Production of Biopigment "Prodigiosin" from *Serratia Marcescens* under Optimized Conditions

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Abstract: ***Prodigiosin, a Biopigment, is a secondary metabolite that is a natural red colored pigment which belongs to the family prodiginines. It has a tripyrrole in its structure and is produced by many strains of bacterium Serratia marcescens and other gram negative and some unrelated organisms. Prodigiosin has a variety of biological activities such as antimicrobial, antifungal, antioxidant, antitumor, antimalarial, antiproliferative and immunosuppressive activity and also has wide application in textile, printing, food, cosmetic and therapeutic industries. Therefore, the present study was aimed to screen different natural samples (soil, water and surface swab) for isolation of the prodigiosin producing Serratia marcescens. Isolates were identified by standard microbiological procedures and production of pigment was carried out on Nutrient broth. Extraction was carried out using acidified methanol and subjected to spectrum scanning in range between 300-700nm using methanol as blank followed by Presumptive test to confirm pigment as Prodigiosin. Various parameters were optimized for highest pigment production. Pigment was used to check Bioemulsification activity, Antioxidant activity, Antimicrobial activity and Antibiofouling activity. In the current study, highest pigmented bacterial isolate obtained from the effluent sample and thus was used for pigment production, extraction and further study. The highest absorption peak was observed at 534nm using methanol as blank and pigment production was found to influence by various condition that used for optimization.***

Keywords: Antimicrobial, Microbial pigments, Optimization, Prodigiosin, Serratia marcescens

1. Introduction

The most important attribute of any article is the color, particular for food [1]. We inevitably eat with our eyes [2]. Color in food makes things appealing and recognizable [3]. The word Biopigment is a combination of the words: Bio- means from living and Pigment- means something which is natural and used for coloring purpose [1]. Today mainly artificial colors are available in the market and are used for a variety of purpose. But the Biopigment are one of the natural sources and can be used ecofriendly. Long term use of artificial colors has many side effects which includes allergy and toxicity to humans, generation of waste and issues in disposal [4].

There are various sources of pigments but the main place among them is occupied by Plants. But this, the most important source is attacked by many pathogens and the most common pathogen is Fungi, which leads to the loss of the plants. Fungicides may be used to reduce the loss but again it poses environmental problems [5].

An alternative to plants are the microorganisms. There are a variety of microorganisms that produce the pigment and hence named as Chromogenic bacteria [6]. Microbial pigments are small molecular weight compounds which are referred as "Biopigments". Higher yields in pigment production and lower residues are obtained from microbial fermentation compared to the use of plants and animals [7]. Pigment production from microorganisms has many advantages over plant pigments: Easy Doubling time, Fast growth rate, Cheap culture medium [7], Stability of microorganisms [8], Availability of cultural conditions throughout the year [9] [10], Colors of different shades [11]. Optimisation of culture medium as well as fermentation conditions plays an important role in maximum pigment production [7].

Pigments which are derived naturally are safe, healthy and biodegradable and have high compatibility with environment. Usually pigments produced by microorganisms are the product of their secondary metabolism means that they occur only in late stages of their growth and are produced in retort to many biological activities such as photosynthesis, UV Protection, Defence mechanism, Stress etc.

One such Biopigment is the Prodigiosin from *Serratia marcescens*. Prodigiosin is a red colored pigment which belongs to the family prodiginines and has a tripyrrole in its structure. It has a low molecular weight (323.4 Da). Prodigiosin is produced by many strains of bacterium *Serratia marcescens* and other gram negative and some unrelated organisms such as *Vibrio psychroerythrus, Streptomyces griseoviridis*[12]. *Serratia marcescens* is agram negative, facultative anaerobic, motile, single short rods which belongs to the family Enterobacteriaceae. In 1819, Bartolomeo Bizio, an Italian pharmacist discovered and named *S. marcescens* in honor of an Italian physicist named Seratti and choose *marcescens* because the bloody pigment was found to deteriorate quickly.

Pigment prodigiosin is also known to have many bioactivities such as antibacterial, antifungal, antioxidant, Bioemulsification, Antibiofouling, etc.

The present study is intended for optimization of various parameters for the enhanced production of the pigment. Explorations ofantioxidant and Bioemulsification activity of prodigiosin were the strinking features of the study [12].

