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Optimization, a Potential Production, GC-MS and Characterization of Dark Green Pigment from New Local Isolate *Streptomyces nigra* Strain GH12

Gehad H. El Sayed [a], Mohamed Fadel [a*] and Mohamed Marzouk [b]

[a] Microbial Chemistry Department, Biotechnology Institute, National Research center, Dokki, Giza, Egypt.
[b] Chemistry of Tanning Materials and Leather Technology Department, Chemical Industries Research Institute, National Research Centre, Dokki, Giza, Egypt

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Due to increasing health awareness among consumers and the identification of novel pharmacological properties of diverse natural pigments, the market for natural pigments is currently seeing rapid growth. *Streptomyces. nigra* GH12, a new Actinomycete isolate identified by 16S rRNA used for dark green pigment production. Manipulations of the factors affecting pigment production are the most effective way to have maximum yield of pigment. Starch 2.5% (w/v), ammonium nitrate 0.2% (w/v), dibasic sodium phosphate 0.1% (w/v), inoculums size 6% (v/v), initial pH level of 8, 150 rpm rotation speed, 37 ºC temperature, and 9 days of incubation were found the optimum growth conditions for the highest green pigment production. The extraction of

**Corresponding author: E-mail: mfadel5455@gmail.com;*

green pigment was performed efficiently with ethanol solvent. Results showed no effect on the pigment content at 40, 50 and 60°C. On heating pigment extract at 70, 80, 90 and 100°C for 60 min, retention of pigment remained as high as 97.4, 93, 86 and 79% of the total pigment density , respectively, even with continuous treatment at 100°C for more 60 min. The results indicated that the pigment extracts exhibited their greatest stabilities at high pH values, i.e. 8.0, 8.5, 9.0 and 10.0, during the entire holding time. The pigment was characterized by full UV-visible spectroscopic screening, where the potential absorption maximum of the target green pigment extract was recorded in the range of 300–360 nm, with a λ_{max} at 340 nm. Moreover, the chemical composition diversity of the pigment extract was further investigated by GC/MS analysis that revealed the presence of 57 metabolites in their silylated form.

Keywords: Actinomycetes; green pigment; culture conditions; extraction; UV and GC/MS characterization.

1. INTRODUCTION

Pigments are defined as finely divided and usually water-insoluble colorants, absorbing and reflecting the visible light to show different colors [1]. They are of larger molecular weights, less water-soluble, and less transparent than dyes. The usage of pigments has a long history, dating back to the beginning of ancient civilizations (ancient China, India, and Egypt), whereby natural plants, insects, and minerals were used to dye textiles, color foods, manufacture paints, color the body in religious ceremonies, and more [2]. Pigments are used to manufacture various products because they can enhance the natural color or replace color lost during the manufacturing process, generating greater consumer appeal by adding a novel sensory aspect. In the field of colors development and production, a recent worldwide trend large-scale research is being done to replace synthetic dyes with natural colors. The colors derived from plants or microorganisms suggest a certain level of safety. Pigments' toxicological issues are less severe than those associated with their synthetic counterparts due to their previous history and usage habits [3,4]. Three primary sources for a sustainable eco-friendly production of bio-safe natural pigments include plants, animals, and microbes [5]. As a result, one of the exciting and developing fields of study that shows promise for a range of industrial applications is microbial pigment synthesis [6-8]. Additionally, some microbial pigments have been found to be stable to light, heat, and pH, contain pro-vitamin A, and exhibit anticancer action [9]. However, from an industrial standpoint, it is important to create a high-tech and economical harnessing for the widespread manufacture of different microbial pigments [10]. Natural pigments are now used more frequently in cosmetics, pharmaceuticals, food, and textiles [11,12]. Additionally, it is important to note that the natural pigment market is expected to grow at a 12.4 CAGR to reach USD 3.5 billion in the food industry by 2027 [13]. One of the most intriguing genera among microorganisms that generate pigments is *Streptomyces*, which has a high capacity for reproduction and the ability to make melanin, one of the most prevalent pigments in the market [14,15]. The intriguing genetic dispersion of this particular species of actinomycetes makes it appealing for replication in the biotechnology sector [16,17]. Additionally, Streptomyces are well known for producing a variety of bioactive compounds, including antibiotics, anti-inflammatory, antioxidants, and cytotoxins, through their secondary metabolism, which is highly abundant and structurally diverse [18,19]. Numerous generated compounds were colored and given the bioactivity potential showed by *Streptomyces* strains [20,21]. Additionally, many of the pigmented substances produced by Streptomyces could be a promising avenue for discovering bioactive pigments. According to Elattaapy and Selim, 2020 [22] chemical, physical, and physiological parameters, such as anaerobic environments and temperatures, have an impact on pigment formation. Actinomycetes produce pigments that contain bioactive agents like antibacterial, antioxidant, and anticancer agents. There is a lot of work being done to identify and use the microbial pigments and their producers due to the growing demand for biological pigments over synthetic colorants. Actinomycetes are regarded as the ideal targets since pigment production appears to be more prevalent in them than in any other microbial group. Accordingly, this research directed to find optimized production conditions and characterization of a green pigment biosynthesized by a new local *Streptomyces* isolate, namely *S. nigra* strain GH12.

2. MATERIALS AND METHODS

2.1 Sample Collection

Rhizosphere soil samples were procured from a depth of 8–10 cm using a sterile spatula. The samples were brought to the laboratory and processed further immediately.

2.2 Pre-treatment of the Soil Sample

The soil samples were pretreated in order to reduce the proportion of other microorganisms differ from Actinomycetes. The soil samples were dried at about 50–60°C for 5–10 min [22].

2.3 Isolation and Screening of Actinomycetes

In order to isolate Actinomycetes from different rhizosphere soil samples gathered from different locations in cultivated fields from Aswan, Red sea, Alfayoum and/or Kafr Elshykh governorates. The samples were dried at 60^{o}C for 1 h and then 1g of the respective sample was suspended in 9 ml of distilled water taken in a pre–autoclaved sterile test tube. The suspension was then serially diluted up to 10^{-5}. The diluted suspension (100µl) of 10^{-3}, 10^{-4}, and 10^{-5} was spread plated on Actinomycetes isolation agar (AIA). The medium contained (g/L) starch (20), KNO_3 (2.0), K_2HPO_4 (1.0), $CaCO_3$, (3.0), $MgSO_4$ (0.5), NaCl (0.5), $FeSO_4$, (0.01), and agar (20) at *p*H (7.8). The culture was sterilized by autoclave at 121^{o}C for 15 min and incubated at 28^{o}C for 5 days. All the plates were screened for Actinomycetes colonies based on morphology and pigmentation. The actinomycete colony that showed significant coloration was sub–cultured onto fresh medium by streaking until a pure culture was obtained.

2.4 Molecular Identification of Isolate

2.4.1 Genomic DNA extraction

A single colony of each bacterium was cultured in a 100 ml conical flask (Pyrex, USA) containing 20 ml of TSB by shaking in an orbital shaker (Thermo Fisher Scientific, USA) at 180 rpm for 18 h at 37°C. Bacterial culture then subjected to genomic DNA extraction using Gene JET Genomic DNA Purification Kit (Thermo scientific, USA) according to the manufacturer's instructions. Genomic DNA was used for16s rDNA gene amplification.

