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LOCAL CULTURE MEDIUM FROM THE LEGUMES MIXTURE AS A NOVEL MEDIA FOR THE GROWTH AND STIMULATION OF PRODIGIOSIN PIGMENT WHICH PRODUCTION FROM *SERRATIA MARCESCENS* THAT ISOLATED ENVIRONMENTALLY

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Abstract

In order to induce rapid division of the bacterial cells and to stimulate the specific metabolic products of these cells, the researcher prefers to use natural culture medium as an alternative to the synthetic medium. This study aimed to stimulate the production of the prodigiosin (red pigment) in the bacterium *Serratia marcescens* that was grown on entirely natural components medium. To investigate the production of prodigiosin pigment in environmental isolates of *Serratia marcescens*, culture medium was prepared from powder by mixing the equal quantities of seven types of dry legumes : white bean, faba beans, mung bean, green peas, chickpea, black-eyed pea and red lentil, these legumes were available at low costs in the local mini markets in the city of Baghdad. Both of the liquid and solid forms of this medium showed remarkable ability for the stimulation of the pigment production after incubation under 25-28°C for 24h. The experiment was also performed by using only one type of the seven legumes for knowledge of each type of legumes individually to stimulate the pigments; pigment production was decreased and the color of the bacterial colonies was light red. The characteristics of this medium compared to other media that naturally prepared are: easily prepared, rarely contaminated due to its dry components and it is simply prepared as both liquid and solid forms because of its dehydrated ingredients. comparing to the commercial media; constituents of this medium are available at low costs and showed the effectiveness of pigments stimulation of *Serratia marcescens* in addition to enhanced bacterial growth.

Key words : Legumes, *Serratia marcescens*, prodigiosin, soil, water.

Introduction

Globally, there is an increasing interest to the use of natural organic pigments (Biopigments) instead of artificial synthetic pigments (Shahitha and Poornima, 2012; Vijayalakshmi and Jagathy, 2016). The biopigments can be obtained from two major sources, plants and microorganisms, Prodigiosin is a multipurpose red pigment, produced by various microorganisms such as *Serratia marcescens*, *Vibrio psychoerythrus*, *Rugamonas rubra*, *Streptomyces lividans*, *Streptomyces coelicolor*, *S. lividans*, *Hahella chejuensi*, *Pseudovibrio denitriccans*, *Pseudo alteromonasrubra*, *Vibrio gazogenes*, *Serratia plymuthica*, *Zooshikella rubidus* and other bacteria (Esabi *et al*., 2015; Chandni *et al*., 2012; Darshan and Manonmani, 2015).

Serratia marcescens is Gram-negative bacteria of family Enterobacteriaceae, motile, non-spore forming, rod shaped. *Serratia*, grow well on synthetic media under anaerobic and aerobic conditions . many strains of *S. marcescens* are produce enzymes such as protease , DNAase, lipase, gelatinase. lectinase , chitinase and hemolysin as virulence factors (Srimathi *et al*., 2017; Ana *et al*., 2006). Environmental isolates of *Serratia marcescens* characteristically produce a prodigiosin that possesses a wide range of advantages and exploits in the

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fields of medicine. The prodigiosin has a variety of biological activities such as antimicrobial, anti-fungal, antimalarial, antiprotozoal, antiproliferative, anti-oxidant, anti-tumor, anti-inflammatory, anti-diabetic properties. Also considered as insecticidal and algicidal, while the clinical isolates are rarely pigmented (Usman *et al*., 2017).

Also, prodigiosin used in industries applications such as: for imparting colors in fabrics, inks, papers, plastics, cosmetics, foods, rubbers, paints, candles and in making soaps and antiseptics, etc. many researchers reported the possibility to replace the synthetic dyes to the colorant which are based on natural sources that are safe because of non-toxic, non-carcinogenic, non-irritating and would be eco-friendly (Meenakshi and Anjali, 2018). Pigment production from microorganisms has many advantages like: fast growth rate, stability of microorganisms ,easy extraction of pigment and cheap culture medium. (Sorathiya and Manisha, 2018). Hence, many environmental factors should be available in alternative culture medium includes: a suitable temperature, pH, incubation time, essential components of bacteria growth in culture medium composition and oxygen amount etc. (Taif and Sawsan, 2015).

