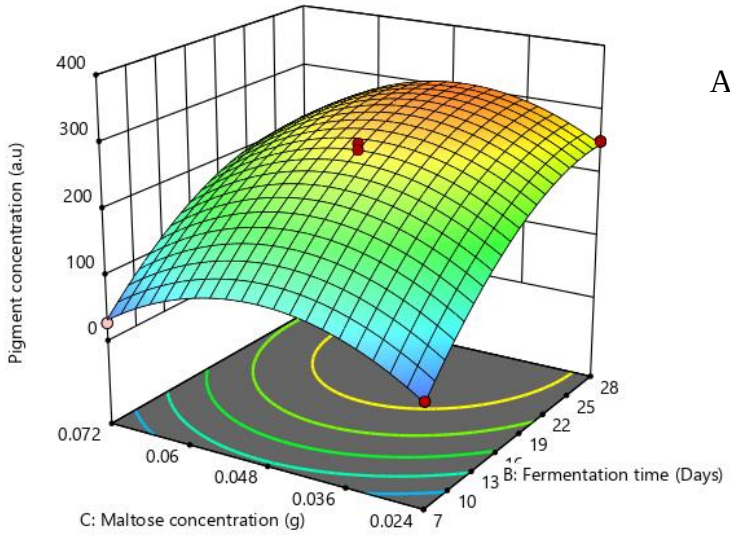


Figure 4. 9: Three-dimensional response plot showing interaction effects of Maltose concentration and fermentation time on pigment production

The interaction effect of pH and maltose concentration

The interaction effect of pH and maltose concentration was found to be insignificant, where in the pH range investigated, inconsequential dependence of pigment concentration on the amount of maltose concentration was supplemented (Figure 4.10). Additionally, increasing maltose concentration and pH from 0.024-0.048 g and 3-7 respectively enhanced the pigment production and then decreased gradually. Thus, the change in levels of pH does not have significant interaction

with maltose concentration. The lines are parallel. The interaction effect between pH and maltose concentration at 17.5 fermentation time was shown in figure 4.10.

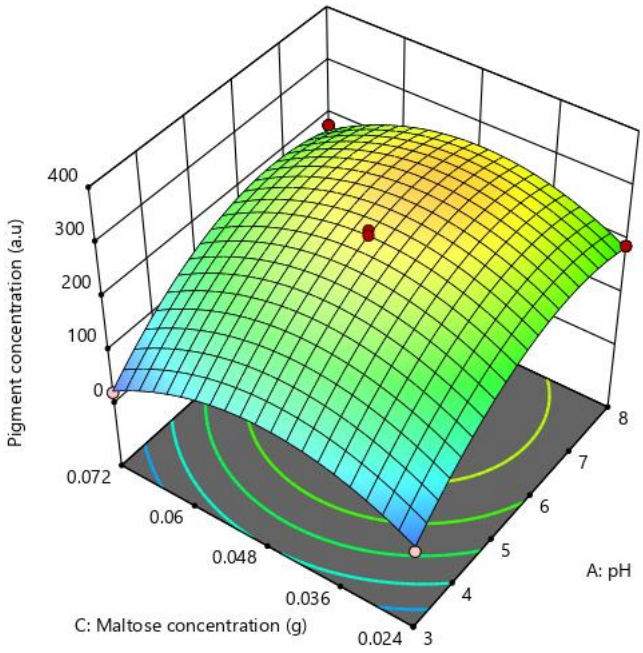


Figure 4. 10: Three-dimensional response plot showing interaction effects of Maltose concentration and pH on pigment production

4.7 Fermentation Conditions Optimization

For response, the second-order polynomial developed models in this study were applied in order to achieve the desired optimum values. Numerical optimization by the design expert was used to determine the best conditions for Aspergillus niger to produce maximum pigment during submerged fermentation.

The acquired model was applied to provide a solution that optimized pigment production while keeping the target pH, fermentation time, and maltose concentration in the given range and the response to be maximized. The software recommended values pH = 7.678, maltose concentration = 0.045 g, and fermentation time =27.953 days for achieving of a pigment concentration of 357.425 a.u..

The optimal condition was tested in a triplicate experiment. The experimental result and predicted values agreed well. The relative error between the experimental (349.93) and predicted values (357.425) were 2.1%. The verification value is within 98 % of the predicted values, indicating that the model effectively optimized pigment yield from Aspergillus niger within the given range of process parameters and fitted the experimental data very well.

4.8 Model Validation

The predicted values for the pigment concentration were compared to the experimental values. The proposed model was shown to be reliable and valid because there was a strong correlation between experimental results and predicted values (Figure 4.11c).

The normal probability plot of the residuals given in Figure 4.11a indicates the residuals are generally along a straight line, and this implies the residuals have a normal distribution in the given probability range. The residuals are the difference between the actual and predicted values of experimental runs by the design expert. Thus, one can generalize that there is not a problem with the normality in the data. Hence, there are no severe outliers to be found. The points in the plots for this experimental data fit in to the straight line in the figure, demonstrating that the quadratic polynomial model satisfies the analysis of variance (ANOVA) assumptions that the error distribution is estimated normally.

As shown in figure 4.11c, the predicted vs. actual figure represents that all points are near to the straight line, thus the regration is best fit to the model. On the other hand, the residual vs. run also shows that there are no outliers of data which verify the system’s stability and the points scattered are dispersed inside the red limited lines (Figure 4.11b).