2. Materials and Methods

A. Isolation and identification of*S. marcescens*

For the isolation of *Serratia marcescens*, samples from various natural sources such as soil[13], water[7], sewage

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sludge, effluent water[7] and surface swab[12] were collected. The collected samples were serially diluted in sterile distilled water and 0.1 ml aliquots were plated on sterile Nutrient agar plates and incubated at 30^0C for 24-48 hours. After incubation red pigmented colonies were picked up from the plate and sub cultured until pure cultures. Identification was done by Colony characterization, morphological identification and Standard biochemical tests. Cultures were preserved on Nutrient agar slants at 4^oC.

B. Selection of potential isolate for maximalpigment production

Among all the obtained isolates, isolate with the maximum pigment production capacity was selected. For this purpose, all the isolates were inoculated in different flasks containing Nutrient broth and incubated at 30^0C on a rotary shaker for 24-48 hours and prodigiosin production was estimated. The selected isolate was then used for further study.

C. Selection of Media for maximal pigment production by *S. marcescens*

Various media were tested in order to determine the media that supports the maximum production of prodigiosin by *S. marcescens*[12]. Media used included Nutrient broth, Nutrient broth supplemented with 2% glycerol, Peptone glycerol broth and Luria Bertaini broth. Selected isolate was inoculated in these different media and incubated at 30^0C for 24-48 hours.

D. Extraction, Confirmation and Quantification of Prodigiosin

Bacterial cell absorbance of the broth was taken at 620nm [12]. The pigment was extracted using liquid-liquid extraction method [3]. The culture broth was then transferred to centrifuge tubes and centrifuged at 9000 rpm at 4^0C for 10 minutes. Supernatant was discarded and cell pellet was collected. Acidified methanol was added to the cell pellet and vortexed for 2 minutes and centrifuged under the same condition. Supernatant was collected. Presumptive test for confirmation of Prodigiosin was carried out by transferring 2mlsupernatant in two different test tubes. A drop of concentrated HCl was added to one test tube and a drop of concentrated ammonia was added to another tube. Spectral analysis of pigment was carried out in the range of 300-700nm using acidified methanol as blank [12].

Estimation of extracted prodigiosin was done using following formula [14]:

$$\text{Prodigiosin unit/cell} = ([OD534 - (1.381 \times OD620)]) \times 1000/OD620 \quad (1)$$

Where,
OD534 – pigment absorbance
OD620 – bacterial cell absorbance
1.381 – constant

E. Chromatographic separation

Thin layer chromatography was performed to characterize the pigment. The extracted pigment was loaded on silica gel plate and was placed in the solvent chamber containing methanol, ethyl acetate and chloroform in the ratio of 6:3:1 [15]. The Rf value of the extract was determined.

F. Enzyme profile of the isolate

Isolate was studied for different enzymes such as Gelatinase, Caseinase and Lipase [16] by using appropriate media.

G. Optimization of different parameters

Optimization of various process parameters is necessary in order to determine the optimum condition for the maximum production of prodigiosin [17].Optimization was carried out using Nutrient broth.

(1) Effect of Carbon sources on prodigiosin production

Different carbon sources used were glucose, sucrose, fructose and lactose. 1 ml overnight culture of *S. marcescens* was inoculated in Nutrient broth supplemented with 1% w/v concentration of carbon sources and incubated at 30^0C on a rotary shaker for 24-48 hours and prodigiosin production was estimated [12].

(2) Effect of Nitrogen sources on prodigiosin production

Organic and inorganic nitrogen sources were used which included tryptone, yeast extract, ammonium chloride and ammonium sulfate. 1 ml overnight culture of *S. marcescens* was inoculated in Nutrient broth supplemented with0.5% w/v concentration of nitrogen sources and incubated at 30^0C on a rotary shaker for 24-48 hours and prodigiosin production was estimated [18].

(3) Effect of Natural substrates on prodigiosin production

Natural sources such as Peanut seed powder, Sesame seed powder and coconut powder can increase the yield of prodigiosin production and are also cost effective. 2% w/v Peanut seed powder, Sesame seed powder and coconut powder were added to distilled water[16].1 ml overnight culture of*S. marcescens* was inoculated in the medium and incubated at 30^0C on a rotary shaker for 24-48 hours and prodigiosin production was estimated.