This study aims to prepare cheap and natural components culture medium for prodigiosin stimulate production in the bacterium *Serratia marcescens*. Besides that consider a safe and alternative for the growth of bacteria.

Materials and Methods

Four isolates of *Serratia marcescens* were diagnosed from water and soil samples

Isolation of *Serratia marcescens* from soil

Different soil samples were collected in sterile containers. 1 gm of each sample was homogenized with 9 ml of D.W in test tubes and the suspensions were serially diluted up to 10^{-5}. A loopful from the last dilution of homogenated soil samples is streaked onto sterile nutrient agar plates. Red pigmented colonies were isolated after incubation and further purified by a serial sub-culturing method (Ruqayya *et al*., 2016).

Isolation of *Serratia marcescens* from water

Different water samples were collected in sterile containers. 1 ml of each sample was homogenized with 9 ml of D.W in test tubes and the suspensions were serially diluted up to 10^{-5}. A loopful from the last dilution of homogenated water samples is streaked onto sterile nutrient agar plates. Red pigmented colonies were isolated after incubation and further purified by a serial sub-culturing method (Phatake and Dharmadhikari, 2016).

Fig. 1 : *Serratia marcescens* on MacConkey agar.

Diagnostic studies of *Serratia marcescens* isolates

The cultural characteristics of *S. marcescens* colonies were red on nutrient agar and late lactose fermenter on MacConkey agar (fig. 1). Then were carried out the staining technique under a microscope. The definitive identification of *Serratia marcescens* was done using the VITEK® 2 GN ID card.

Collection of the legumes

The seven types of dry legumes (fig. 2) {white beans, faba bean, mung bean, green peas, chickpea, black-eyed pea and red lentil} were collected from local mini markets in the city of Baghdad.

Preparation of a local culture medium of each type of legumes

The mentioned legumes were completely ground into a fine powder by an electric blender and with the use of sieve. Then the powdered legumes were kept in sterile containers until its use. Seven different culture media were prepared as follows : 1.4 gm of each type of legumes dissolved in 100 ml of distilled water (for liquid medium) & add of 1.5 gm of agar-agar to solidification (for solid medium) .the pH of the culture media were measured and recorded 7±0.5. The culture media were sterilized by an autoclave at 121°C for 20 minutes under the pressure of 15 lbs/inch^2 and were poured into sterile Petri dishes separately.

Preparation of local culture medium from the mixture of seven types of legumes

Two grams of each seven types of legumes powder were taken and they were well mixed to become 14 gm (fig. 3) then dissolved in 1000 ml of distilled water [for liquid medium] (fig. 4 A-B) then distributed into plane

Fig. 2 : Some of the dry legumes.

Fig. 3 : The powder of seven dry legumes.

tubes (5 ml) & sterile glass flasks (200 ml). As for the solid medium, 15 gm of agar-agar/L is added to solidification(fig. 5). The pH of the culture medium was measured and recorded 7±0.5. The culture medium were sterilized by an autoclave at 121°C for 20 minutes under the pressure of 15 lbs/inch^2. The solid medium poured into Petri dishes in sterile conditions.

Growing the isolates of *Serratia marcescens* in liquid legumes mixture and on solid legumes mixture

- Legumes alternative culture medium was prepared and distribute into 5 plane tubes, each tube is filled with 5 ml of liquid legumes mixture medium and sterilized then allowed the medium to cool. The five tubes inoculate with various volumes of a fresh culture broth of *Serratia marcescens* (0.5%, 1%, 1.5%, 2%, 2.5%) sequentially by using a sterile inoculation loop.
- While the 500 ml of conical flasks were filled with 200 ml of liquid legumes medium then sterilized, after cooling, inoculated by micropipette with various volumes of a fresh culture broth of *Serratia marcescens* (0.5%, 1%, 1.5%, 2%, 2.5%) sequentially, the flasks plugged with cotton or gauze before incubation.

