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Isolation of fluorescent pseudomonas from soil, medium optimization and designing of diagnostic media.

Dnyaneshwari M. Joshi[1], Pallavi S. Deshmukh[2],

S.P. Dushing[3], Dr. S. S.Mohorekar[4], Dr. S.T.Mohorekar[5].

Asst. Professor[1], Image Analyst[2],Asst. Professor[3]
Head of Department[4],Assistant Professor[5]
Department of Biotechnology
New arts commerce and science college,
Ahmednagar 414001.

(dmj1257@gmail.com, deshmukhp177@gmail.com)

ABSTRACT: The isolation of the Fluorescent *Pseudomonas spp* .from soil is difficult and therefore the present study is focused on the isolation , identification and the optimum requirements for its growth and pigment production. The biosynthesis of yellow green fluorescent water soluble pigment by *Pseudomonas spp.* occurs under iron deficient conditions
.The isolated organism *Pseudomonas aeruginosa JCM 5962T* shows the presence of two types ofsecondary metabolite pigments: Pyocyanin and Pyoverdine .The antagonistic property of organism is due to Pyocyanin whereas pyoverdine is a siderophore which forms stable Fe III complex and fluoresces under UV light.By a simple modification common commercial dehydrated laboratory media can be used for the evaluation of fluorescent pigment excretion by *Pseudomonas*. The addition of sufficient sterile Egg white or iron binding egg white protein Albumin to bind all the iron in these media converted them to efficient diagnostic media .The absorption maxima of pyoverdine were obtained at 405 nm whereas pyocyanin absorbs more at 520 nm.

Keywords: *Pseudomonas aeruginosa JCM 5962T,* Pyocyanin, Pyoverdine, Evolutionary relatedness, Egg white, Albumin.

Introduction

Several studies on bacterial population within the root environment of plants have shown that the fluorescent pseudomonas constitute a major group of rhizobacteria.Their benifical effect is mainly exerted by antagonism on deleterious soil bacteria and phytopathogenic fungi (*kloepper et al 1980 ;Lamberts and Joos,1989;Weller 1988*). In most cases, this biocontrol effect has been attributed to the production of antibiotics or siderophore in rhizosphere of the pseudomonas colonized roots *(Leong, 1986; tomashow et al., 1990*). Pyoverdine binds to fe^{3+} (Journal of general microbiology vol107,319-328 : The fluorescent pigment of *Pseudomonas fluorescence* : Biosynthesis , purification and physicochemical properties.*J.M*

MEYER etal.1978) and functions as acellular iron carrier or siderophore. However,it is somewhat unstable in aqueous solution and formed degradation products. The pigments produced by pseudomonas species synthesis under certain growth conditions.These pigments are yellow green, fluorescent and water soluble. Many environmental factors affect the synthesis of these pigments.Notably the chemical nature of energy source (*Hepierre*, 1895,*Sullivan.1905,Balanchetiere.1920,Giral* 1936.), pH, light and the cations like mg^{2+}(*Georgia* and *Poe 1931*.) Zn^{2+} (*Baghtiantz*, 1952.) and Fe^{3+} . The pigment produced by the pseudomonas species varies according to physicochemical conditions such as pH, temperature. The concentration of pigments production by organism depends on the cations present in the environment and in the medium. The composition of medium may affects the bacterial growth and the pigment production. Optimization of the medium is important to determine the optimum temperature, pH, and the cation concentration required for the growth andpigment production. The growth of bacterialcells was determined by spectrophotometrically.The *pseudomonas aeruginosa* is a gram negative , rod shaped, facultative anerobic bacteria which is a opportunistic pathogen amd produces two pigments i.e. Pyocyanin and the pyoverdine. The molecules are not only powerful iron(III) scavengers but efficient iron (III) transporters as well. *P. aeruginosa* attracts attention by producing a variety of extra- cellular phenazine pigments. Pyocyanin is produced abundantly in media with low iron content and plays an important role in iron metabolism which considered as a crucial requirement for the growth of *P. aeruginosa.* pocyanin is a secondary metabolite with the ability to oxidise and reduce other molecule and therefore can kill microbes competing against *P. aeruginosa* (*Sudhakar et al., 2013*).)By conventional methods the diagnosis of *Pseudomonas* in various hospital acquired diseases is difficult but if that organism has fluorescent capacity thenit can be possible to design such a medium which enhances its ability to fluoresce. The medium must be cost effective and contains some of the raw sources so that it can be easy to made. After studying the growth and pigment production ability of bacteria in conventional medium which are King's B, Nutrient broth and Succinate – citrate medium , The succinate citrate media was taken for the further enhancement purpose because it shows more pigment production in less growth . Albumin is a protein which have capacity to chelate the FeIII ions in the free form so the different concentrations of albumin were taken to check the effect of competitive pigment production . Egg white contains approximately 56% of Albumin so it can be used as raw source of albumin and also act as easy and cheap source for medium preparation.