(4) Effect of Incubation time on prodigiosin production

1 ml overnight culture of *S. marcescens* was inoculated in Nutrient broth and incubated at 30^0C on rotary shaker and prodigiosin production was estimated at intervals of 24, 48, 72 and 96 hours [15].

(5) Effect of Agitation on prodigiosin production

1 ml overnight culture of *S. marcescens* was inoculated in two different flasks containing Nutrient broth. One flask was incubated at 30^0C on rotary shaker while another flask was kept in static condition in an incubator at 30^0C for 24-48 hours and prodigiosin production was estimated [17].

(6) Effect of pH on prodigiosin production

pH plays an important role in maintaining an environment for the microorganisms to grow luxuriantly[18].1 ml overnight culture of *S. marcescens* was inoculated in Nutrient broth having different pH such as 3,5,7,9 and 11 and incubated at 30^0C on rotary shaker and pigment production was estimated.

(7) Effect of temperature on prodigiosin production

Temperature influence the growth of microorganisms and also pigment production [18]. 1 ml overnight culture of *S. marcescens* was inoculated in different flasks containing

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Nutrient broth and were incubated at 4^0C, 25^0C, 30^0C and 37^0C for 24-48 hours and prodigiosin production was estimated.

(8) Effect of salt concentration on prodigiosin production

1 ml overnight culture of *S. marcescens* was inoculated in Nutrient broth with 0.5%, 1%, 2%, 4% and 6% w/v NaCl concentration [3] and incubated at 30^0C on a rotary shaker for 24-48 hours and prodigiosin production was estimated.

H. Bioemulsification activity

Emulsification activity of *S. marcescens* was studied. 24 hours old culture of *S. marcescens* was inoculated in 100 ml Nutrient broth and incubated at 30^0C for 24 and 48 hours. After incubation, the broth was centrifuged and the supernatant was further used to check emulsifier activity. To measure emulsifier activity, 3 ml of kerosene, toluene, peanut oil and coconut oil was taken in different tubes and 2 ml of supernatant was added to each tube. Tubes were then vortexed at high speed for 2-5 minutes. Tubes were kept in refrigerator and measurements were made 24 hours later. The emulsion index is the height of emulsion layer, divided by the total height, multiplied by 100 [16].

I. Total Antioxidant activity of Prodigiosin

Antioxidant is the substance that prevents the oxidation of other molecules. The total antioxidant capacity of the methanolic extract was evaluated by phosphomolybdenum method [19]. 0.1 ml of the extract solution was mixed with 1 ml reagent solution (6 M Sulphuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95^0C for 90 minutes in water bath. The absorbance of the solution was measured spectrophotometrically at 695nm using a blank. Blank was prepared by using 0.1 ml of the solvent used for extraction andmixed with 1 ml reagent solution and treated in the same way as above. Ascorbic acid (100µg/ml) was used as standard. The antioxidant capacity of the extract was evaluated as equivalents of ascorbic acid (µg AE/ml extract) [12].

3. Results and Discussions

Biopigments obtained are the natural sources of pigment and have a wide application in many fields. Prodigiosin, obtained from *S. marcescens*, is a red pigment which also exhibits variety of bioactivities along with its applications.

A. Isolation and identification of *S. marcescens*

Different natural samples were collected and screened which included 14 soil samples, 3 water samples and 2 surface swab samples and 2 effluent samples. Out of all 21 samples screened fpr prodigiosin production, six isolates that produced red pigment were (S_8, S_{14}, S_{15}, S_{17}, S_{18}, S_{19}). All the isolates were identified by Morphological, colonial and biochemical characterization as *Serratia marcescens.*

Figure 1: Isolate S_8 on nutrient agar plate

B. Selection of potential isolate for maximalpigment production

All the six isolates were tested for maximum pigment production. Pigment production by all isolates is represented in figure below. Isolate S_8 showed maximum pigment production and was selected for further study.