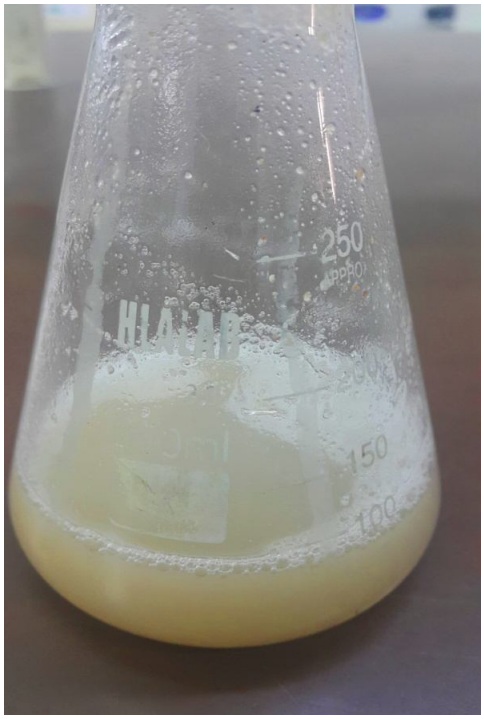

Fig. 4-A : Liquid of seven legumes in a flask.

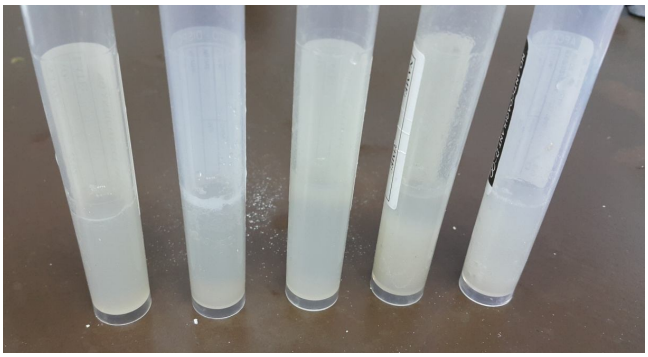

Fig. 4-B : Liquid medium of seven legumes in a plane tubes.

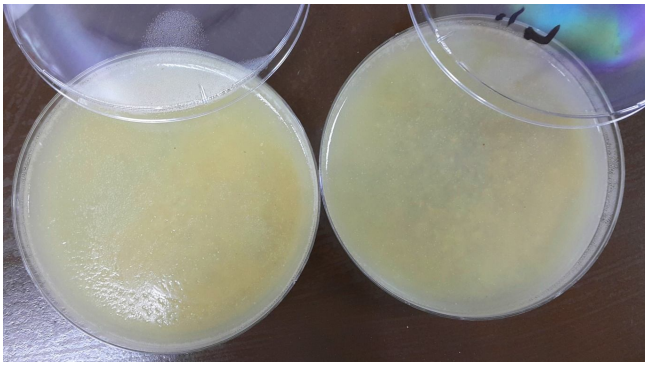

Fig .5 : The solid medium of seven legumes in Petri dishes.

- All *Serratia marcescens* isolates cultured on legumes mixture solid growth medium by streaking. Then all the plane tubes, conical flasks & plates were incubated aerobically at 28°C. After the incubation, the *Serratia marcescens* growth and the prodigiosin production were observed for 72 hours.

Results and Discussion

The results showed that all seven types of legumes supported the growth of *Serratia marcescens* and stimulated to prodigiosin production but in various rates. The mung bean, black-eyed pea, broad bean & green peas supported the maximum stimulation rate of pigment production, while the minimum stimulation rate of prodigiosin was on chickpea, white beans & red lentil. The highest of prodigiosin production was observed when the seven legumes were combined in one culture medium (fig. 6). This increase in the color intensity of prodigiosin may be attributed to varying kinds amino acids in legumes thus causing to rising the concentrations of C & N sources of the medium (Aremu *et al*, 2006).

Our study confirms that both of the liquid and solid legumes mixture medium are able to growth *Serratia marcescens* and enhanced stimulate prodigiosin production, it should be noted that the beginning of the present study, some bacteria, which are characterized by its colorful colonies were cultured on the local culture medium such as *Salmonella*, *staphylococcus aureus* and *Pseudomonas aeruginosa* (fig. 7).