2.5 16s rDNA Amplification and Sequencing

The fragment of 16s rDNA gene was amplified in a reaction volume of 25 µl containing 12.5 µl of PCR Master Mix 2x conc. (Thermo scientific, USA). One microliter of each forward and reverse primer sets 8 F/1492 R, 9.5 µl of sterile deionized water, and 1 µl of the bacterial genomic DNA were mixed. The reaction was achieved in Gene Amp PCR system 9600 (Applied Biosystems, Bedford, MA, USA). A temperature cycling program [23] was applied with a slight modification by a hot initiation as 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 10 min. PCR product was analyzed on a 1% agarose gel by electrophoresis using a 100-bp ladder DNA marker (Invitrogen, California, USA). The gel was visualized and photographed using ™XR + Gel Documentation System (Bio-Rad, California 94547, USA). Purified PCR product was subjected to sequencing by Sanger sequencing method using sequencer 3500 genetic analyzer, big dye X terminator kit (Thermo Fisher, USA) for forward and reverse directions in biomedical laboratory of colors (Clinilab, Egypt). BLASTn was used to detect the evolutional relationship with other relatives (https://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Multiple sequence alignment of 16s rRNA sequence from this study plus 16s rRNA sequences retrieved sequences from Gene Bank was performed using MUSCLE algorithm [24] available in MEGA X [25]. The evolutionary history was inferred using the neighbor-joining method [26].

2.6 Inoculum Seed Preparation

Five days old Actinomyceses culture slants were obtained by addition 10 ml sterilized water, where the growth was crushed with culture loop. 1% of spore suspension was used to inoculate 250-ml conical flasks containing 50 ml of pigment production medium constituted (g/L) starch, 20, KNO_3, 2.0, K_2HPO_4, 1.0, $MgSo_4$, 0.5, NaCl, 0.5, $CaCO_3$, 3.0, $FeSO_4$, 0.01, *p*H, 7.8. Afterward, incubation was done in a rotary shaker at 150 rpm for 5 days at 30^{o}C. The culture growth was used to inoculate the experimental flasks at 2% v/v.

2.7 Standardization of Culture Conditions for Optimum Pigment Production

The effect of various cultural conditions were studied like *p*H (6, 6.5, 7, 7.5, 8, 8.5 and 9),

different carbon, nitrogen, and phosphorous sources and their concentrations, different inoculum size (2-8) %, different culture medium volume (25-125) ml of broth medium in 250 ml conical flask, different heavy metals ions (Zn^{++}, Mn^{++}, Cu^{++} and Cr^{+++}), different incubation temperatures (25, 28, 31, 34, 37 and 40ºC) and different incubation period (3-12 days). The pigment production was studied separately by inoculating bacterial suspension of *S. nigra* GH12.

2.8 Extraction of the Pigment

At the end of fermentation period, the whole biomass of culture filtered off through a Whatman No. 1 filter paper. The filtrate was then centrifuged at 10,000 rpm for 10 min. The supernatants were considered the source of extracellular pigment. The analysis of pigment production was conducted by measuring the absorbance of the filtered extract at 340 nm using spectrophotometer (UV spectrophotometer V-630, JASCO, Japan). Extraction of the pigments was carried out using different solvents ethanol, methanol, acetone, propanol, hexane, water and supernatant samples was taken and mixed well. The mixture was centrifuged at 8,000 rpm for 10 min; the supernatant was monitored at 340 nm to check the optical density. Finally, the extract was lyophilized and used for further characterization studies [27,28].

2.9 Characterization of the Pigment by GC/MS analysis

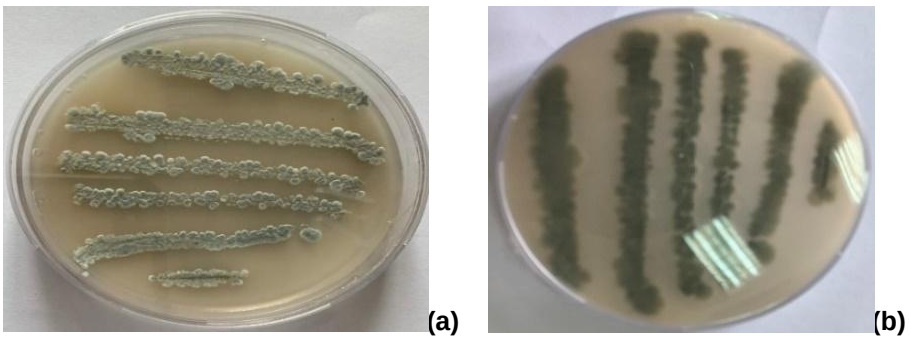
2.9.1 Sample derivatization

To the dried residue, add 100µl of derivatization reagent (80 µl BFSTA+20 µl TMCS) and incubate for 1hr at 65°Cand inject into GC/MS. The most critical point is to avoid any water or moisture during derivatization step especially the silylating step is highly vulnerable. Chemical composition of the sample was performed using Trace GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 50°C for 5 min, then increased by 5°C /min to250 °C, followed by holding for 2 min, raised to the final temperature 300°C at a rate of 30°C /min and finally maintained for 2 min. The injector and MS transfer line temperatures were kept at 270, 260°C respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 4 min and diluted samples of 1 µl were injected automatically using Auto sampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50–650 in full scan mode. The ion source temperature was set at 200 °C. The components were identified by comparison of their mass spectra with those of WILEY 09 and NIST 14 mass spectral database [29].

3. RESULTS AND DISCUSSION

3.1 Isolation

Actinomycetes are group of prokaryotic micro-organisms gram-positive filamentous bacteria. Actinomycetes are widely distributed in the natural habitats, like different cultivated lands, riveres and seas waters as well as various natural eco-system. A number of 225 Streptomycetes colonies were randomly chosen and isolated from different soil sources of Egypt on the basal starch nitrate medium. Out of the 225 Streptomycetes isolates, *S. nigra* GH12 strain was sharp pigment producer with green pigmentation, which then selected for further studies (Figs. 1&2).



(a) **(b)**

Fig. 1. (a) Morphology of isolated colony; (b) Pigment production of *S. nigra* GH12

Fig. 2. Diffusible green pigment in the medium produced by *S. nigra* GH12

3.2 Molecular Identification of the Actinomycetes Isolate

The Actinomycetes isolate's ribosomal internal transcribed spacer (ITS1-8.5SITS2) rDNA was effectively amplified using conventional PCR, and it was detected as a clear band of about 600 bp (Fig. 3). The NCBI Gene Bank database accepted the *Streptomyces* isolate identified in the current study and registered it with accession number OQ145630. The analysis of the S. nigra GH12 phylogeny was done using the sequence of the ITS region. By comparing this isolate to the ITS recorded sequences that were retrieved from NCBI, it was possible to identify the isolate at the genus and species levels. The obtained phylogenetic tree showed that GH12 isolate belongs to the genus of *Streptomyces*, had a tight relationship with each other. The cluster showed a close relationship in the same clade, with a bootstrap value of 100%, as presented the cluster (Fig. 3).

3.3 Optimum Culture Conditions for Highest Pigment Production

In general, the production of pigment is related to cell growth and is influenced by nutritional influences (such as carbon and nitrogen sources), microbiological parameters (such as spore, seed, and inoculum ages), and environmental variables (such as spore, seed, and inoculum ages). In terms of environmental conditions, many important parameters should be eximined and improved [2]. These parameters include medium's humidity, the substrates' physical and structural characteristics, temperature, *p*H and agitation [30] (de Castro and Sato 2015). Due to the low moisture content, only a few microorganisms, primarily yeasts and fungi, are able to perform this cultivation procedure. Submerged fermentation (SmF), which depends on liquid culture for most microbes, is the second cultivation method. It is significantly influenced by variables such as temperature, pH, and agitation [31].