Material and methods:

The laboratory media were used : Nutrient Broth (NB), King's B media (KB), Succinate citrate medium *J.M MEYERetal.1978*. The media were prepared as directed by different isolation protocols for fluorescent *pseudomonas*. Rhizospheric soil was added into sterilized broth and then incubated for 24 hrs, at 37°C. Previously incubated culture was taken and inoculated into sterilized King'S B medium. Streaking was carried out on King'S B agar plate, the plates were observed under UV transilluminator.

Optimization studies were performed by using succinate citrate medium as illustrated by J.K. Meyer 1978 *et al* for appropriate growth and pigmentation .

The parameters studied was :pH – 4, 7, and 9,Temperature -- 4°C, 20°C, 37°C, and 45°C, Fecl3 concentration– 10, 20, 30 mg.

Analysis of conventional medium

100 ml of Nutrient broth, king's B broth and succinate citrate broth were prepared according to above composition and isolated bacterial culture were inoculated and kept for incubation at 37°C for 48 hrs.The results were checked under UV transilluminator and by taking O.D The broth were centrifuged at 10,000 rpm for 15 min and
O.D. was taken at 405 nm to check pigment production. Trypticase soy agar (TSA), Plate count agar, pseudomonas agar F (PsAF), Nutrient agar. Were traditionally used for the pigment enhancement purpose according to reference .(J.A. Garibaldi 1967 : media for enhancement of fluorescent pigment production by pseudomonas spp. Journal of bacteriology vol.94, 1296-1299 American society for microbiology)But as per our modification the second medium used was **succinate citrate medium** along with albumin powder with different concentrations i.e. 5,10,15,20,25,30 mg/100ml broth and 10% egg

white to the

100 ml of succinate broth was taken andbacterial culture were added in broth andkept for incubation at 37°C for 48 hrs.The results were checked under UV transilluminator and by taking O.D. for bacterial growth. The broth were centrifuged at 10,000 rpm for 15 min and O.D. was taken at 405 nm to check pigment production.

Result and Discussion:

Medium optimization :Optimization of pH:

For the medium optimization purpose thesuccinate – Citrate medium is used with the pH of 4, 7,and 9 and they were kept forincubation at 37°c for 48 hrs.After 48 hrs. it shows significant change in growth and pigment production along with change in color of pigment , the results were checked by using spectrophotometer for bacterial growth analysis the OD were and forpigment production analysis OD were taken at 405nm(in Graph 1,2).At pH 4 the bacteriawas unable to grow hence it also not produces pigment in that broth .At pH 7 bacterial growth and pigment production was observed to be maximum and also fluoresces with maximum intensity. At pH 9the bacterial growth was intermediate and at alkaline conditions it shows differentpigment color formation ie Blue color ofmedia because , the alkaline pH causes formation of digradative products of pigment which fluoresces with less intensity as compare to pH 7 .So according to study itsuggests that the optimum pH for Growth of bacteria and for pigment production is 7.(Journal of general microbiology vol107 ,319-328 : The fluorescent pigment of *Pseudomonas fluorescence* : Biosynthesis , purification and physicochemical properties.J.M MEYER etal.1978)