Also, their other factors contributed to the increase of prodigiosin production are: prolonged incubate until 72 hrs at room temperature (25-28^{0}C), pH at 6-7, nutrients amount in culture medium and its type (Zara, 2016; Darshan and Manonmani, 2015). This study as well proves the media containing legumes as substrates are alternative to commercial culture media that could be used for bacterial growth. Legumes are excellent source of protein, carbohydrates and dietary minerals; the nutritional values of legume used in this study as following (Yvonne and Victoria, 2017): the mung bean contained an average of 53 - 67 g carbohydrates, 14.6-33 g protein, 0.7- 1.85 g fat per 100 g (Dahiyaabcd *et al*, 2015). For broad beans the carbohydrates 51-68 g, protein 20-41 g and fat 2 to 3 g per 100 g (Concepcion *et al*., 1998; Larralde *et al*., 1991). While each 100 grams of red lentil contained 1.1 - 2.2 g of fat, 25 - 25.8 g of protein and 59.2 - 60.1 g of carbohydrates (Shahwar *et al*., 2017) whereas the chickpeas, 59 - 67 g of carbohydrates, 16 - 21 g of protein, 3 - 7 g fat per 100 g (Jukanti *et al*., 2012; Al-Snafi, 2016). The values were for green peas 24 g protein, 58 g carbohydrates, 3 g fat per 100 g (Polesi *et al*., 2011), white beans (fava), contains 54- 64 g carbohydrate, 23 g protein, 1.5 g Fat per 100 grams 22) and finally every 100 grams of black-eyed pea contained 25 g protein, 1.9 g fat and 64 g carbohydrates (Hangen and Bennink *et al*., 2002). With many minerals and vitamins in each type of legumes required to growth of bacteria and pigment production. Furthermore, the pH value was kept constant at 6-7 in the liquid & solid medium (Martin *et al*., 2013; Oyebiodun, 1980).

Serratia marcescens that isolated from the soil samples when cultured on a solid and liquid legumes mixture medium showed dark pinkish-red colonies (fig. 8A-B). Besides, appeared lysis zones around the colonies of *Serratia marcescensin* in the solid medium of legumes mixture (fig. 9) the microorganisms from soil samples possess mixed – enzymes that are capable of lysis of starch molecules with the proteolytic enzymes; because of the containment the legumes to carbohydrates and proteins According to Basam *et al.* (2019), Yang *et al*. (2012), Kamble and Hiwarale (2012). While the color of prodigiosin that produced from Isolates *Serratia marcescens* of water samples was orange on the solid medium of legumes mixture (fig. 10).

The results proved that there is a relationship between the volume of bacterial inoculum in liquid legumes and the rate of pigment production the broth medium like as a

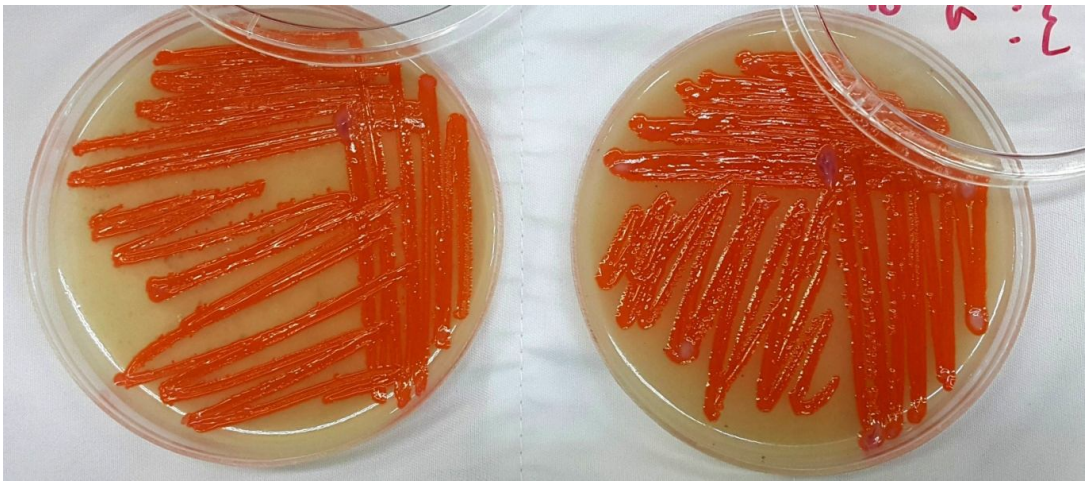

Fig. 6 : *Serratia marcescens* on a solid medium of legumes mixture after incubation.