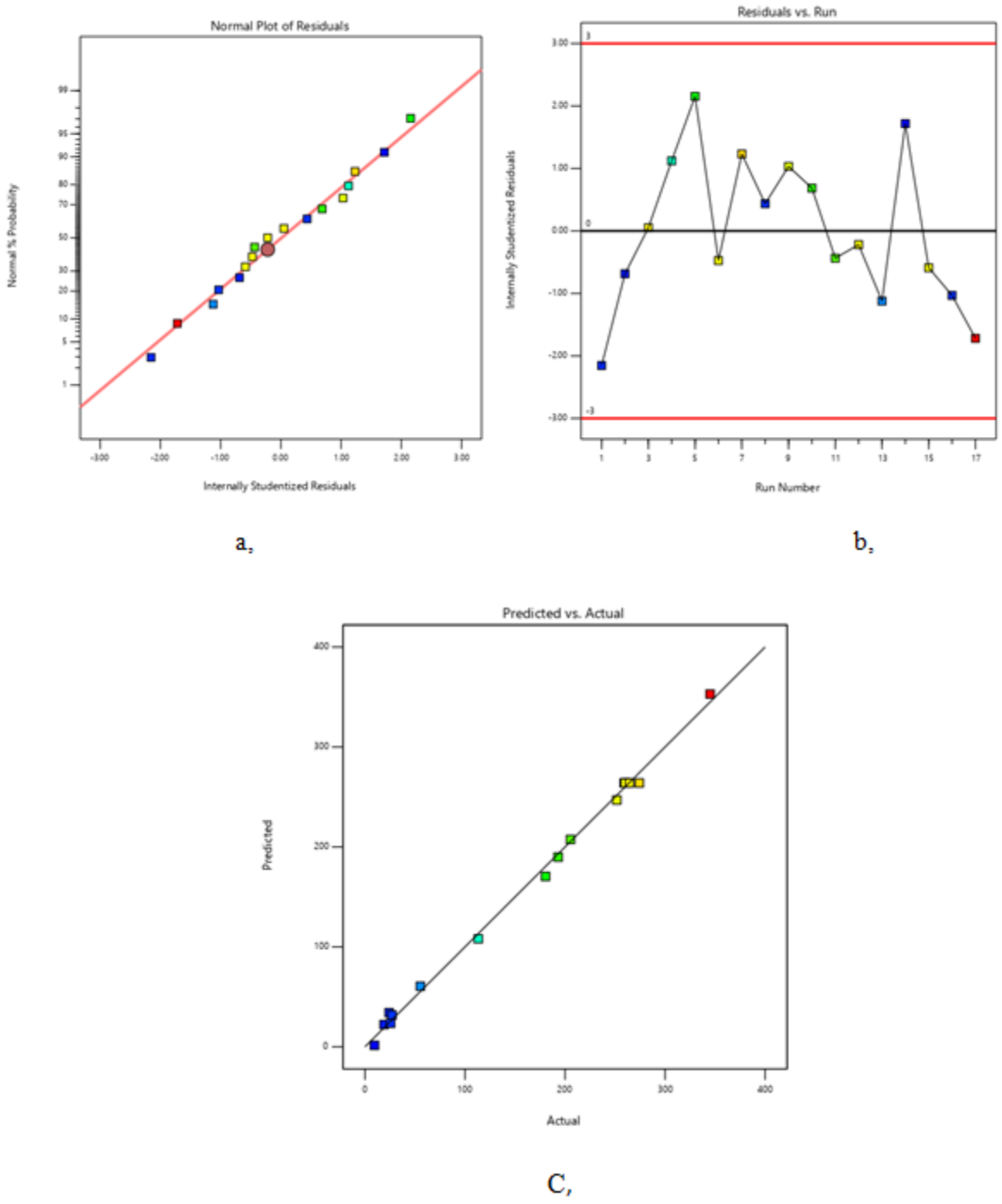


Figure 4. 11: Diagnostic plots for the adequacy of proposed model for pigment concentration

4.9 Effect of different carbon source on pigment production

After optimization was performed, the effect of substitution of another carbon source (starch soluble and glucose), instead of maltose and PDB only (without any carbon source supplemented) as a control group, was studied. The study revealed that maltose supplementation resulted in higher pigment concentration as shown in Figure 4.12, which may be because the rate of assimilation of maltose by the species is different than other carbon sources (Aftab et al., 2021; Singh et al., 2017). In this study it was observed that a fermentation process supplemented with maltose (349.93±4.65 a.u.) has enhanced pigment intensity than a fermentation process with PDB (253.85±1.5 a.u.) only, glucose (253.13±1.4 a.u.) and starch soluble (265.6±2.2 a.u.).

Figure 4. 12: Submerged fermentation of Aspergillus niger species with PDB only (a), supplemented with maltose (b), Supplemented with starch soluble (c), and supplemented with glucose (d)

4.10 Purification of the microbial pigment using TLC

TLC was used to analyze the polarity and purity of the pigment. Under the TLC analysis, different polar and non-polar types of solvents were used to separate the pigment. In the present study the pigment were concluded that, it is a high polarity compound thus because of its more stickiness to the silica of the TLC plate during the use of chloroform solvent (RF=0). The pigment was moved out with the polar solvents (methanol, ethanol, and ethyl acetate); this shows the solvents used were the same property with the pigment. But they are weak solvents to separate the components with in the pigments.

In addition, hexane and toluene were used to separate the components in the pigments. During the experiment, hexane was found to be the best solvent with RF value of 0.647 and toluene 0.70175 to resolve the extracted pigment. As a result many experiments must be done to ensure and select the best solvent used to separate the components in the pigment to purify and characterize the single component that is essential to make that color.