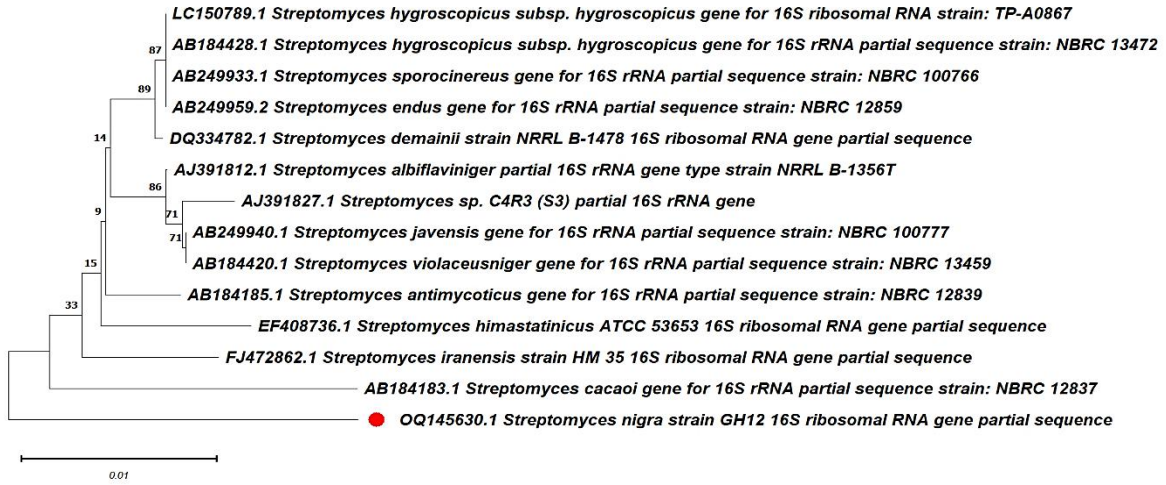


Fig. 3. Phylogenetic tree of *S. nigra* GH12 based on 16S rRNA gene sequences. The 16S rRNA sequences were obtained from Gene Bank

3.4 Determination of Suitable Initial *p*H Value

Results in Fig. 4 illustrated that the new local *S. nigra* GH12 strain produced its maximum pigment concentration at *p*H 8, the concentration production of green pigment is highly affected by low or high values than *p*H 8. The *p*H of fermentation medium may be altering the color of pigment produced by the same organisms. Despite the growth rate not being affected in the same way, the results show that the initial pH has a substantial impact on pigment synthesis [22]. According to Sethi et al. [32] *Penicillium purpurogenum* BKS9 produced the most red pigment at a pH of 6.0. Additionally, *Penicillium aculeatum* ATCC 10409 produced the most yellow pigment when grown at pH 6.5, according to Afshari et al. [33]. The highest biomass and pigment synthesis by *Penicillium sp*. were noted at an initial pH of 9.0, according to Gunasekaran and Poorniammal's 2008 research. On the other hand, Méndez et al. [34] found that *P. purpurogenum* GH2 produced the most red pigment at pH 5 and 24°C.

3.5 Selection of Proper Carbon Source

Data presented in Fig. 5 showed that insoluble starch was more stimulating carbon source for secretion of the green pigment in the growth medium. However, derivatives of starch, maltose, glucose and galactose, lactose, mannitol can produce about 25-40 % comparable to starch. Other various carbon sources failed to stimulate the green pigment production by *S. nigra* GH12 i.e sucrose, farctouse, xylose and dextran (Fig. 5). Santos-Ebinuma et al. 2013 [35] reported similar results for *P. purpurogenum* that has shown the capability to utilize starch and sucrose for the best pigment production. In addition, starch was the best for red pigment production by *Paecilomyces sinclairii* [36,37]. Mannitol was used as the best carbon source for production of red microbial pigments [38] Moreover, Sankhyayan et al. [39] found that lactose and fructose strongly decreased pigment production.

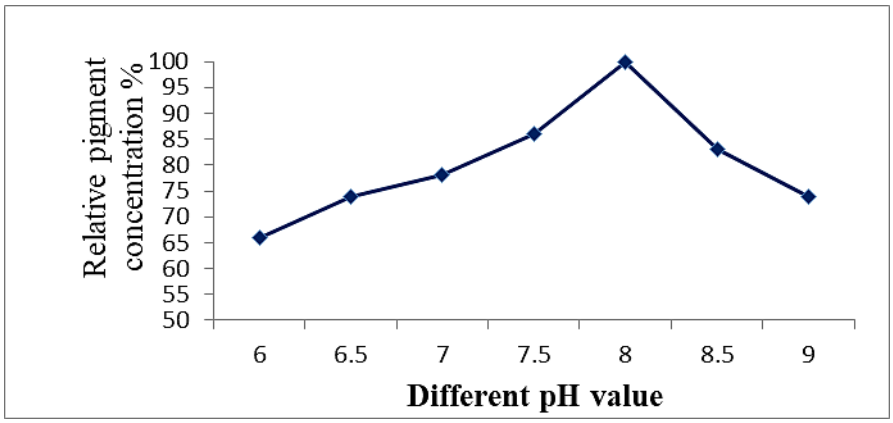


Fig. 4. Effect of different initial *p*H value on the green pigment production by *S. nigra* GH12

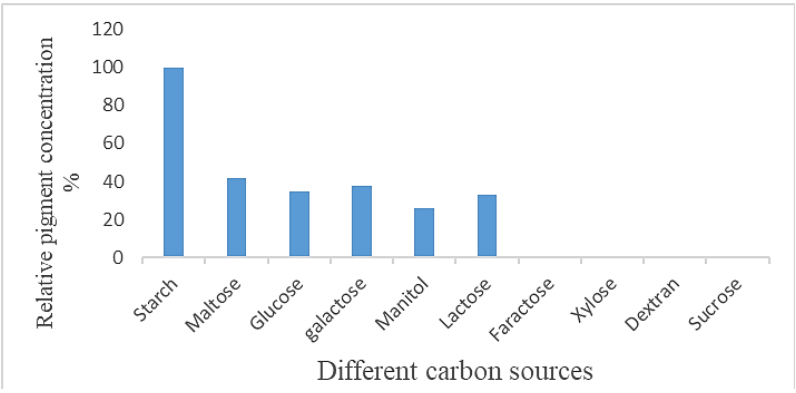


Fig. 5. Effect of different carbon sources on the green pigment production by *S. nigra* GH12

3.6 Effect of Starch Concentration on the Green Pigment Production by *S. nigra* GH12

The green pigment secretion in the growth medium inoculated with *S. nigra* GH12, was increased by increasing the introduced starch in the growth medium from 0.5% up to 2.5% (w/v) then affected negatively by adding more starch in the medium (Fig. 6). Starch is more widely applied as carbon source for pigment production by Actiomycetes, while glucose and fructose were reported as more suitable carbon source for pigment production by *Sarcina* sp. and *Exiguobacterium aurantiacum* FH [40,41].

3.7 Effect of Inorganic Nitrogen Source on the Green Pigment Production

It is well known the importance of nitrogen for growth of all alive organisms and its essential for metabolism that affected greatly by the presence of nitrogen source in the growth medium. The consumption of the nitrogen source and the metabolism associated to a favorable metabolic way both are necessary for the formation of pigments [35]. Previous studies have proved that the effect of a nitrogen source on pigment production is strain dependent, while using one of nitrogen sources could promote pigment production, using another nitrogen source could inhibit pigment production by the same isolate [22]. For instance, ammonium nitrate is more suitable than other inorganic nitrogen sources for more green pigment secretion in the growth medium inoculated with *S. nigra* GH12 (Fig. 7).

3.8 Effect of Different concentration of Ammonium Nitrate on the Green Pigment Production

Fig. 8 illustrated the green pigment secretion in the growth medium inoculated with *S. nigra* GH12 contained the optimum above level of starch and adding different concentration of ammonium nitrate as a suitable inorganic nitrogen source. It seems that 2.0 g/l of ammonium nitrate is the more suitable concentration to give the high green pigment production and above or below 2.0 g/l decrease pigment production. The presence high nitrogen concentration in fermentation medium has terminal effect. Joshi, et al. [41] found also potassium nitrate is the best for microbial pigment production by *Sarcina* sp isolated from water. Furthermore, potassium nitrate (0.5%) was found also the more probe nitrogen source for production of pigment by *Pseudomonas* sp.