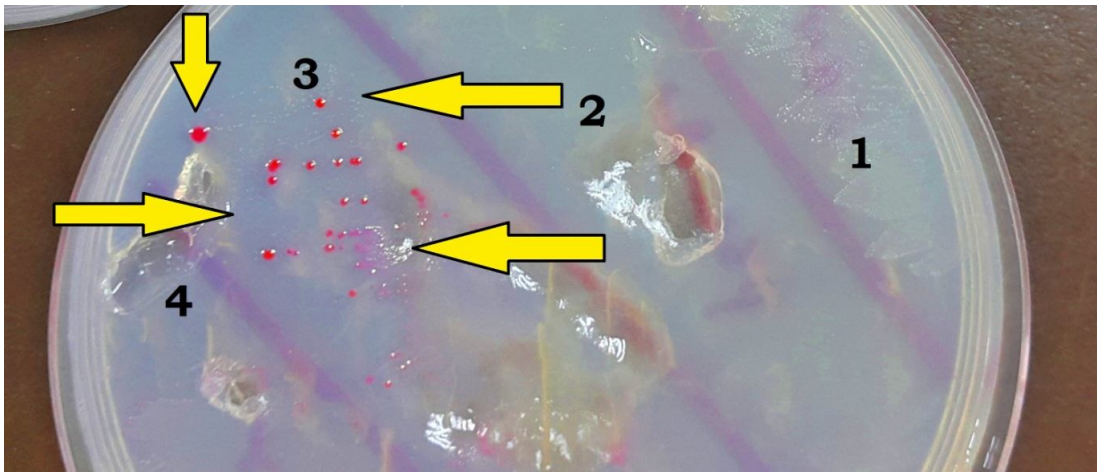

Fig .7 : 1) *Salmonella*, **2**) *Pseudomonas aeruginosa*, **3**) *Serratia marcescens*, **4**) *Staphylococcus aureus* on the first experimental solid medium of legumes.

Fig. 8-A : *Serratia marcescens* from soil samples after 24 hours.

closed and limited environment for inoculated bacteria: that means non-renewable of nutrients for survival for a long period or reproduction of bacterial cells or removal of toxins from it (Manuel *et al*., 2010). So when the tubes and flasks of legumes mixture broth was inoculated with bacterial suspension concentrations of *Serratia marcescens* by the following percentage : [0.5%, 1%, 1.5%, 2%, 2.5%] (v/v) showed that the less volume of

Fig. 8-B : *Serratia marcescens* from soil samples after 72 hours.

Fig. 9 : The formation of clear zones around the *Serratia marcescens* colonies by proteolytic action.

bacterial inoculum led to the elevated pigment production rate in broth (figs. 11, 12, 13).

According to these results, interpreted this as follows:

1. The consistency of bacterial culture media used : unlike solid medium, the diffusion of toxic waste products in the liquid medium will be faster &the accumulation speed of these materials they have negative effects on other living bacterial cells in the culture medium (Kathryn *et al*., 2005; Wardani *et al*., 2017).
2. Optimum conditions for growth & competition principle : the bacterial cells require the best environmental factors in culture medium to grow and to maintain the bacteria activities up to steady-state the cells, so the prodigiosin (secondary metabolite) will appear, if the growth requirements in a culture medium are insufficient; the bacterial cells will oblige to do competitive behavior toward nutrient components & other requirements. this competition may affect on disappearing the pigment due to the death of cells before entering to the Lag or Log phase or even incomplete these phases (Erica and Michiko, 2014; Reed and Paul, 2016).
3. pH when is lowed: increasing the numbers of bacteria cells it is meaning the rise of enzymes secretion and metabolism & thus accumulating more toxic end-products that convert the local culture medium to an acidic environment, prodigiosin production is inhibited (Bhathini *et al*., 2013).