4.11 FT-IR analysis

The FT-IR spectrum of pigment sample is shown in figure 4.13. It was measured the spectral range between 400 and 4000 cm^{-1}. The main absorption peaks observed include 3417.48, 2924.7, 2852.7, 1634, 1454, 1311, 1266, 773.6, 612, 532 cm^{-1}. The very broad band that extends from about 2500 to 3800 cm^{-1} and centered at 3417.48 cm^{-1} can be attributed to the carboxylic acid hydroxyl group (O-H) absorption. The two shoulder bands at 2924.7 cm^{-1} and 2852.7 cm^{-1} correspond to C-H asymmetric and symmetric stretching vibrations, respectively. The peaks at 1634 cm^{-1} and 1454 cm^{-1} are due to the presence of alkene C=N (C=C) and methyl N-H groups. The bands at 1311 and 1266 cm^{-1} may be assigned to C-N stretching and C-O stretching frequencies.

The FT-IR spectrum of the pigment indicates some similarities with the prodigiosin pigment produced by Serratia marcescens NPLR1 (Sumathi et al., 2014). According to Sumathi et al. (2014) the red pigmented prodigiosin was found to have a wide band between 3600 and 3300 cm^{-1} that was centered at 3416 cm^{-1} and attributed to the N-H stretch and the peaks 2916 and 2852 cm^{-1} are caused by methylene groups being stretched symmetrically and asymmetrically, respectively. The presence of –C=N and methyl groups is what cause the peaks at 1652 and 1445cm^{-1}, respectively. The C-O groups and carbon-carbon double bonds in prodigiosin are responsible for the peaks at 1379 cm^{-1} and (1293 cm^{-1} and 718 cm^{-1}) respectively. This suggests that the pattern of the Aspergillus niger pigment is comparable to that of prodigiosin since the functional group constituents in the pigment resembles to the prodigiosin pigment. In addition, the spectral analysis of the produced pigment were showed similarities with the researchers reported functional group constituents of prodigiosin pigment (Elrazak & Osman, 2017; Suryawanshi & Patil, 2014; Tunca et al., 2022).

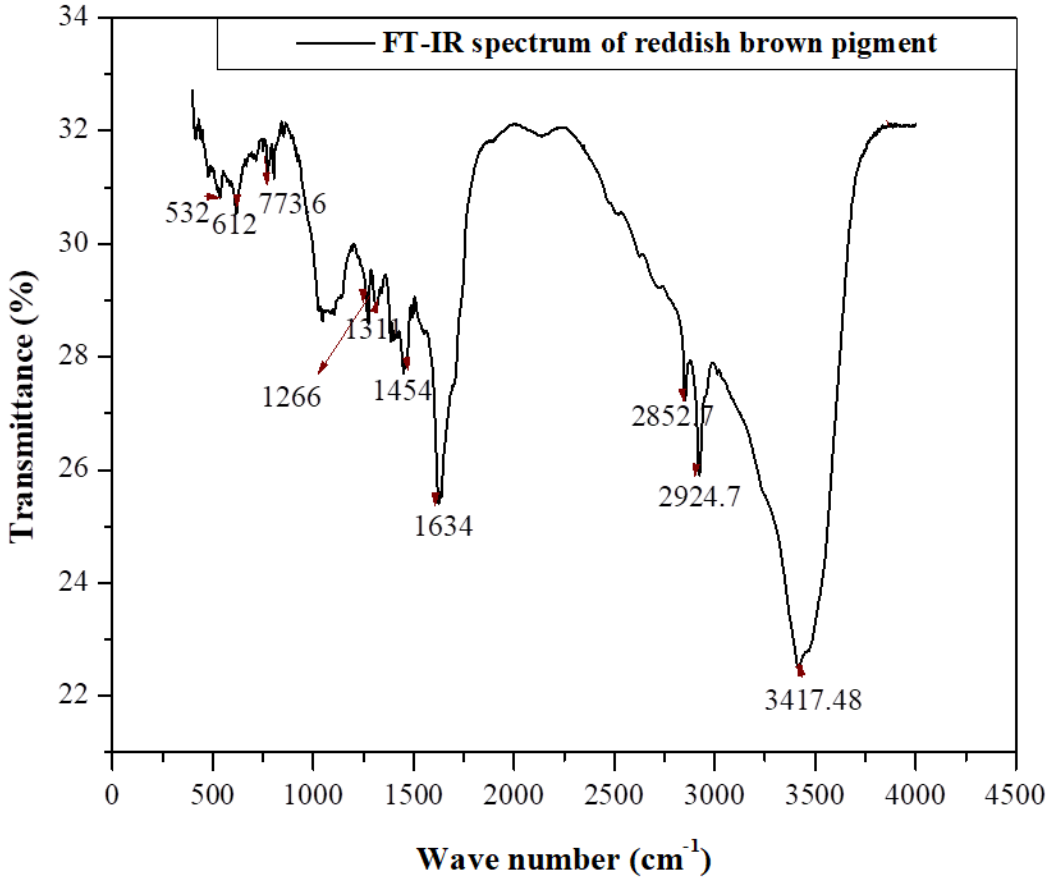


Figure 4. 13: FT-IR spectrum of reddish brown pigment prepared under optimum condition

4.12 Application of pigment on preparation of bio-paint

In the present study, the potential pigment for use as wall paint was investigated. When the (pigment + binder) mixed with a limestone ($CaCO_3$), it turns its color to a light purple red color as shown in figure 4.14. Sastry (2016) reported that when a red microbial pigment mixed with lime, it changes its color to a shade of brown. The measured pH, specific gravity, viscometer, and drying time of bio-paint were listed in the table. These measured characteristics were compared with the synthetic paint characteristics (the data was gathered from current Ethiopian paint factories, BEETAR Paint factory, Bahir Dar).

