

Optimization of media parameters for pigment production in bacteria from effluent water samples

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ABSTRACT

In last few decades, there has been an increasing trend towards replacement of synthetic colorants with natural pigments because of the strong consumer demand for more natural products. These pigments have been extensively used in food production, fish industries, textile industries, paper production, agricultural practices and researches, water science and technology and also having biological activities as antioxidants and anticancer agents. The present investigation was aimed at Prodigiosin and Carotenoid producing *Serratia marcescens*, *Brevibacterium maris* and *Micrococcus luteus* which were isolated from different effluent samples and cultured in Nutrient Agar Medium. In the present study conducted maximum pigment production was observed at pH 7.5, temperature of 25°C and 16°C was optimum, concentration of carbon source was at 0.5% and the concentration of nitrogen source was at 0.5% and 2.0%, respectively. The supernatant after centrifugation was used as the pigment sample. The samples (pigments) were subjected to Thin Layer Chromatography (TLC) for identifying the spots on the TLC plate and the R_f value was calculated for different spots produced by the pigments. *Serratia marcescens* and *Micrococcus luteus* produced maximum amount of the pigments under these optimised conditions, which could be used to increase the production of Prodigiosin and Carotenoid pigments, respectively.

Keywords: Prodigiosin, Carotenoid, *S. marcescens*, *M. luteus*, *B. Maris*, Pigment.

INTRODUCTION

Colour plays a special role with the food we eat. Colours of foods create physiological and psychological expectations and attitudes that are developed by experience, tradition, education and environment: We inevitably eat with our eyes (Stich *et al.*, 2002).

Colouring agents in processed foods has been a common practice for many years. Due to the possible toxicity of artificial colouring agents,

natural colouring alternatives have been increasingly sought. The huge international market for natural pigments has been met mainly by synthetic pigments with structures identical to those of natural pigments, but there is growing demand for natural sources. Natural colorants from plant sources, however, suffer from a diminishing or unstable supply of raw materials, subject to climatic conditions, as well as varying colorant level and quality of the final product. Microbial colorants have attracted must attention in recent years. The main reason for the interest in using microorganisms to produce compounds that can otherwise be isolated from plants and animals or synthesized is the ease of increasing production by environmental and genetic manipulation.

The use synthetic dyes in food are a controversial issue and have been discussed for many years. The increase in the scrutiny and

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negative assessment of synthetic food dyes by the modern consumer has given rise to a strong interest in natural colouring alternatives. This has led to the Penetration of the fermentation-derived ingredients into the food industry is increasing year after year. Some fermentation-derived pigments, such as β -carotene from the fungus *Blakeslea trispora* in Europe or pigments from *Monascus* in Asia are been produced in commercial level to meet the demands of natural dyes (Wissgott *et al.*, 1996; Carroll 1999; Downham *et al.*, 2000).

Many different environmental factors affect the synthesis of these pigments, notably the chemical nature of the carbon and energy source (Lepierrec 1895; Sullivanm 1905; Blanchetirae 1920; Giralf *et al.*, 1936; Goudas and Greppinh 1965; Goudas and Chodatf 1963), the degree of aeration of the culture medium (Elliotr 1958; Lenhoffh 1963) , pH and light and the cations Mg^{2+} (Georgiaf 1931), Zn^{2+} (Baghdiantaz 1952; Chakrabartay *et al.*, 1964) and Fe^{3+} (King *et al.*, 1948; Totterj *et al.*, 1953; Lluch *et al.*, 1973).

In the present investigation microorganisms producing pigments were isolated from different sources and identified as *Brevibacterium maris*, *Arthrobacter sp.* and *Serratia marcescens*. These microorganisms were optimised for different parameters to increase the growth and production of pigments. *Brevibacterium maris*, *Arthrobacter sp* produced β -carotene, *Serratia marcescens* produced prodigiosin. The optimisation was carried out for mass production of β -carotene and prodigiosin by respective micoorgnism so that they could be produced at commercial level and replaces the synthetic pigment.

MATERIALS AND METHODS

Isolation and Identification of the microorganisms:

Microorganisms were isolated from different sources like soil samples, air exposure samples, sewage samples and fruits. The microorganisms from soil were isolated by serial dilution of the sample. Air exposure plate method was followed to isolate air microorganisms. For all the other samples they were serially diluted and plated on to Nutrient agar plates. The microorganisms producing higher intensity of colour pigmentation were selected and identified using Gram's staining, morphology and biochemical tests and

molecular characterization. The pure culture of the isolates was maintained in nutrient agar slants for further investigation.