Most studies of researchers were concentrating on using the plants or seeds that have abundant content of fatty acids or oils as culture media to grow and stimulate

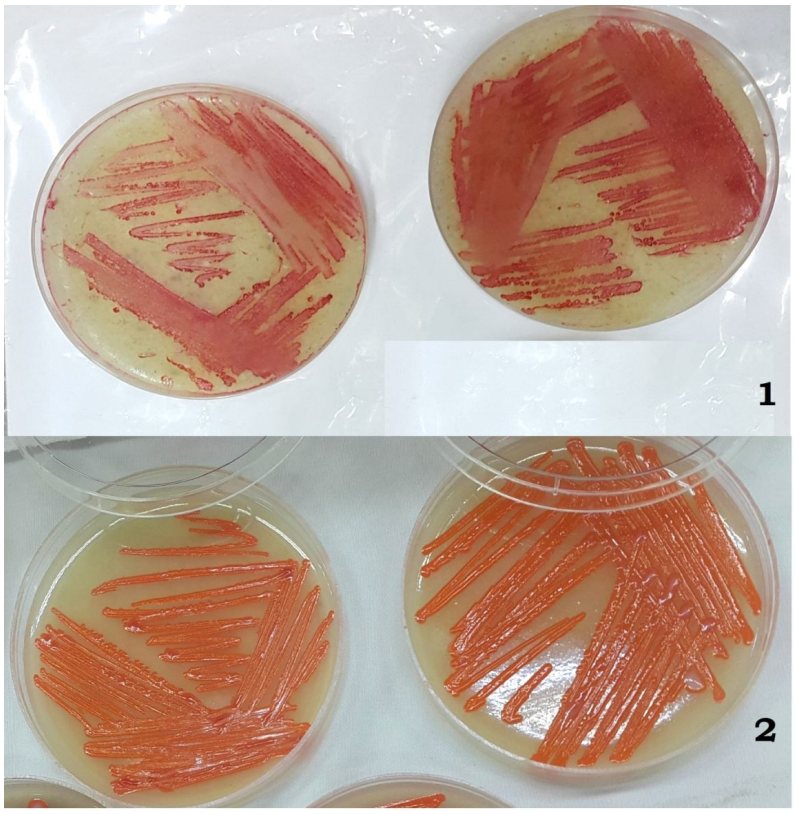

Fig. 10 : Prodigiosin color of *Serratia marcescens* on a solid legumes mixture: **1**) The isolates of soil samples, **2**) The isolates of water samples.

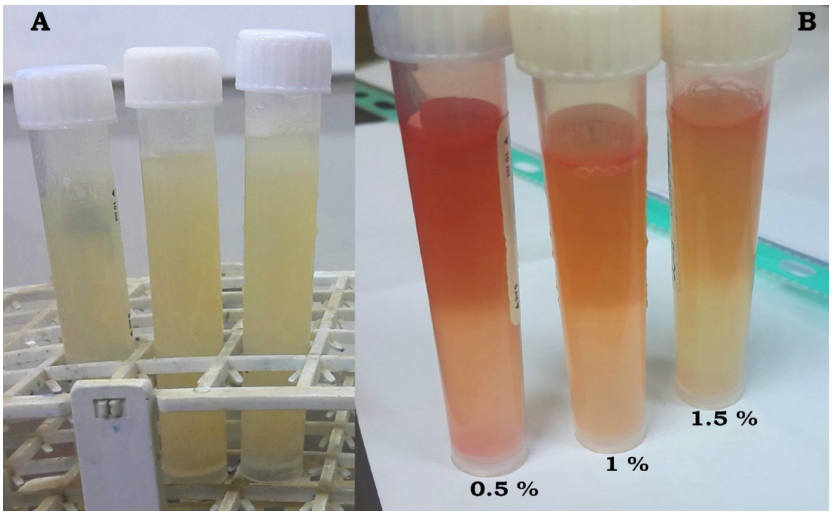

Fig. 11 : Before and after inoculation of legumes medium with *Serratia marcescens*.

the pigment such as powder of peanut, sesame, coconut or oils like: sesame oil, coconut oil, peanut oil, palm oil & olive oil as well as using the sunflower or castor seeds because it's considered as an essential source of carbon and nitrogen that bacteria need to stimulate prodigiosin (Pankaj *et al.*, 2015; Chidambaram and Perumalsamy, 2009). But in this study, we proved it is possible to stimulate the pigment by a non-fatty or oily substrates (fig. 14).

The quantified estimation of prodigiosin (UV spectrophotometer) it was difficult to calculated by equation or formula [O. D499 – (1.3831 x O.D620)]/ O. D 620 ×1000 (Mansi and Gaurav, 2015) because the optical density of legumes mixture medium is unknown.