Figure 4. 14: Illustrations of the application of bio-paints made with fungal dyes

Table 4. 4: Measured parameters of bio-paint

Measured parameters	Synthetic paint (Super water paint)	Bio-paint
pH	6.5-8.5	6.8 $\pm$ 0.57
Viscosity	110-120 ku	55 mPas $\pm$ 1.34
Specific gravity	1.18-1.44	1.3296 $\pm$ 0.06
Density	1.18-1.44 g/ml	1.3272 g/ml $\pm$ 0.04
Drying time	30 min	30 min $\pm$ 1.63

5. Conclusion and Recommendation

5.1 Conclusion

These thesis findings suggest that microbes in general and fungus (Aspergillus niger spp.) in particular, are reliable sources of natural colorants. In this study, a reddish-brown-colored pigment was successfully extracted from the broth. The synthesis of these fungal colors and media in general, maltose concentration in particular and carbon source, the parameters, *i.e*. (pH, and fermentation time are found to significantly affect the intensity and color of the hues. The effect of pH, fermentation time and maltose concentration were investigated and optimized using RSM using design expert version 13, whereby a quadratic model was developed to describe the process by the design expert. It was found, using submerged fermentation, that pH = 7.678, fermentation time = 27.95 Days and maltose concentration = 0.045 g using to give higher pigment production of 357.425 a.u... Further, a carbon source, maltose, was found to enhance the pigment intensity.

Fungal dyes can circumvent the problems associated with synthetic dyes including environmental toxicity, high treatment costs and secondary wastes formed during disposal activities. Even though various dyes are used by the industry, this thesis mainly focused on the potential of the pigment as a paint colorant. Utilizing scientific information based on the history of paints, ecofriendly paints can be prepared using fungal colorants from readily accessible natural resources. The prepared bio-paint has pH of 6.8$\pm$0.57, a viscosity of 55 mPas $\pm$ 1.34, a density of 1.3272 g/ml $\pm$ 0.04 and a specific gravity of 1.3296 $\pm$ 0.06 and a drying time of 30min $\pm$ 1.63. In the pigment purification, hexane found to be the best solvent in resolving the pigment compound using TLC with R_F value of 0.647. The pigment was further characterized using FT-IR spectroscopy. The FT-IR characteristic absorption peaks are qualitatively comparable with the literature spectral data, and therefore, the main constituents of the pigment produced are carboxylic acid hydroxyl group (O-H), C-H asymmetric and symmetric stretching vibrations, alkene C=N (C=C) and methyl N-H groups, C-N stretching and C-O stretching. However, in order to unambiguously determine the chemical nature and purity of the pigment, NMR spectral analysis is required, which was not within the scope of the study.

5.2 Recommendations for Further Research

The following suggestions are made for future works, taking into account the extremely promising findings obtained in this thesis with the aforementioned restrictions: The potential of intracellular pigment production of Aspergillus niger species should be investigated. It is needed to work on the factors affecting pigment yield that are not studied here to enhance pigment yield. The binder used for this study is casein milk, which is expensive, and needs a lot of research to use a good binder to use for a specific industrial purpose. Further research is needed on the replacement of the synthetic media by agricultural waste. The compatibility of extracted pigments from fungal species with various types of binders must be investigated further to determine the success of the pigment's dyeing capability when utilized as a potent coloring agent. More research will be conducted on optimizing different nutrient kinds and their concentration in culture media. Further research is needed on determining the chemical structure of Aspergillus niger's fungal pigment using advanced analytical techniques including HPLC, NMR, and LCMS.

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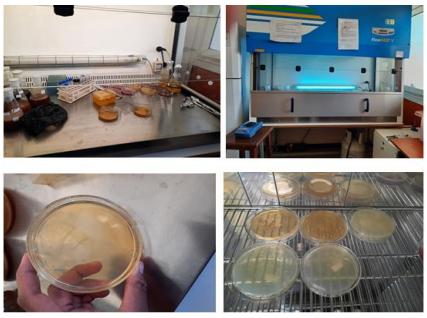
Appendixes A

a,

b,

The microbe (Aspergillus niger species) in a vile, which is taken from EIB (a), Preserve and store Aspergillus species in a freezer (b)



Paint the Aspergillus niger species on a Petridish containing solidified agar with the spreader in the biosafety cabinet, then put it in an incubator to grow

a,

b,

c,

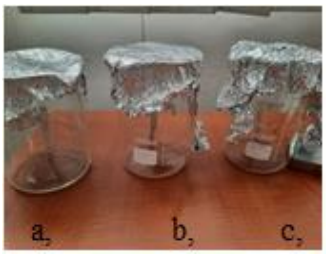
Prepared liquid media using PDB and maltose as nutrient (a), Adjust the pH to the desired pH value (b), sterilizing the prepared media (c)


Submerged fermentation of Aspergillus niger species in liquid media with in a controlled temperature (30^{o}c) and speed (150 rpm)



a, b, c,

Extraction of extracellular pigment from the broth (a), Centrifugation of the pigment to remove cell debris (b), a pigment kept in the refrigerator for further analysis (c)



Left represents the pigment dissolved in hexane (a), toluene (b) and methanol (c); whereas right represents the corresponding TLC results.

Representative pictures of preparation of bio-paints using milk casein and natural ingredients.

Apparatus used in preparing media (a), Measuring 3.9 g of PDA powder using analytical electronic balance (b), A stove used to boil PDA solution (c), An autoclave used to sterilize media containing flask and pippets (d), Working in biosafety cabinets for protection against biocontaminants.