Colour Reactions:

Colour reaction studies were carried out to have a comparative analysis and to characterise the reactions with acid and bases. The acids used were concentrated H_2SO_4 , 12N HCl, Perchloric acid and bases were 5 N KOH, 5N NaOH. The colour reactions was done by placing a loop full of the broth culture on grease free glass slide and one or two drops of the acid and bases were added separately to see the change in the colour. The change in the colour indicated the reaction with the acid and bases.

Optimisation:

The optimisation for the production of pigment from the isolates was carried out with nutrient broth and YM broth.

Optimisation of Temperature:

Incubation temperature is one of the important parameter which helps in the growth of the microorganisms, temperature even plays a role in pigment production as some of the microorganisms have proved to produce pigment only at particular temperature. The isolates were inoculated in nutrient broth and YM broth and incubated at different temperature like $4^\circ C$, $28^\circ C$, $37^\circ C$ and $42^\circ C$ for 7days. After incubation, OD values of the cultures were checked individually against respective blank to identify the optimum temperature for growth of the organisms.

Optimisation of pH:

pH plays a vital role in maintaining the acidic or basic nature of the medium. The pH provides an environment for the microorganism to grow luxuriantly. The selected strains were inoculated in nutrient broth and YM broth having different pH such as 3, 4, 6, 7, 8 and 9 and they were incubated for 7 days at appropriate temperature for the organism. After incubation the OD value of the culture was observed against a blank.

Optimisation of carbon source:

The nutrition for the growth of the microorganisms is mainly by the macronutrients like carbon, nitrogen source and micronutrients like iron, calcium. The macronutrients mainly effect the growth of the microorganism thus the optimisation of different carbon sources was carried out. carbon sources such as fructose, glucose, dextrose and sucrose were supplemented

in the nutrient broth and YM broth separately and inoculated with the isolates. The media containing different carbon sources with the organism were incubated at 28°C for 7 days. After 7 days of incubation OD was checked in order to check the effect of carbon source on the growth and pigment production.

Optimisation of Nitrogen source:

Growth of the selected microbes at different nitrogen sources was investigated in media containing different nitrogen sources like tryptone, peptone, beef extract, yeast extract and ammonium chloride. Optimization for nutrient broth was carried out using tryptone, beef extract, yeast extract and NH₄Cl. Nitrogen sources used for the optimization for YM broth were tryptone, beef extract, NH₄Cl and peptone. The media with different nitrogen sources were inoculated with the respective microbes and incubated at appropriate temperature for 7 days. After 7 days the OD value was determined to check the effect of nitrogen source on the pigmentation and growth rate of the microorganism.

Mass Production:

The mass production for *Brevibacterium maris* was done in YM broth, nutrient broth was used for *Arthrobacter sp.*, *Serratia marcescens* as the pigmentation was seen in the respective media. The starter culture for the isolate was prepared by inoculating the microorganisms in the media's and incubating it for 48 hours. The starter culture was then used for Mass production of the cell.

The pigmentation production for *Brevibacterium maris* was done in YM broth with carbon source glucose, nitrogen source peptone. The pH of the media was set at 6.0 and the fermentation was carried out at 28°C for 48 hours. The pigment extraction was then done using the fermented broth.

The pigmentation production for *Arthrobacter sp* was done in nutrient broth with carbon source glucose, nitrogen source tryptone. The pH of the media was set at 7.0 and the fermentation was carried out at 37°C for 48 hours. The pigment extraction was then done using the fermented broth.

The mass production for *Serratia marcescens* was done in nutrient broth with carbon source dextrose, nitrogen source beef extract. The pH of the media was set at 7.0 and the fermentation was carried out at 28°C for 48 hours. The pigment

extraction was then done using the fermented broth.

Extraction and purification of pigment:

The broth culture was centrifuged at 7000rpm for 10 min at 0°C to harvest the cells. Petroleum ether and acetone in the ratio (1:1) was added to the harvested cells. The petroleum ether layer was evaporated to dryness in a vacuum and pigments were then dissolved in acetone. The carotenoid solution in acetone was allowed to stand at -20°C for overnight incubation. Saponification of the pigments was carried out in a 10% methanolic potassium hydroxide solution for 30 min at 40°C with continuous stirring and the unsaponifiable matters were extracted with petroleum ether. Separation and identification of the pigments was carried out by means of thin layer chromatographic method.

