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# EFFECT OF SURFACE FERMENTATION AND PARAMETERS OF CULTIVATION ON PRODIGIOSIN PRODUCTION

**Relevance.** Biopigment are natural compounds (secondary metabolite) produced by many organisms represent one of the important sources of potential lead compounds. Prodigiosin (5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene) is a red, tripyrrole, water insoluble, bioactive pigment produced by number of different bacteria, actinomycetes and some fungi. Prodigiosin is known to have immunosuppressive, anti-fungal, anti-viral, anti-microbial, anti-malarial, and anti-proliferative properties [1]. Surface fermentation has been gaining renewed interest and focused attention from researchers owing to its importance in recent developments in biomass energy conservation. The solid-state is known to give high nutrient concentration and availability from the substrate for pigment production [2]. Also, various parameters, like temperature, incubation time, pH, nutrient source, and quorum sensing, are the main factors that have been reported to impact prodigiosin production [1]. Attachment to horizontal surfaces stimulates bacterial growth as organic material suspended in liquid settles, is deposited on surfaces, and increases the local concentration of nutrients. Similarly, increasing the substrate surface area provides more area on which nutrients can adsorb, enabling cells to grow at nutrient concentrations that would normally be too low to support growth [3].

**Purpose of the study is** to compare pigment production, both liquid and solid forms of media using *Serratia marcescens* and screening of the main factors contributing to the accumulation of the pigment prodigiosin.

**Materials and methods.** As a pigment producer, we used the species *S. marcescens*, namely the pigment-forming strain, which isolated in the laboratories of the Department of Microbiology, virology, and immunology of Bogomolets National Medical University from the bentonite clays of Kurtsivskyi deposit (Crimea, Ukraine).

In order to compare pigment production, both liquid and solid forms of media were screened for pigment production. Glycerol-ammonia medium was used as the basal medium with the following composition: glycerol – 10, $(NH_4)_2SO_4$ – 1; $MgSO_4$ – 1; NaCl – 1; $CaCO_3$ – 3. Briefly, 10 ml of nutrient broth was inoculated with fresh 1% culture. Similarly, nutrient agar plate was spread with 1% of culture. Both the media were incubated for 60 h and evaluated for pigment production. In case of solid medium, the culture was scraped and suspended in 10 ml distilled water. For the extraction process, 2.5 ml from above broth was taken in a test tube, and 1 ml of ethanol was added. The solution was then centrifuged for 10 min at 6000 rpm. Following the above step, 0.8 ml supernatant was further mixed with 0.2 ml of ethanol. The absorbance of the resulting solution was then measured at 530 nm. For the extraction process, 2.5 ml from above broth was taken in a test tube, and centrifuged for 10 min at 6000 rpm.

The extraction of prodigiosin pigment from biomass of bacteria was carried out by double processing of biomass with 96% ethanol. The resulting preparation dried in air and reextracted. The procedure was repeated several times before the release of insoluble admixtures. The resulting homogeneous solution was designated as a crude pigment complex or ethanol extract. The ethanol extract was evaporated dry in a drying oven at a temperature of +45-50 °C and, the residue dissolved in chloroform (10 mL/g of precipitate). The resulting solution was mixed with an equal volume of a water-ethanol mixture (4:1) and emulsified on a magnetic stirrer for 1 hour at room temperature. A water-ethanol mixture containing water-soluble admixture separated by a separating funnel. The procedure was repeated by increasing the volume content of ethanol by half. The drug was then redried in a vacuum oven and redissolved (10 mL/g of precipitate) in ethanol.

The purity of prodigiosin isolated from the pigmented strain was determined by high-performance liquid chromatography (HPLC-MS) on the Agilent 1200 device (Agilent Technologies, USA) with diode-matrix and mass-selective detectors. The quantitative determination of the red pigment was done by measuring the absorbance at 530 nm using double beam UV-Visible spectrophotometer.

Important parameters, like pH (5, 5.5, 6, 6.5, 7, 7.5, and 8), temperature (20, 25, 28, 30, 35 and 40°C), and incubation time (24, 48, 72 h) were checked for optimum pigment production. The initial pH and the temperature at which maximum production of prodigiosin was observed was chosen and maintained in the following studies. The effect of carbon source (glycerol, glucose, starch, glucose, maltose and rhamnose), nitrogen source (peptone, yeast extract, beef extract, tryptone, fish meal, soybean powder, casein hydrolysate) were also studied, respectively. After optimizing each parameter for maximum prodigiosin production, the same was selected and maintained throughout the study.