Left: Appearance of Aspergillus niger species from white to black conidia color formation during cultivation (a and c) and Right: The petri pelets shows no microbial growth which is a control group (b)

Progress of fermentation

Preservation and storage of cultivated fungal species and sub cultured fungal species for further experiment

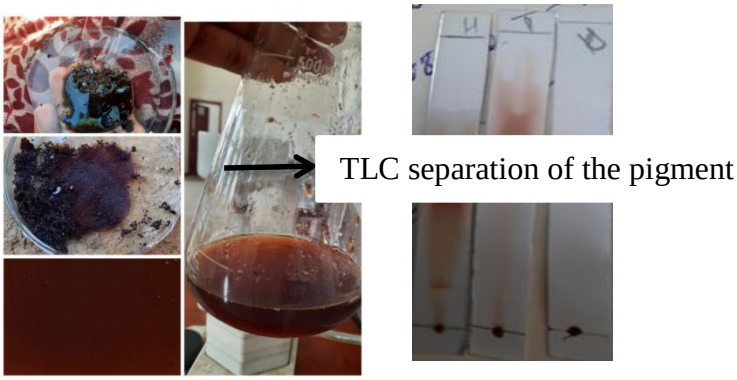
Overall picture representation of pigment production process

Fermentation with pH 3,5.5,5.5 and 8 respectively for 28 days

Fermentation with pH 5.5 for 17.5 days

A submerged fermentation under different condition (carbon source (maltose, glucose, and starch soluble)), and pH=7.678, fermentation time=27.953 days, and 0.045 g of carbon sources.

Reddish brown pigment produced under pH (5.5-8) and an orange pigment produced under pH of (pH<5.5) in submerged culture from Aspergilllus niger species.

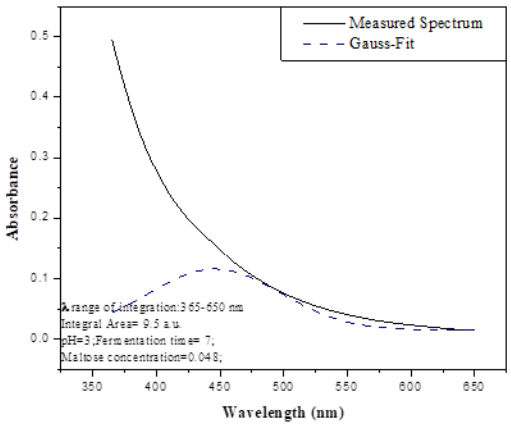


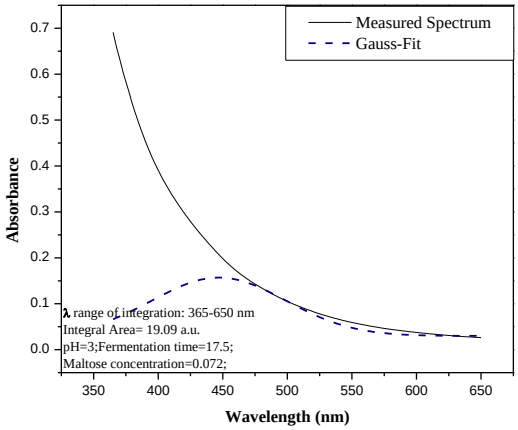
A reddish brown pigment produced under submerged fermentation from Aspergillus species

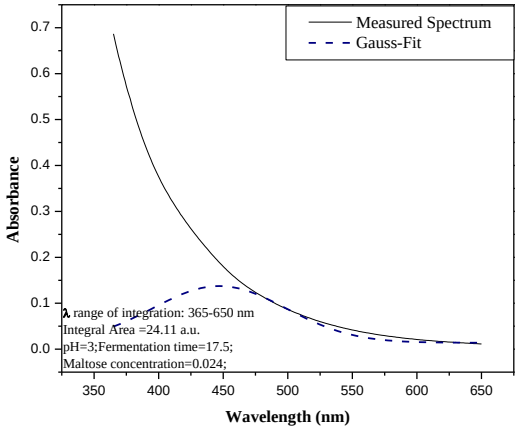
Representative pictures of some characteristics of a bio-paint with pH (6.8±0.57), density (1.3272 g/ml±0.04), specific gravity (1.3296±0.06) and viscosity (55 mPas±1.34)

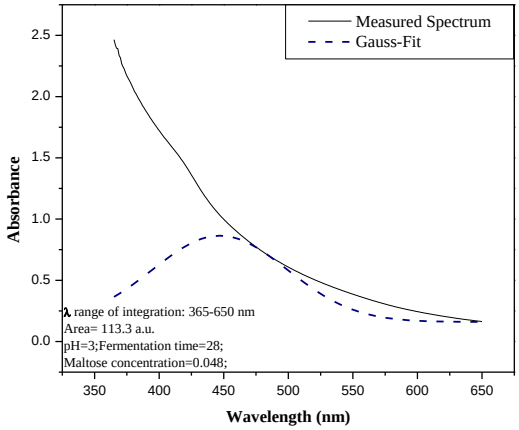
Appendixes B

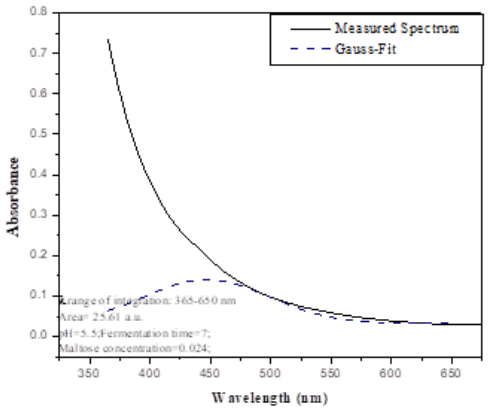
Visible spectrum and gauss fit of the pigment under different parametric conditions