3.9 Influence of Organic Nitrogen Source and its Concentration on the Green Pigment Production

Four organic nitrogen sources i.e. casein, malt extract, yeast extract and peptone at three different concentrations of 0.5, 1.0 and 1.5 % were examined (Fig. 9). The presence of 0.5 g/l malt extract in the medium was the more suitable organic source for production the green pigment by *S. nigra* GH12. This result was similar to the findings obtained by Elattaapy and Selim, [22] had enhanced red pigment production using malt extract by *Penicillium* sp. Many researchers reported that the presence of organic source in the culture media resulted in higher accumulation of pigment [42] during studying the optimization factors for maximum pigment production by *Rhodotorula glutinis, Exiguobacterium sp*. and *Sarcina* sp. [41]. According to Santos-Ebinuma et al. [35] yeast extract followed by malt extract were both effective in inducing *P. purpurogenum* to produce pigment.

3.10 Effect of Phosphorous Source on the Green Pigment Production

Among the five phosphorous sources examined, i.e. as supplement in growth medium, the dibasic sodium phosphate was the prefilled source of phosphate to produce the maximum green pigment concentration by *S. nigra* GH12 in the growth medium. However, sodium dihydrogen phosphate was lowest one (Fig. 10).

3.11 Effect of Phosphorous Concentration on the Green Pigment Production

Different five concentrations from dibasic sodium phosphate were tested in the fermentation medium of *S. nigra* GH12. The obtained results revealed that the maximum relative concentration of the green pigment at the concentration of 1.0 (g/L).

3.12 Effect of Inoculums Size (v/v) on the Green Pigment Concentration Production

Study of inoculums percent was taken into consideration, when the pigment produced from microorganisms [43.44] Fig .12 illustrated that the inoculation the medium with 6 % (v/v) inoculums recorded the maximum green pigment production by *S. nigra* GH12 than other lower or higher sizes of inoculums. The maximum pigment synthesis was recorded by Elattaapy

and Selim, 2020 [22] at a spore concentration of 106/ml. The ideal inoculum size for pigment formation, according to Babitha et al. [45] was 3 ml of 9x104 spores/ml. The ideal inoculum size for pigment formation, according to Velmurugan et al. 2011 [46] was 4 ml of spore suspension. With 108 spores/ml, Santos-Ebinuma et al. [35] has produced the higher pigment concentration with 108 spore/ml. Arora et al. 2012 [46] reported that high inoculum size led to increases biomass and decreases pigment production, due to the inhibition the affiance of utilization of essential nutrients of culture medium by increased biomass.

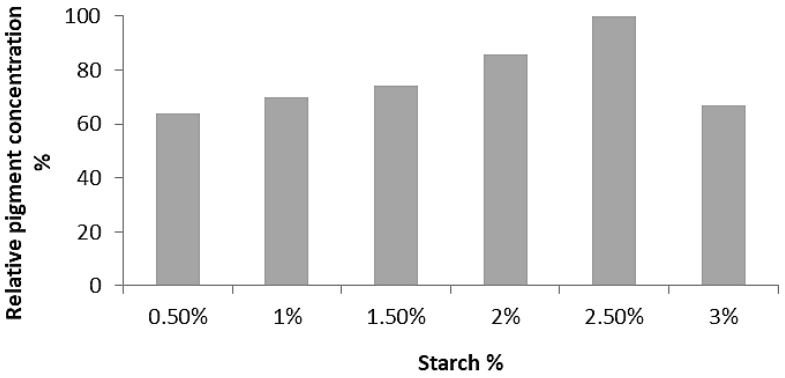


Fig. 6. Effect of starch concentration on the green pigment production by *S. nigra* GH12

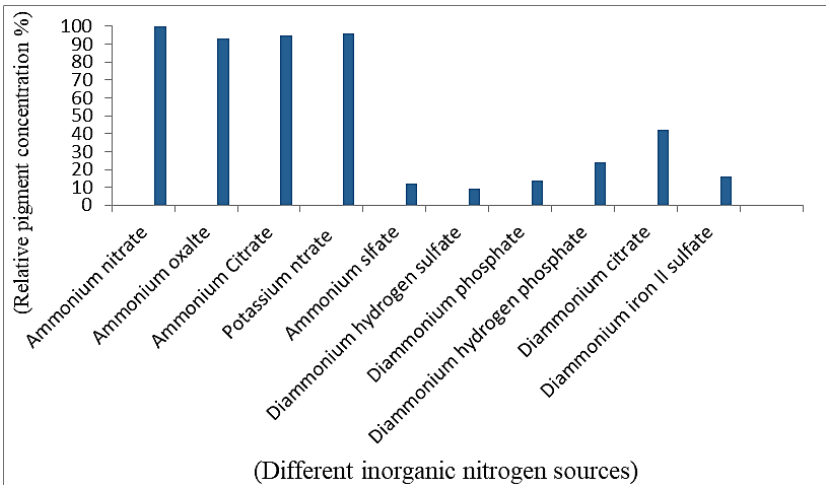


Fig. 7. Influence of inorganic nitrogen source on green pigment production by *S. nigra* GH12

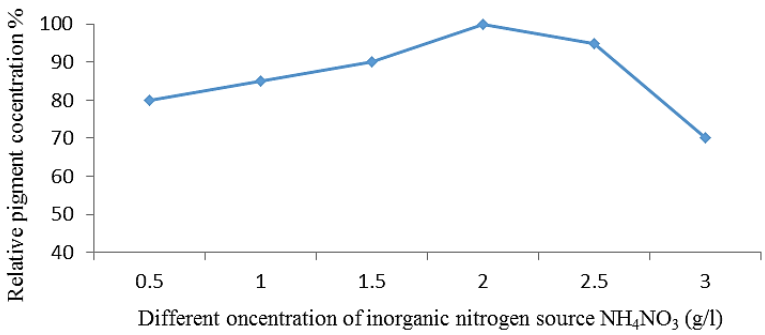


Fig. 8. Influence of different concentrations of ammonium nitrate on the green pigment production by *S. nigra* GH12

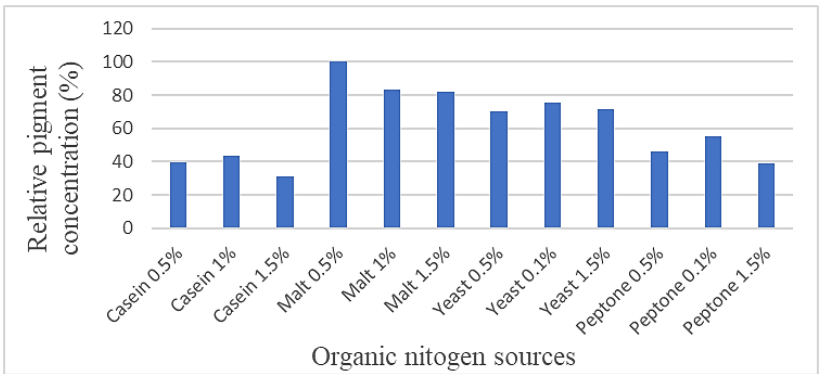


Fig. 9. Influence of organic nitrogen sources on green pigment production by *S. nigra* GH12

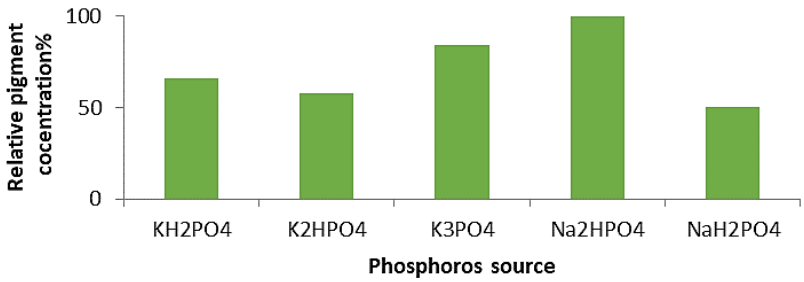


Fig. 10. Effect of phosphorous source on green pigment production by *S. nigra* GH12