RESULTS AND DISCUSSION

Isolation and Identification of the microorganisms:

A total of 20 isolates were obtained on YM agar plates in which three isolates showed luxuriant growth and high colour intensity. These isolates were selected and identified as *Brevibacterium maris* (orange colony) and *Arthrobacter sp.*, (pink colony) *Serratia marcescens*.



Figure-1: *Brevibacterium maris*



Figure-2: *Arthrobacter sp.*



Figure-3: *Serratia marcescens*

Colour Reactions:

The comparison study was carried out to check the reactions of acid and bases with the cultures. The cultures reacted differently with the acids and bases. The colour change is given in Table-1.

Optimisation:

The optimisation was carried out to increase the pigment production as well as growth rate of the isolates. *Brevibacterium maris* produced higher intensity of pigment when cultured on YM broth, *Arthrobacter sp.*, *Serratia marcescens* showed pigmentation when cultured on nutrient broth. Thus, the optimisation was further carried out with the respective broths. The maximum growth and intensity of colour production for *Brevibacterium maris* was observed at 28°C, for *Serratia marcescens* at 28°C and for *Arthrobacter sp.* at 37°C (Figure 4). The colour intensity for pigmentation was seen maximum when *Brevibacterium maris* was cultured with YM broth having pH of 6.0.

Arthrobacter sp. and *Serratia marcescens* showed optimum growth and colour intensity at pH of 7.0 (Figure 5).

Brevibacterium maris and *Arthrobacter sp* produced optimum growth and colour production

in the presence of glucose as its carbon source. *Serratia marcescens* showed maximum colour intensity with dextrose as its carbon source (Figure 6). The optimum nitrogen source for the isolates is given in Figure 7. Yeast extract proved to be a better source of nitrogen for the growth of *Brevibacterium maris* similarly tryptone and beef extract were the nitrogen source for *Arthrobacter sp.* and *Serratia marcescens* respectively.

Figure-4: Optimization of incubation temperature

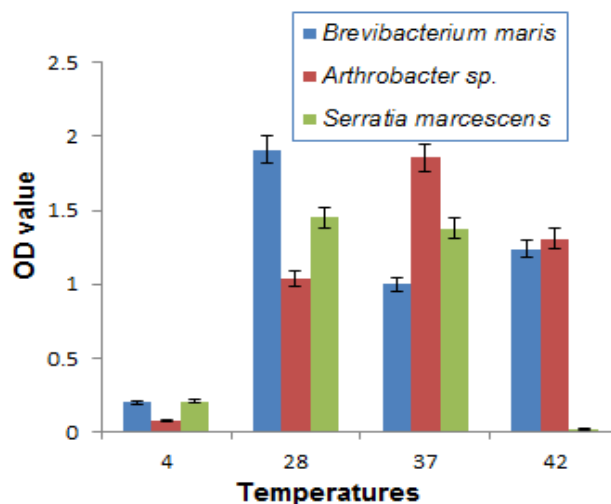
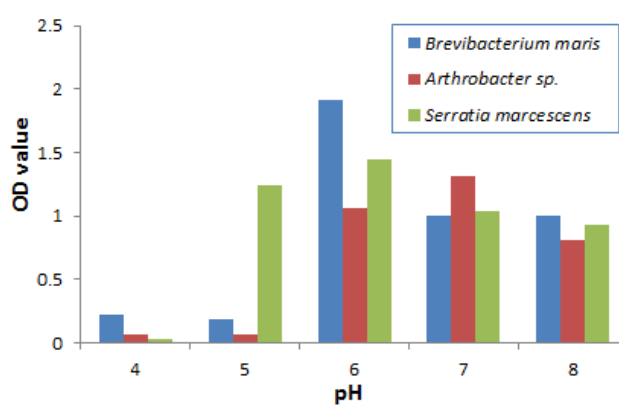
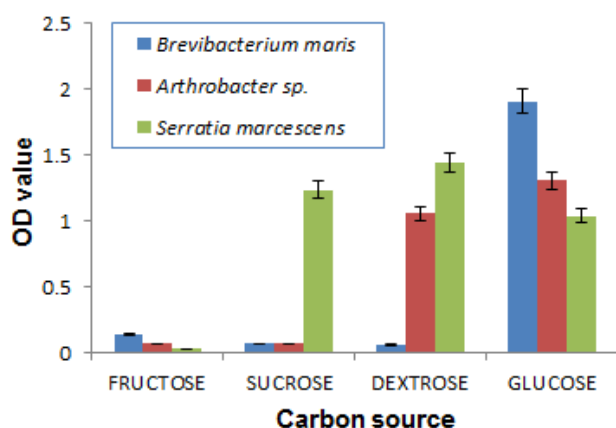
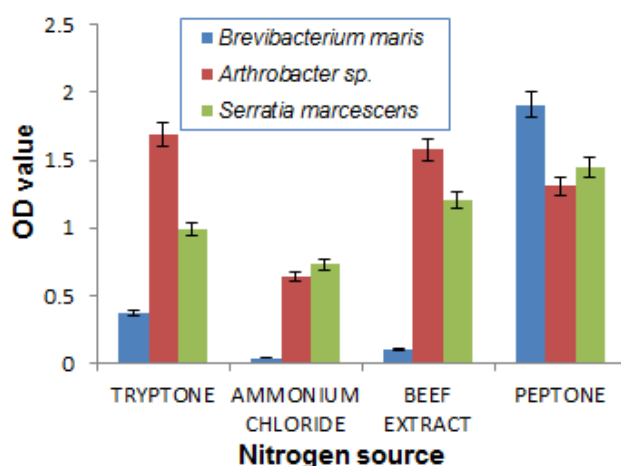