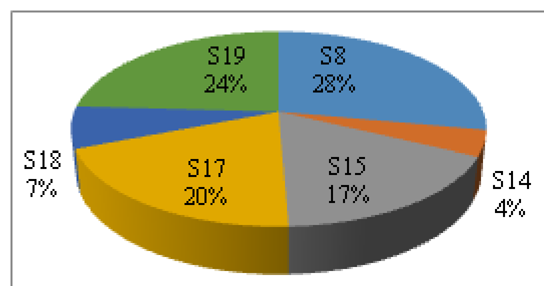

Figure 2: Selection of potential isolates

C. Selection of Media for maximal pigment production by *S. marcescens*

Among all the different media used for optimization, Nutrient broth (6528.81 prodigiosin unit/cell) (Figure-3) was found to be the best medium for maximum pigment production by *Serratia marcescens.*

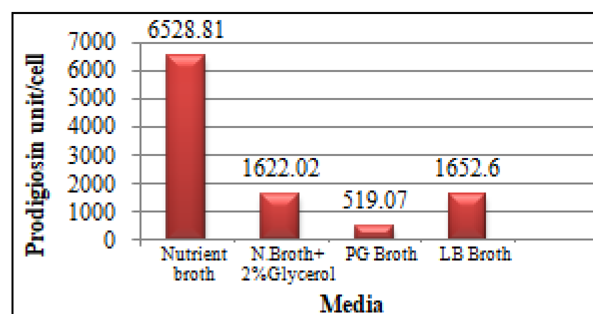

Figure 3: Selection of media

D. Extraction, Confirmation and Quantification of Prodigiosin

The extraction of the pigment was carried out using acidified methanol. Presumptive test for pigment was the carried out in which red/pink color in acidic condition and yellow/tan

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color in alkaline condition (Fig-4) indicated the positive presumptive test for prodigiosin and confirmed the pigment as prodigiosin. Pigment when subjected to spectrum scan showed maximum absorbance peak at 534nm using acidified methanol as blank.

Figure 4: Presumptive test for prodigiosin

E. Chromatographic separation

Seperation of the extracted pigment was done using TLC. The Rf value of the pigment was 0.97. Ahmed et al., 2017 obtained a single band with Rf value 0.9 which are near to our results.

F. Enzyme profile of the isolate

Enzyme profile of the isolate was studied and isolate gave the positive results for all the enzymes tested. It was able to produce gelatinase, caseinase and lipase enzyme. Picha et al., 2015 carried out the enzyme profile of *Serratia marcescens* PP1 which gave positive results for all the enzymes tested. Our results matched with their results.

G. Optimization of different parameters

Various parameters were checked during optimization study to increase pigment production and growth rate of the *S. marcescens.*

(1) Effect of Carbon sources on prodigiosin production

Different carbon sources were used among which maximum prodigiosin production was observed in Nutrient broth supplemented with fructose (2016.49), which was followed by glucose (1491.94) and sucrose (1235.42) while no pigment production was observed in Nutrient broth supplemented with lactose (Figure-5). Prasad, 2015 found that *Serratia marcescens* showed highest pigmentation with dextrose as carbon source in the medium.

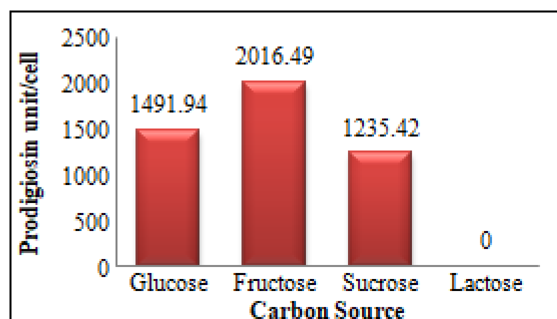

Figure 5: Optimization of Carbon sources

(2) Effect of Nitrogen sources on prodigiosin production

Organic and Inorganic nitrogen sources were used for optimization. Maximum prodigiosin production was observed with Tryptone (854.11) followed by yeast extract (722.74), ammonium chloride (327.33) and ammonium sulphate (141.89) (Fig-6). Prasad, 2015 found that *Serratia marcescens* showed highest pigmentation with beef extract as nitrogen source in the medium.