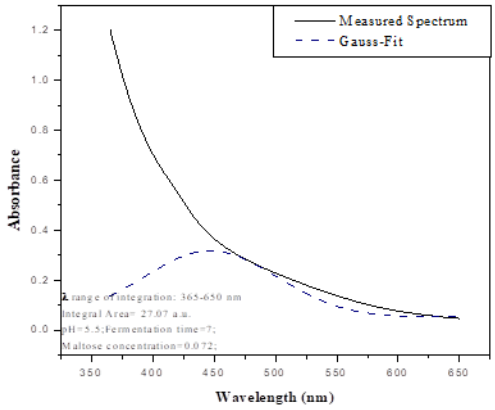


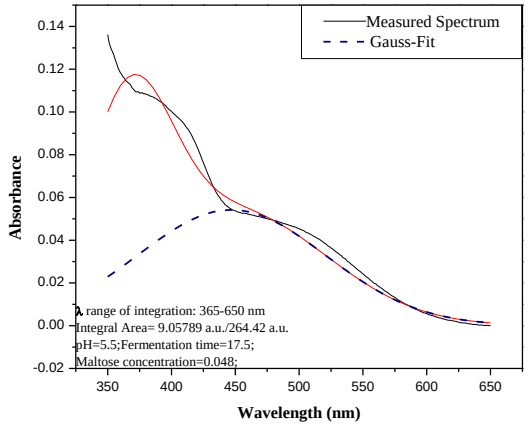


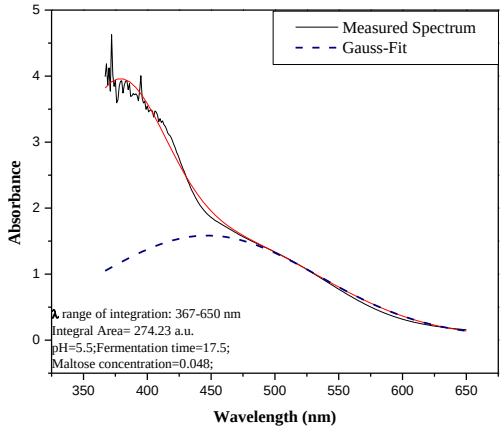


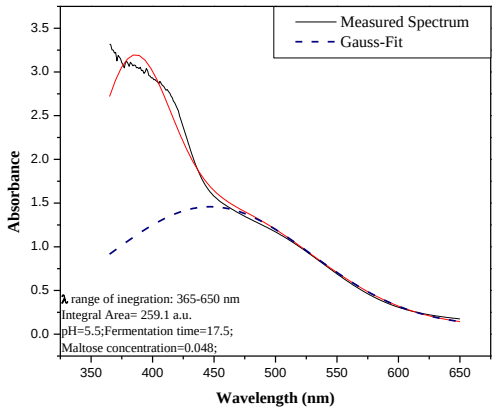


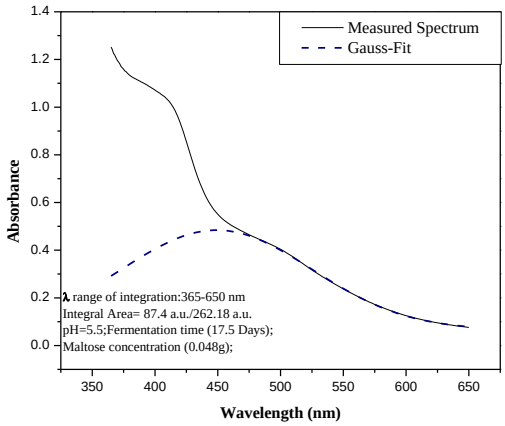

Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 365-650 nm
Area= 25.61 a.u.
pH=5.5;Fermentation time=7;
Maltose concentration=0.024;

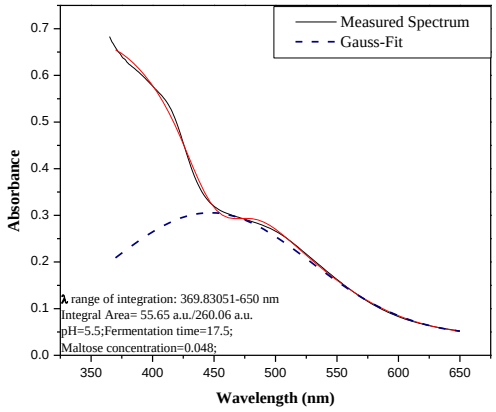

Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 365-650 nm
Integral Area= 27.07 a.u.
pH=5.5;Fermentation time=7;
Maltose concentration=0.072;

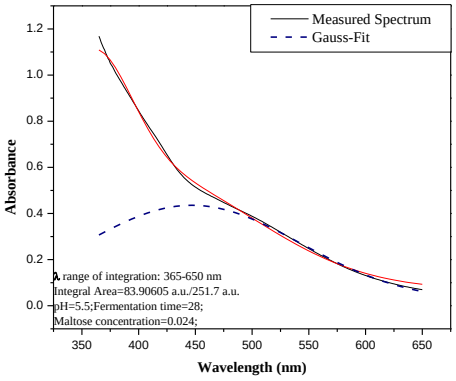

Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 365-650 nm
Integral Area= 9.05789 a.u./264.42 a.u.
pH=5.5;Fermentation time=17.5;
Maltose concentration=0.048;

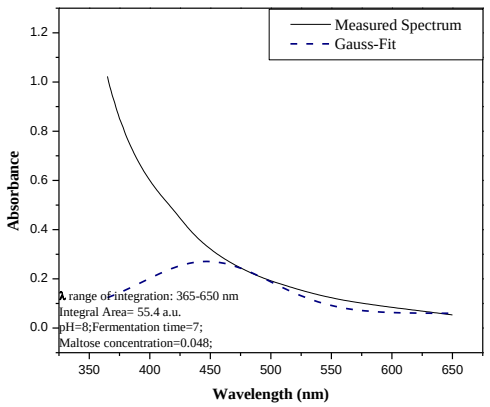

Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 367-650 nm
Integral Area= 274.23 a.u.
pH=5.5;Fermentation time=17.5;
Maltose concentration=0.048;