Figure-5: Optimisation of pH



Microorganisms	Colour Changes With The Indicated Reagents				
	NaOH	KOH	HClO ₄	H ₂ SO ₄	HCl
<i>Brevibacterium maris</i>	Red	Red	Blue	Green	Decolouration
<i>Arthrobacter sp.</i>	No colour change	No colour change	Decolouration	Decolouration	Decolouration
<i>Serratia marcescens</i>	Decolouration	Slight decolouration	Dark brown	Orange-brown	Slight decolouration

Table-1: Colour reactions of the isolates with acid and bases.

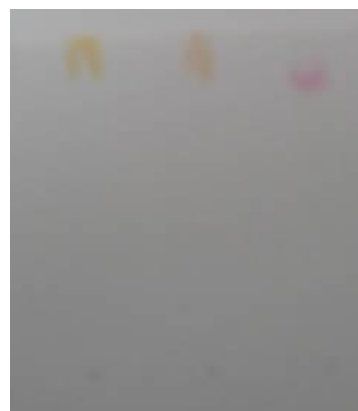
Figure-6: Optimisation of Carbon source**Figure-7: Optimisation of Nitrogen source**

Mass Production, purification and Identification of the pigment:

The mass production was carried out for the isolates individually in their optimised conditions for 48 hours. The pigment was extracted from cell harvest by solvent extraction method using petroleum ether and acetone (1:1). The pigment moved along with the solvent. Thin layer chromatography was carried out for the separation and identification of the pigment. Thus the pigment produced by *Brevibacterium maris* and *Arthrobacter sp.* were identified as β -carotene (Rf value: 0.95) and the pigment produced by *Serratia marcescens* was identified as prodigiosin (Rf value: 0.83) by TLC method.

Flavobacterium sp. in a nutrient medium containing glucose or sucrose, sulphur-containing amino acids such as methionine, cystine or cysteine, pyridoxine and bivalent metal ions selected from the group consisting of Fe^{2+} , Co^{2+} ,

Mo^{2+} or Mn^{2+} were able to produce up to zeaxanthin (Shepherd *et al.*, 1976). Isorenieratene and hydroxyl derivatives were produced by bacteria like *Brevibacterium linens*, *Streptomyces mediolani* or *Mycobacterium aurum* (Dufossé *et al.*, 2004; Rekha Sethi and Prasad. M.P, 2014; Guyomarc'h *et al.*, 2000). β -carotene were produced by *Blakeslea trispora* (Lampila *et al.*, 1985), *Fusarium sporotrichioides* (Jones *et al.*, 2004), *Mucor circinelloides* (Iturriaga *et al.*, 2005; Negin Adavoudi Jolfaei, Ceera. M and Prasad. M.P, 2014) and *Phycomyces blakesleanus* (Cerdá-Olmedo 2001).

**Figure-8: Thin layer chromatography of the pigments extracted from the isolates**

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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