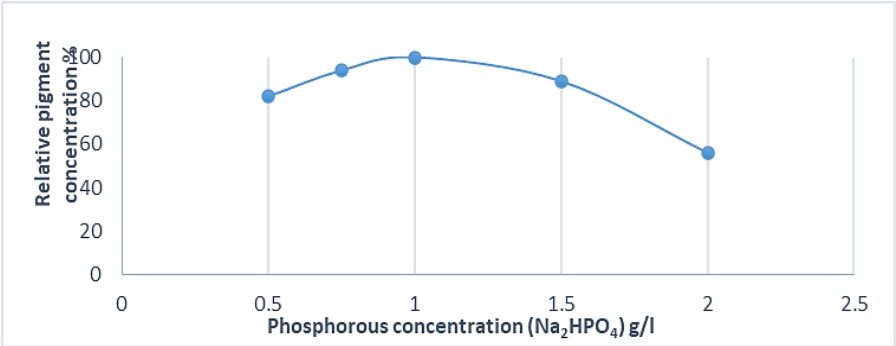


Fig. 11. Effect of phosphorous level on the green pigment production by *S. nigra* GH12

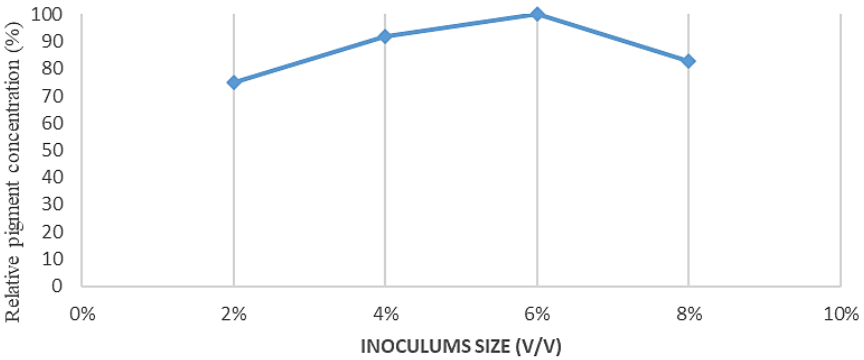


Fig. 12. Effect of inoculums size (v/v) on the green pigment production by *S. nigra* GH12

3.13 The Effect of Medium Volume

Different volumes of pigment production medium from the range of 25-125 ml were introduced in 250 ml conical flasks capacity. The findings of this study demonstrated that the introduction 75 ml of the growth medium in 250 ml conical flasks capacity was more suitable to produce maximum concentration of green pigment (Fig. 13). The culture volume affects the amount of oxygen available for organism growth, utilization nutrient present in culture, and consequently biosynthesis of pigment.

3.14 Effect of Metal Ions Addition

Metal ions are very essential for biosynthesis pathways in cells as enzymes, coenzymes and bioactive compounds production. Data plotted in Fig. 14 revealed that Cr^{+++} ion is very essential in the culture medium for green pigment production by *S. nigra* GH12 followed by Mn^{++}.

3.15 Effect of incubation temperature on the green pigment production

Temperature is a one of vital factors for growth, nutrients metabolism and scan dry bioactive agent production, as well. It is showed that the green pigment concentration secreted in the growth medium by *S. nigra* GH12 strain was optimum at an incubation temperature of 37°C (Fig 15). Above temperature 37°C the pigment affected sharply negative. Joshi et al. 2011 [41] reported 35°C as an optimum temperature for the pigment production by *Sarcina sp.* cultivated on apple pomace. However, according to Zahan et al. [47], *Penicillium minioluteum* ED24 pigment synthesis requires an incubation temperature of roughly 30°C. Furthermore, according to Afshari et al., [33] temperature affects the metabolic activity of fungi and their growth. The regulation of enzymatic activity inside fungal cells may be influenced by the temperature of the incubation process. The highest pigment production obtained at the optimum temperature because the enzyme activity for pigment synthesis seems to be enhanced at this temperature.

3.16 Effect of Incubation Period on the Green Pigment Production

On verification of Fig. 16, it is clear that the density of green pigment liberated by *S. nigra* GH12 in fermentation medium increased by increasing the age of fermentation period till ninth day then achieved a steady state**.** After 8 days, Elattaapy and Selim, 2020 [22] observed their highest pigment synthesis; nevertheless, as more incubation time passed, the production markedly reduced. Santos-Ebinuma et al. [35] found that *Penicillium purpurogenum* DPUA 1275 produced the most pigment after 12 days of incubation, but Chadni et al. 2017 [48] found that *Talaromyces verruculosus* produced the most pigment after 24 days of incubation. On the other hand, many authors have reported shorter incubation times. Méndez et al. 2011 [34], found that *P. purpurogenum* GH2 produced the highest yield of red pigment after 150 hours (6.25 days) of incubation, and Babitha et al. 2006 [43], found that *Monascus purpureus* produced the most pigment after 6 days [49], however, achieved maximum pigment production by the same method. Previous studies reveal that the optimum incubation time for maximum pigment production varies from one strain to another.

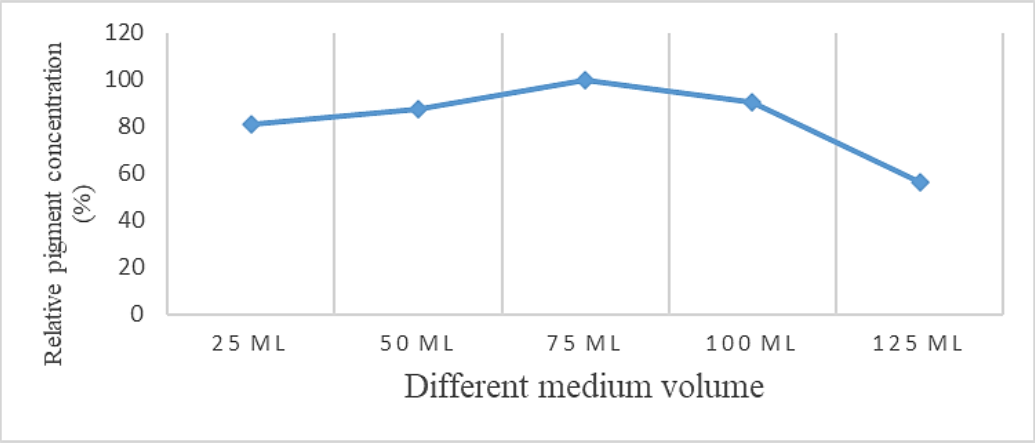


Fig. 13. Effect of culture volume (v/v) on the green pigment production by *S. nigra* GH12