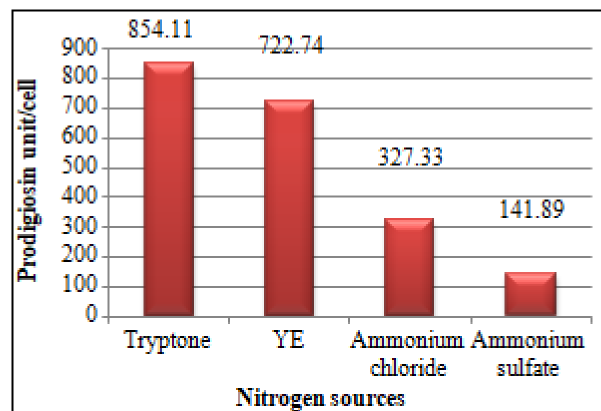

Figure 6: Optimization of Nitrogen sources

(3) Effect of Natural substrates on prodigiosin production

Natural substrates were used for maximum prodigiosin production that included Powdered peanut seed broth, Powdered sesame seed broth and Powdered coconut broth. Highest amount of pigment production was observed with powdered peanut seed broth (295.22) followed by powdered sesame seed broth (291.84) (Fig-7). Jose et al, 2017 in their study found that maximum pigment production was observed in peanut powder broth.

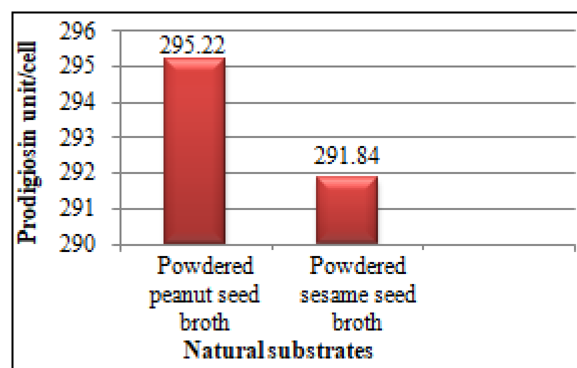

Figure 7: Optimization of Natural sources

(4) Effect of Incubation time on prodigiosin production

Production medium was incubated for different time periods. Maximum prodigiosin production was observed after 48 hours (233.16) of incubation period (Fig-8). Jose et al., 2017 observed that increased pigment production was observed until 48 hours and reduces afterwards. Our results matched with their results. This showed that prodigiosin is produced in late stages of growth and hence is a secondary metabolite.

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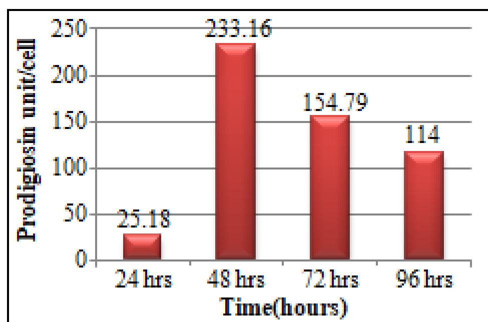

Figure 8: Optimization of Incubation time

(5) Effect of Agitation on prodigiosin production

Maximum prodigiosin production was observed with shaking condition (1070.42) rather than static condition (842.71) (Fig-9). Jose et al., 2017 found that maximum pigment production was observed in shaker conditions rather than static. Our results matched with their results.

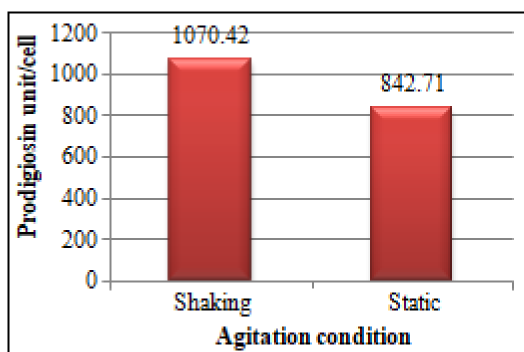

Figure 9: Optimization of Agitation conditions

(6) Effect of pH on prodigiosin production

Medium was adjusted to different pH(3,5,7,9,12). Maximum prodigiosin production was observed at pH 7 (6528.81). No pigment production was observed at pH 3 and pH 12 (Fig-10). Prasad, 2015 found that *Serratia marcescens* showed optimum growth and pigmentation at pH 7. Our results matched with their results.