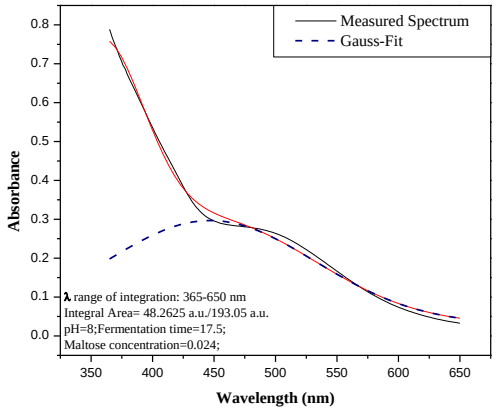

Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of inegration: 365-650 nm
Integral Area= 259.1 a.u.
pH=5.5;Fermentation time=17.5;
Maltose concentration=0.048;


Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration:365-650 nm
Integral Area= 87.4 a.u./262.18 a.u.
pH=5.5;Fermentation time (17.5 Days);
Maltose concentration (0.048g);


Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 369.83051-650 nm
Integral Area= 55.65 a.u./260.06 a.u.
pH=5.5;Fermentation time=17.5;
Maltose concentration=0.048;


Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 365-650 nm
Integral Area=83.90605 a.u./251.7 a.u.
pH=5.5;Fermentation time=28;
Maltose concentration=0.024;


Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 365-650 nm
Integral Area= 55.4 a.u.
pH=8;Fermentation time=7;
Maltose concentration=0.048;


Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 365-650 nm
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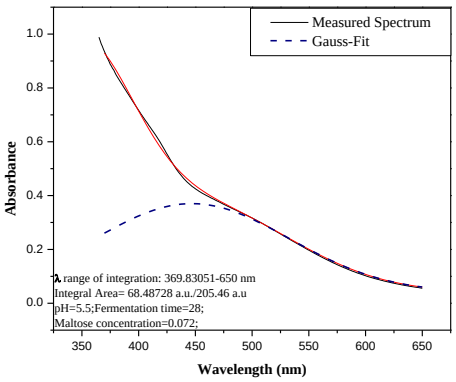
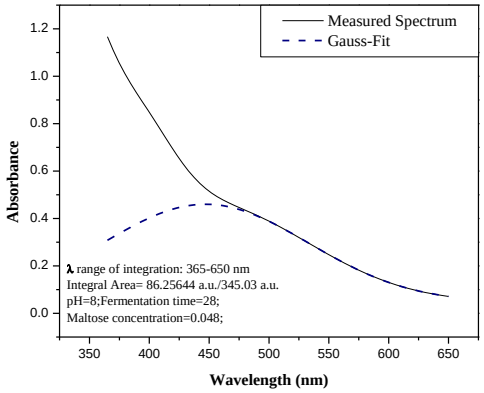
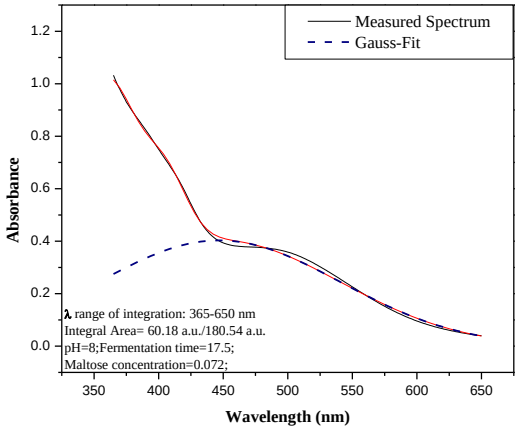
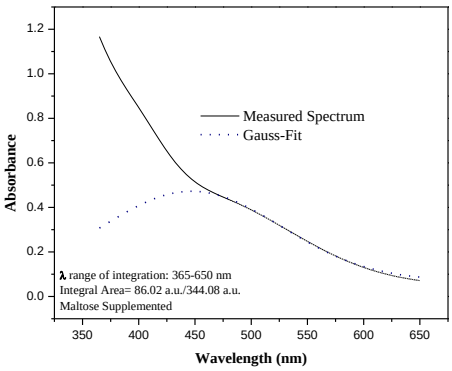
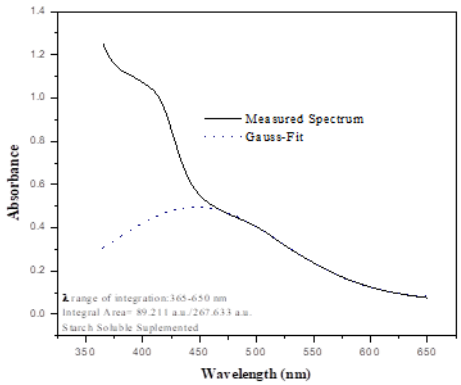
pH=8;Fermentation time=17.5;
Maltose concentration=0.024;

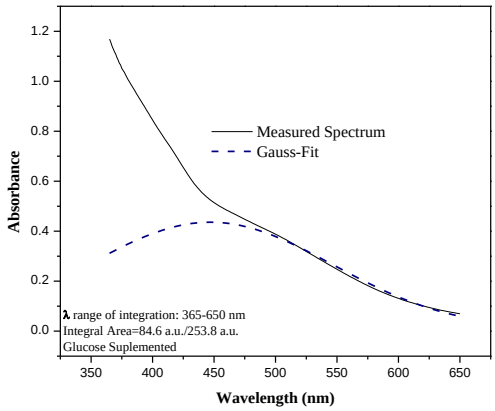

Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 369.83051-650 nm
Integral Area= 68.48728 a.u./205.46 a.u
pH=5.5;Fermentation time=28;
Maltose concentration=0.072;