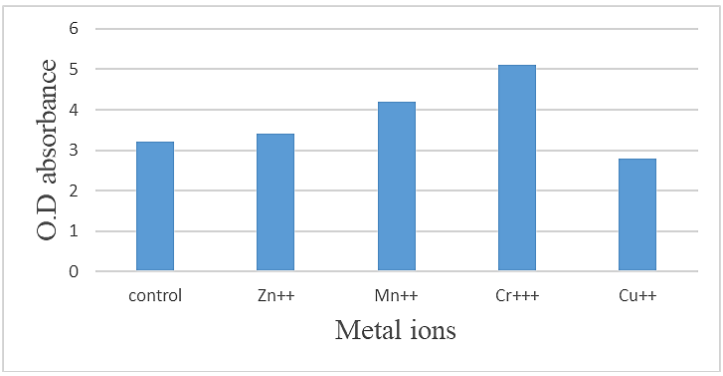


Fig. 14. Effect of metal ions on the green pigment production by *S. nigra* GH12

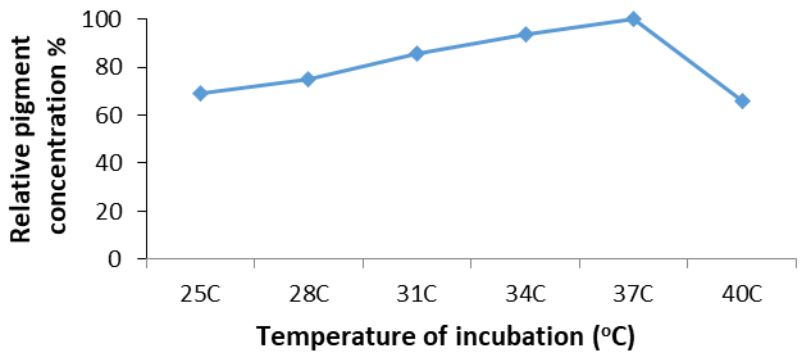


Fig. 15. Effect of incubation temperature on the green pigment production by *S. nigra* GH12

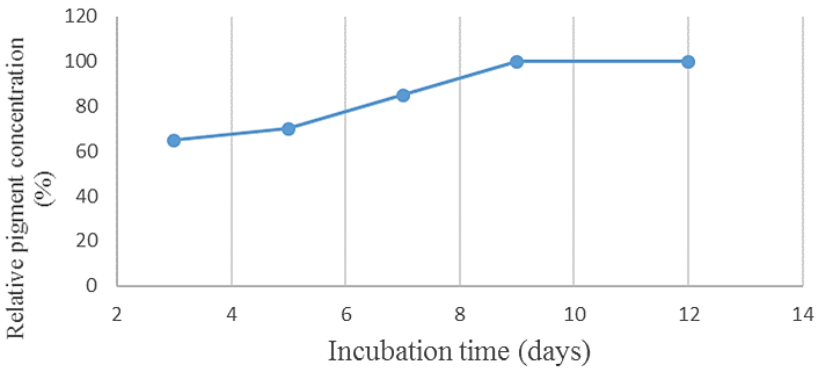


Fig. 16. Effect of incubation period on the green pigment production by *S. nigra* GH12

3.17 Extraction of the Pigment of *S. nigra* GH12

In fact, the highest yield green pigment extract is produced by using ethanol as an extracting solvent via liquid-liquid extraction method that was found to be different from the reports by Abraham and Chauhan 2018 [50]. Thereafter, the recovered extract was vacuum dried at a lower pressure (Fig. 17).

3.18 Effect of Heat Treatment on Green Pigment Stability

Thermal stability of extracted pigment was studied by heating the extracts at 40, 50, 60, 70, 80, 90 and 100 °C for 60 min (Fig. 18). Based on the absorbance values measured for green extracts before and after heat treatments, retentions of pigment as related to heating temperature were calculated. The stability of

green pigment was markedly influenced by heat treatment. At 40, 50 and 60°C, no significant loss occurred in pigment content. When the heating temperature was elevated up to 70, 80 and 90^{o}C, retention of pigment was still as high as 97.4, 93 and 86%, respectively after 60 min. Even when heat treatment was carried out at 100 °C for 60 min, green pigment retained more than 79% of its pigment content.

3.19 Effect of *p*H on the stability of green pigment produced by *S. nigra* GH12

Color stability of pigment extracts was determined at a wide range of *p*H values between 4 and 10 at a temperature of 37°C by measuring the absorbance values of green pigment at λ_{max} of 340 nm after 1hr. The results obtained are illustrated in Fig. 19. Data showed that green pigment were almost stable under aqueous conditions. It was observed that the green color of the extracts retained its high stability at *p*H 8.5.

3.20 UV-absorption spectrum of extracted green pigment produced by *S. nigra* GH12

The UV absorption spectrum of green pigment extract produced by *S. nigra* GH12 is shown in Fig. 20. Data indicated that absorption maximum peak of green pigment extract was range from 300 to 360nm, with λ_{max} at 340 nm. However, Kazi et al. 2022 [28] has reported a λ_{max} 247 for the produced pigment from *Streptomyces* species on full UV-scanning. On the other hand, Selim et al. 2008 [51] has demonstrated a λ_{max} 520 for roselle pigments.

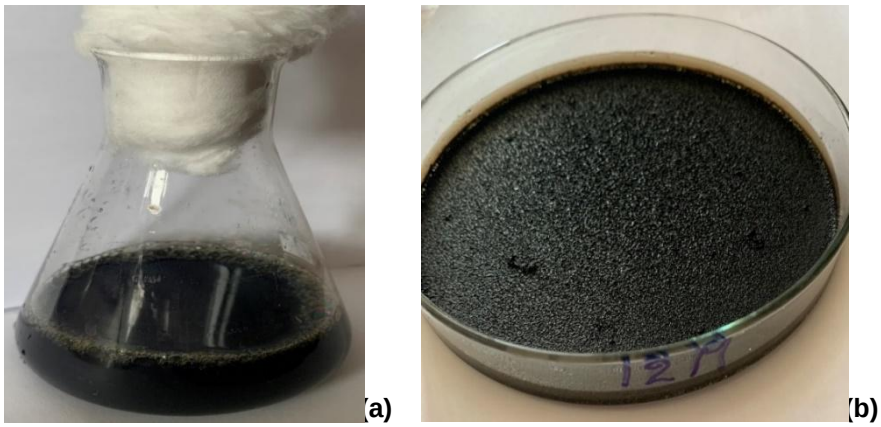


Fig. 17. (a). Pigment extracted by ethanol solvent; (b). Dried pigment after ethanol extraction

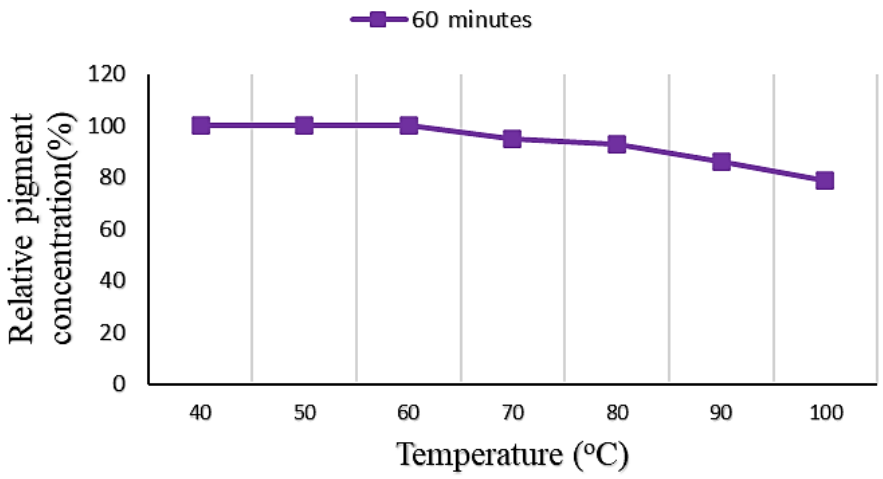


Fig. 18. Effect of heat treatment on the retention of green pigment produced by S. *nigra* GH12 heated for 1 hr at different temperatures

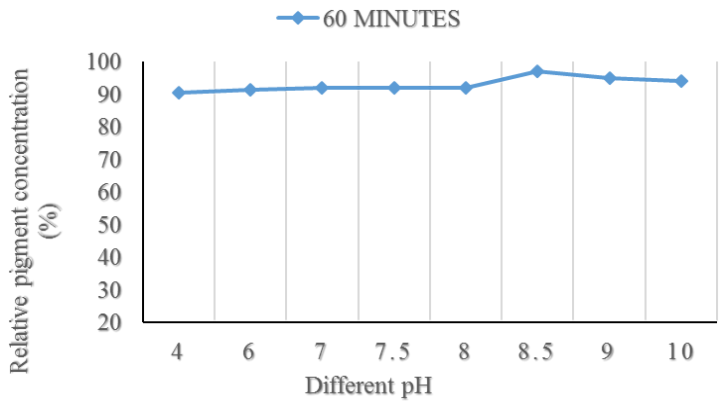


Fig. 19. Effect different *p*H on retention of green pigment produced by S. *nigra* GH12