Conclusion

Presence of the main sources of carbon & nitrogen in this medium is suitable for the overstimulation of pigment production used for especially industrial

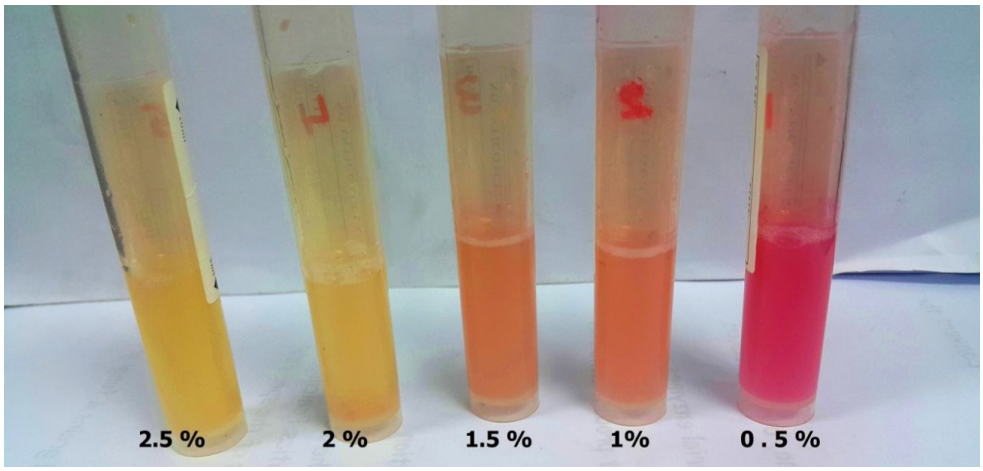

Fig. 12 : Different volumes of *Serratia* inocula (0.5%, 1%, 1.5%, 2%, 2.5%) in test tubes which contains 5 ml of legumes medium after incubation.

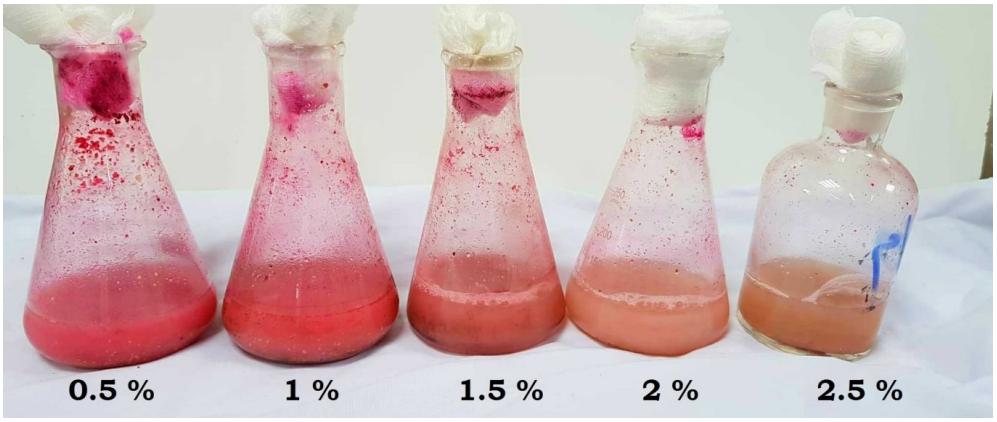

Fig. 13 : Different volumes of *Serratia* inocula (0.5%, 1%, 1.5%, 2%, 2.5%) in flasks, which contains 200 ml of legumes medium after incubation.

Fig. 14 : Pigmentation of gauze after 72 hours by prodigiosin.

applications from environmental isolates of *Serratia marcescens*. The cost of 500 gram of the nutrient medium is approximately 30 $ in Baghdad, whereas the cost of 1 kg of each type of legumes in Baghdad markets 2$ to prepare alternative culture medium. The legumes mixture medium can even be used to grow various bacteria like the nutrient broth or agar but, it cheaper when compared to commercially culture media. Legumes are suitable and inexpensive, which represents a source of plant protein. moreover when the legumes types were combined as one component; the essential amino acids will complete and it could resemble the culture medium containing the

animal protein ingredients.

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