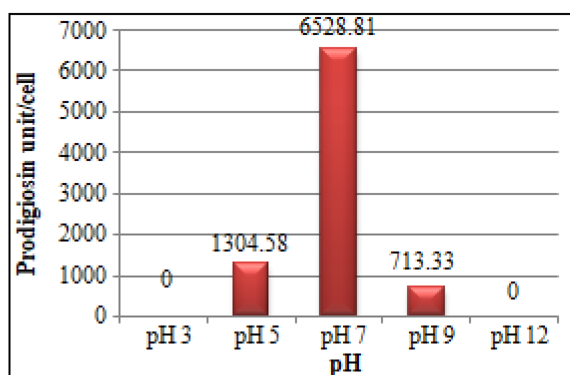

Figure 10: Optimization of pH

(7) Effect of temperature on prodigiosin production

Maximum prodigiosin production was observed at 30^0C (6528.81) followed by 25^0C (2550.88). No pigment production was observed at 4^0C and 37^0C (Fig-11). Jose et al., 2017 in their study to determine optimum temperature for pigment production used temperature 28°C and 37^OC and observed that maximum prodigiosin production was obtained at 28^oC while only slight amount was obtained at 37^0C.

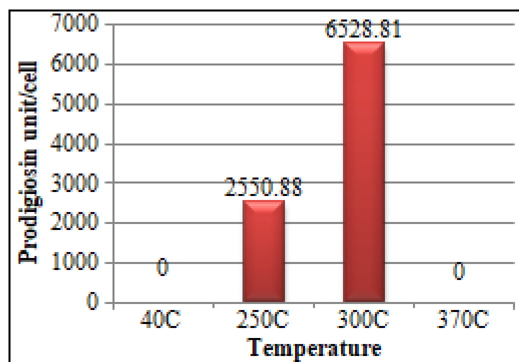

Figure 11: Optimization of Temperature

(8) Effect of salt concentration on prodigiosin production

Different concentration of NaCl salt (0.5%, 1%, 2% ,4%, 6%) was used for optimization. Maximum prodigiosin production was observed at 0.5% salt concentration (6528.81) while no pigment production wasobserved at 4% and 6% salt concentration (Fig-12). Samyuktha and Sayali, 2016 showed that among their 3 isolates maximum pigment production was observed at 4% salt concentration for pb1, 4% and 8% for pb2 and 0.5% for pb3.

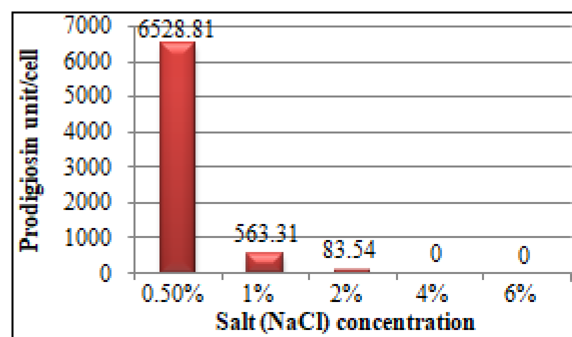

Figure 12: Optimization of Salt concentration

H. Bioemulsification activity

Bioemulsification activity with different hydrocarbons and oils was checked. Highest emulsification activity was observed with Toluene.

Table 1: Bioemulsification activity

Hydrocarbon/Oils	E_{24}	E_{48}
Kerosene	0	5
Toluene	22.5	73.68
Peanut oil	25	45
Coconut oil	60	65

I. Total Antioxidant activity of Prodigiosin

Total antioxidant activity was noted as ascorbic acid equivalents. Basic principle of phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) in the presence of antioxidant compound and a green colored phosphomolybdate complex is formed at acidic pH and at higher temperature. The total antioxidant capacity of the methanolic extract was 200µg AE/ml. The antioxidant activity of the extract was obtained higher as compared to that obtained by Chandni et al which was only 22.05µg AE/ml.

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4. Conclusion

The bacterium isolated from effluent sample, Chaprabhatha area, Surat, Gujarat was identified by morphological characters, colony characterization and standard biochemical tests as a member of Enterobacteriaceae family. The isolate also has potential ability to produce Gelatinase, caseinase and lipase enzymes and shows Bioemulsification as well as antioxidant activity.

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