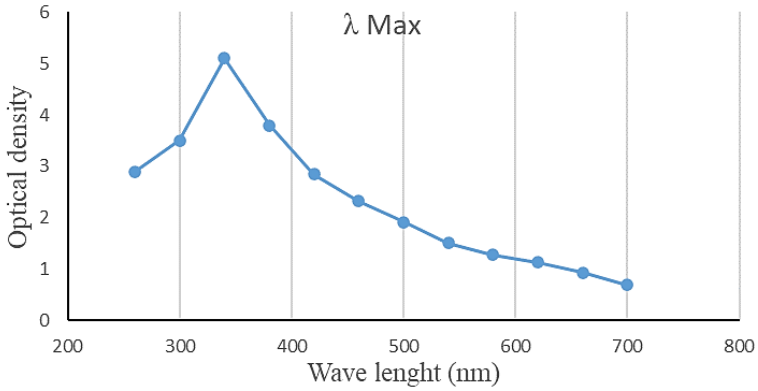


Fig. 20. The UV absorption spectrum of extracted green pigment produced by *S. nigra* GH12

3.21 GC/MS characterization of green pigment produced by *Streptomyces nigra* GH12

Different physicochemical techniques were used for characterization of many *Streptomyces* derived bioactive pigments such as UV-, IR-, Raman- and/ or NMR spectroscopy together with one or more of MS-spectrometric ionization tools [51,52,53,54,55]. GC/MS hyphenated technique is one of the most powerful analytical profiling tools for a fast and precise chemical characterization of the natural extracts, and microbial bio-pigments, as well. Chen et al., 2018 used both GC/ and LC/hyphenated chromatographs with MS-spectrometry for identification of > 500 and 38 compounds, respectively in a potent antitumor active extract from *Streptomyces nigra* sp. nov. Sinapyl alcohol, phloroglucinol, azelaic acid, hydroxyurea, rosteron, shikimic acid, spermidine, 2-deoxy-D-glucose, and dehydroepiandrosterone have been detected among major identified metabolites. In the current study GC/MS analysis identified 57 compounds in the form of their silylated derivatives (Table 1) from the green pigment of the investigated new *S. nigra* GH12 on the basis of matching the corresponding MS-spectra and other output parameters with those of WILEY 09 and NIST 14 mass spectral library database [29]. Fig. 21 displayed the corresponding total ion current (TIC), that showed how much crowded the target pigment with different types of natural organic metabolites. It is worth mentioning that nine major silyl derivatives recorded for Lactic acid (**3**, 6.18 min, 19.72%), 3-Methylbutanoic acid (**5**, 8.73 min, 11.82%), Carbamic acid (**2**, 6.03 min, 7.80%), Ethylene (**13**, 14.78 min, 6.35%), *meso*-Erythritol (**23**, 17.97 min, 4.98%), 2-Methylpropanoic acid (**25**, 19.02 min, 4.54%), Palmitic acid (**42**, 28.12 min, 2.54%), 4-Hydroxy-*N*-valeric acid (**15**, 16.20 min, 2.20%) and 1-Monopalmitin (**51**, 37.29 min, 2.11%). In addition, twelve metabolites, i.e. **6, 9, 17-19, 21, 24, 26, 28, 34, 35** and **49** demonstrated more than 1% relative concentration. Their MS spectra were presented in Fig. 21.

Table 1. Identified silylated derivatives of the constitutive metabolites in the green pigment of *S. nigra* GH12 isolate

P.No.	R_t, min	RC, %	MF	MW	Chemical name
1	4.02	0.38	$C_8H_{19}F_3N_2Si_2$	256	*N,N'*-Bis(trimethylsilyl)trifluoroacetamiine
2	6.03	7.80	$C_7H_{19}NO_2Si_2$	205	Carbamic acid, *N*-(trimethylsilyl)-, trimethylsilyl ester
3	6.18	19.72	$C_9H_{22}O_3Si_2$	234	Lactic acid, bis(trimethylsilyl) derivative
4	8.55	0.61	$C_{10}H_{24}O_3Si_2$	248	Propanoic acid,2-methyl-3-[(trimethylsilyl)oxy]-, trimethylsilyl ester
5	8.73	11.82	$C_{11}H_{26}O_3Si_2$	262	Butanoic acid,3-methyl-2-[(trimethylsilyl)oxy]-, trimethylsilyl ester
6	10.62	1.39	$C_{12}H_{28}O_3Si_2$	276	2-Hydroxyisocaproic acid, bis(trimethylsilyl) derivative
7	10.73	0.95	$C_{12}H_{28}O_3Si_2$	276	Pentanoic acid 3-methyl-2-[(trimethylsilyl)oxy]-, trimethylsilylester
8	11.83	0.88	$C_{12}H_{32}O_3Si_3$	308	Glycerol, tris(trimethylsilyl) derivative
9	11.90	1.02	$C_8H_{16}O_3Si$	188	Levulinic acid, trimethylsilylester
10	12.45	0.31	$C_{13}H_{34}O_3Si_3$	322	1,2,3-Butanetriol, tris(trimethylsilyl) derivative
11	14.28	0.57	$C_{11}H_{24}O4Si_2$	276	3,4-Dihydroxy-5-methyl-dihydrofuran,-2-one,(D)-, bis(trimethylsilyl) derivative
12	14.38	0.76	$C_{17}H_{42}O_5Si_4$	438	*Erythro*-pentonic acid, 2-deoxy-3,4,5-tris-O-(trimethylsilyl)- trimethylsilyl ester
13	14.78	6.35	$C_{11}H_{28}O_3Si_3$	292	Tris(trimethylsiloxy)ethylene
14	15.68	0.63	$C_{13}H_{32}O_4Si_3$	336	(R,S)-3,4-dihydroxybutanoicacid trimethylsilylester
15	16.20	2.20	$C_{11}H_{26}O_3Si_2$	262	4-Hydroxy-N-valeric acid bis(trimethylsilyl)
16	16.35	0.38	$C_{13}H_{34}O_3Si_3$	322	1,2,3-Butanetriol,tris(trimethylsilyl) derivative
17	16.45	1.18	$C_{18}H_{46}O_4Si_4$	438	3,8-Dioxa-2,9-disiladecane,2,2,9,9-tetramethyl-5,6-bis[[(trimethylsilyl)oxy]methyl]-
18	16.63	1.47	$C_{11}H_{28}O_3Si_3$	292	Tris(trimethylsiloxy)ethylene
19	16.81	1.93	$C_{10}H_{24}O_3Si_2$	248	Butanoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilylester
20	17.43	0.40	$C_{14}H_{34}O_4Si_3$	350	D-Erythro-pentofuranose, 2-deoxy-1,3,5-tris-O-(trimethylsilyl)-
21	17.59	1.67	$C_{10}H_{24}O_2Si_2$	232	2-Butene-1,4-diol, bis(trimethylsilyl) derivative
22	17.75	0.90	$C_{16}H_{42}O_4Si_4$	410	L-Threitol, tetrakis(trimethylsilyl) derivative
23	17.97	4.98	$C_{16}H_{42}O_4Si_4$	410	*Meso*-Erythritol, tetrakis(trimethylsilyl) derivative
24	18.63	1.29	$C_{16}H_{40}O_5Si_4$	424	L-Threonic acid, tris(trimethylsilyl ether, trimethylsilylester
25	19.02	4.54	$C_{13}H_{32}O_4Si_3$	336	Propanoic acid, 2-methyl-2,3-bis[(trimethyl silyl)oxy]-,trimethylsilylester
26	20.23	1.42	$C_{17}H_{44}O_4Si_4$	424	Pentitol 3-desoxy-tetrakis(trimethylsilyl)
27	21.20	0.32	$C_{17}H_{42}O_5Si_4$	438	Ribonic acid, 2-desoxy-tetrakis-O-(trimethylsilyl)-
28	21.36	1.27	$C_{14}H_{31}BO_6Si_2$	362	α-D-Galactopyranoside,methyl 2,6-bis-O-(trimethylsilyl)-,cyclicmethylboronate
29	21.52	0.34	$C_{18}H_{44}O_5Si_4$	452	1,5-Anhydrohexitol, tetrakis-O-(trimethylsilyl)- derivative
30	21.94	0.52	$C_{14}H_{36}O_3Si_3$	336	3,9-Dioxa-2,10-disilaundecane,2,2,10,10-tetramethyl-5-[(trimethylsilyl)oxy]-
31	22.03	0.65	$C_{13}H_{32}O_4Si_3$	336	((4,5-bis[(trimethylsilyl)oxtris-O-(trimethylsilyl)-
32	22.14	0.26	$C_{14}H_{36}O_3Si_3$	336	2,2,10,10-tetramethyl-5-[(trimethylsilyl)oxy]-3,9-dioxa-2,10-disilaundecane