**Research results.**

Growth on nutrient agar (119 mg/L) resulted in higher pigment production than nutrient broth (69 mg/L). The main effects plot revealed that pH was a significant factor affecting the pigment production, pH 6.5 supported better pigment production than pH 5 or 8 as it was proved that pH of the media possess a crucial role in the synthesis of secondary metabolites and it was proved that the decrease or the increase in pH caused negative influence on pigment production. The analysis of significance for temperature as a variable revealed that temperature was a very significant factor where maximum prodigiosin production by *S. marcescens* was observed at 28°C. Prodigiosin production increased with incubation time at the beginning of 60 h and further incubation showed reduction in its production.

Prodigiosin production on glycerol-ammonia medium was 39 ±1 mg/L but increased by 25 % (52 ±1.5 mg/L), with 10 g/L of peptone (as the sole nitrogen sources) instead inorganic nitrogen source. The combination peptone with yeast extract and inorganic compounds of strongly promoted prodigiosin production. Prodigiosin

production reached 62 ±1.8 mg/L and 79 ±2.3 mg/L with yeast extract, and inorganic compounds ($K_2SO_4$+$MgCl_2$), respectively. By employing a medium containing the required salt balance, it has been possible to obtain 21 % increase in prodigiosin production (Fig.1).

***Table 1***

**Summary of optimized parameters for prodigiosin production**

| Sr. no. | Parameters | Optimized parameter | Concentation of pigment (mg/L) |
|---|---|---|---|
| 1 | Carbon source | 1 % glycerol | 39 ±1 |
| 2 | Nitrogen source | 1% pepton | 52 ±1.5 |
| | | 0.2 % yeast extract | 62 ±1.8 |
| 3 | Inorganic ions | $K_2SO_4$ + $MgCl_2$ | 79 ±2.3 |
| 4 | pH | 6.5 | 102 ±5.1 |
| 5 | Temperature | 28 °C | 108 ±5.4 |
| 6 | Optimized liquid medium | 1 % glycerol + 1% pepton + 0.2 % yeast extract, 10 g/L $K_2SO_4$ + 1.4 g/L $MgCl_2$, pH 6.5, temp. 28 °C, incubation time 60 h | 69 ±2 |
| 7 | Optimized solid medium | | 119 ±5.9 |

The pure pigment extracted from the culture broth analysed by mass spectrophotometry showed a molecular weight of 324 and wavelength scanning using UV spectrophotometer gave an absorbance peak at 535 nm.

Conclusions. The present investigation provides the scope for effective production of pharmaceutical important secondary metabolite, prodigiosin by surface fermentation using *S. marcescens*. As the pigment was highly hydrophobic, it could not produce much pigment in liquid medium, solid media was used for its production. Peptone and yeast extract were used as a nitrogen source when used with glycerol and gave the highest production of prodigiosin, other organic nitrogen sources didn't support prodigiosin production. Maximum prodigiosin production can be obtained by the addition of increased amounts of either $Mg^{2+}$ or $SO_4$.

The parameters, like carbon source, nitrogen source, pH, temperature, and incubation time, were optimized to obtain maximum production of prodigiosin. The optimum pH for pigment production was determined to be 6.5. The bacterial isolate elaborated pigment production at 28 °C, and the rate decreased as temperature increased. The pigment production increased after incubation for 60 h while it decreased after 72 h.

Could be suggested that glycerol play an important role as a substrate for higher pigment production and stimulate the cell density, which in turn resulted in a higher concentration of positive regulators inside the cell, thus triggering excessive pigment production. The combination of natural and chemical nutrients in the medium can play a vital role by increasing the bacterial density and would produce maximal amounts of prodigiosin pigment. There is a strong influence of pH and temperature on pigment production because a slight difference in pH and temperature decrease the pigment production. However, produce much more prodigiosin pigment under cultivation on the surface of solid nutrient media may indicate the absence of a direct relationship between biomass concentration and pigment production by *S. marcescens*.

**References:**

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