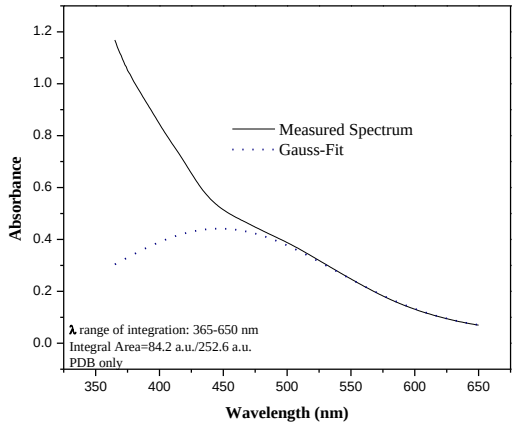

Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 365-650 nm
Integral Area= 86.25644 a.u./345.03 a.u.
pH=8;Fermentation time=28;
Maltose concentration=0.048;


Measured Spectrum
Gauss-Fit
Absorbance
Wavelength (nm)
λ range of integration: 365-650 nm
Integral Area= 60.18 a.u./180.54 a.u.
pH=8;Fermentation time=17.5;
Maltose concentration=0.072;









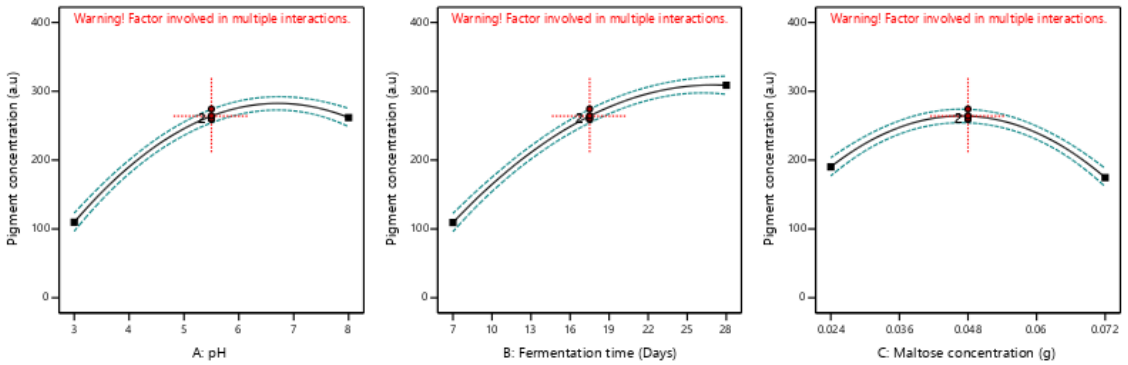
Visible spectrum and gauss-fit of three fold diluted pigment of fermentation with maltose supplemented, glucose supplemented, starch soluble supplemented and PDB only.

Appendixes C

Pictures that are taken from the design expert analysis (Box-Behnken design under Response methodology)

The effects of main effects on the response

Actual Factors
A = 5.5
B = 17.5
C = 0.048



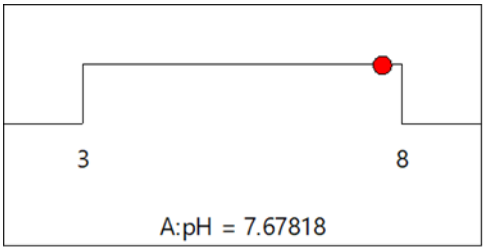
Constraints

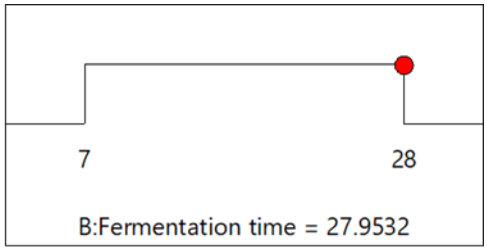
Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:pH	is in range	3	8	1	1	3
B:Fermentation time	is in range	7	28	1	1	3
C:Maltose concentration	is in range	0.024	0.072	1	1	3
Pigment concentration	maximize	16.53	345.03	1	1	3

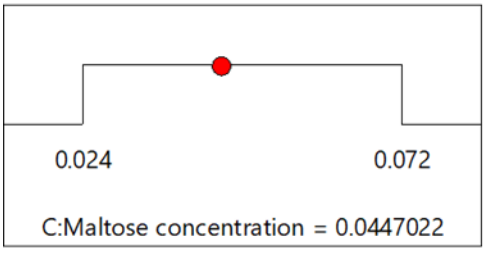
Solutions

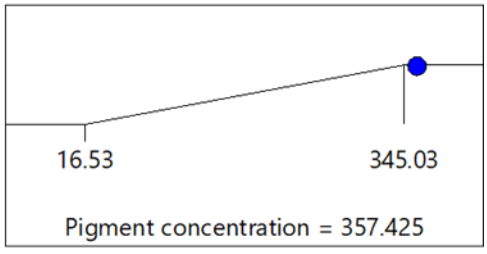
1 Solution was selected among 100 solutions found according to the software

No	pH	Fermentation time	Maltose concentration	Pigment concentration	Desirability	
1	**7.678**	**27.953**	**0.045**	**357.425**	**1.000**	**Selected**









Desirability = 1.000
Solution 1 out of 100