P.No.	R_t, min	RC, %	MF	MW	Chemical name
33	22.42	0.58	$C_{20}H_{52}O_5Si_5$	512	Xylitol, pentakis(trimethylsilyl) derivative
34	23.02	1.47	$C_{20}H_{52}O_5Si_5$	512	Adonitol, pentakis(trimethylsilyl) derivative
35	23.39	1.96	$C_{16}H_{37}BO_6Si_3$	420	α-D-Galactopyranose,1,2,3-tris-O-(trimethylsilyl)-, cyclic methylboronate
36	25.03	0.54	$C_{17}H_{42}O_5Si_4$	438	α-Lyxopyranose, tetrakis-O-(trimethylsilyl) derivative
37	25.11	0.48	$C_{22}H_{54}O_6Si_5$	554	D-Pinitol, pentakis(trimethylsilyl)ether
38	26.14	0.59	$C_{17}H_{42}O_5Si_4$	438	α-D-Xylopyranose, tetrakis(trimethylsilyl) derivative
39	26.51	0.36	$C_{21}H_{52}O_6Si_5$	540	α-D-Allopyranose, pentakis(trimethylsilyl) derivative
40	27.00	0.43	$C_{18}H_{44}O_5Si_4$	452	1,5-Anhydrohexitol, tetrakis(trimethylsilyl) derivative
41	27.67	0.64	$C_{21}H_{52}O_6Si_5$	540	D-Allofuranose,pentakis(trimethylsilyl)ether
42	28.12	2.54	$C_{19}H_{40}O_2Si$	328	Palmitic acid, trimethylsilyl derivative
43	31.05	0.85	$C_{21}H_{42}O_2Si$	354	11-Octadecenoicacid, (E)-, trimethylsilyl derivative
44	31.59	0.38	$C_{21}H_{44}O_2Si$	356	Stearic acid, trimethylsilyl derivative
45	32.35	0.64	$C_{15}H_{34}O_5Si_3$	378	Levoglucosan, tris-(trimethylsilyl) derivative
46	32.82	0.35	$C_{27}H_{66}O_8Si_6$	686	Glyceryl-glycoside trimethylsilyl ether
47	32.92	0.50	$C_{18}H_{44}O_5Si_4$	452	1,5-Anhydrohexitol, tetrakis(trimethylsilyl)- derivative
48	33.01	0.39	$C_{36}H_{86}O_{11}Si_8$	918	2-à-Mannobiose, octakis(trimethylsilyl) ether
49	33.53	1.81	$C_{19}H_{38}N_2O_6Si_3$	474	5-Methyluridine, tris(trimethylsilyl)- derivative
50	36.81	0.42	$C_{25}H_{54}O_4Si_2$	474	2-Palmitoylglycerol, bis(trimethylsilyl) derivative
51	37.29	2.11	$C_{25}H_{54}O_4Si_2$	474	1-Monopalmitin,bis(trimethylsilyl) derivative
52	38.28	0.46	$C_{36}H_{86}O_{11}Si_8$	918	Maltose, octakis(trimethylsilyl) derivative , isomer 1
53	38.81	0.83	$C_{36}H_{86}O_{11}Si_8$	918	α-D-Lactose,(isomer 2), octakis(trimethylsilyl)
54	40.01	0.79	$C_{26}H_{36}O_5Si$	456	(+-)-(6-*Endo*-7-exo)-7-[4-[(*tert*-butyldimethylsilyl) oxy]-3-methoxyphenyl]-3-methox y-6-methyl-5-(2-propenyl)bicyclo[3.2.1]oct-3-ene-2,8-dione
55	40.19	0.31	$C_{35}H_{74}N_4O_{13}Si_5$	898	L-Asparagine,Nç-[2-(acetylamino)-4-O-[2-(acetylamino)-2-deoxy-3,4,6-tris-O-(trimethylsilyl)- α-D-glucopyranosyl]-2-deoxy-3,6-bis-O-(trimethylsilyl)-α-D-glucopyranosyl]-
56	41.02	0.49	$C_{27}H_{52}N_2O_{10}Si_3$	648	α-D-Glucopyranosiduronicacid,3-(5-ethylhexahydro-2,4,6-trioxo-5-pyrimidinyl)-1,1-dimethylpropyl2,3,4-tris-O-(trimethylsilyl)-,methylester
57	41.83	0.68	$C_{17}H_{42}O_5Si_4$	438	α-Arabinopyranose, tetrakis(trimethylsilyl) derivative

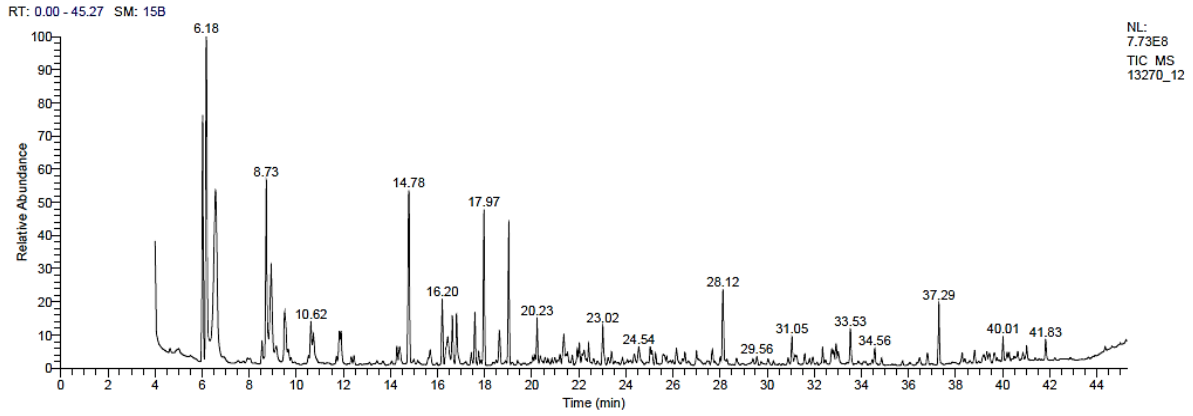


Fig. 21. TIC chromatogram for the total green pigment of *S. nigra* GH12 isolate

4. CONCLUSION

This research directed to find optimized production conditions and characterization of a green pigment biosynthesized by a new local *Streptomyces* isolate, namely *S. nigra* strain GH12. the chemical composition diversity of the pigment extract was further investigated by GC/MS analysis that revealed the presence of 57 metabolites in their silylated form.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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