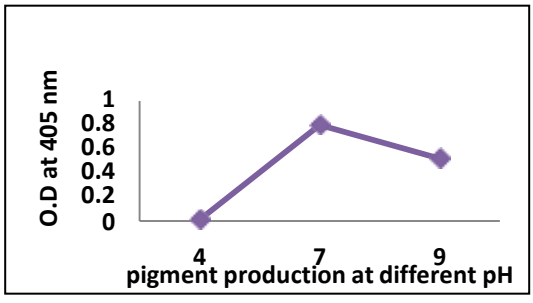
Optimization of Temperature:

For the medium optimization purpose the succinate– Citrate medium is used with thetemperature of 4°,20° ,37°,and 45° C and they were kept for incubation where their corresponding temperature can be maintained for 48 hrs.

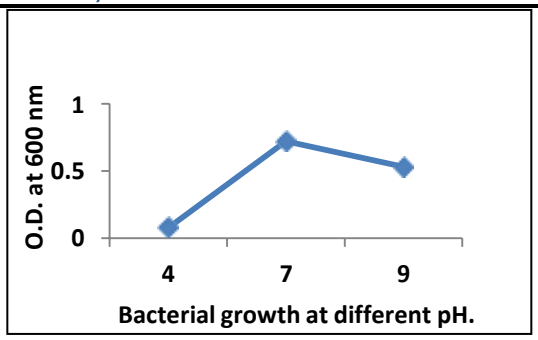
After 48 hrs. it shows significant change in growth and pigment production along with change in color of pigment , the results werechecked by using spectrophotometer for bacterial growth analysis the OD were takenat 600nm and for pigment production analysis OD were taken at 405nm(in graph 3,4). At temperature 4° ,20 ° and 45° C therewas no significant growth was observed and also it shows minimum or very less pigment production.At temperature 37°C the bacterial growth and pigment production were maximum which shows that the optimum temperature for bacterial growthand pigment production is 37°C. Journal of general microbiology vol107 , 319-328 : The fluorescent pigment of *Pseudomonas fluorescence* : Biosynthesis , purification and physicochemical properties. J.M MEYER et al.1978.

Optimization of $FeCl_3$ concentration

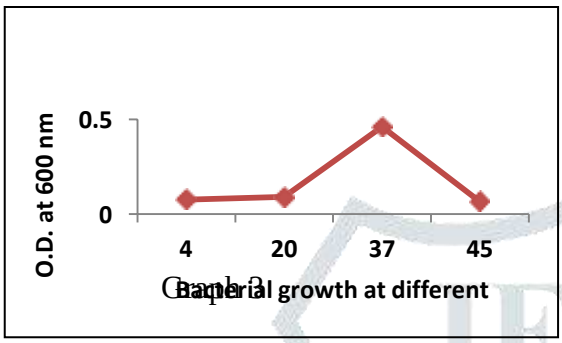
For the medium optimization purpose the succinate – Citrate medium is used with the three different concentration of $FeCl_3$:10 mg , 20mg. ,and 30 mg they were kept forincubation at 37° C for 48 hrs. After 48 hrs. it shows significant change in growth and pigment production along with change in color of pigment , the results were checked by using spectrophotometer for bacterial growth analysis the OD were taken at 600nm and for pigment production analysis OD were taken at 405nm(in graph 5,6). The results shows that the growth of bacteria increases with increase in $FeCl_3$ concentration whereas it decreases the capacity to produce pigment as the FeIII ions get readily available for bacteria.The absorption maxima obtained was 360 nm for10mg, 368 nm for 20mg and 373nm ,and 536 nm for 30mg FeCl3 concentration.



Graph 1.

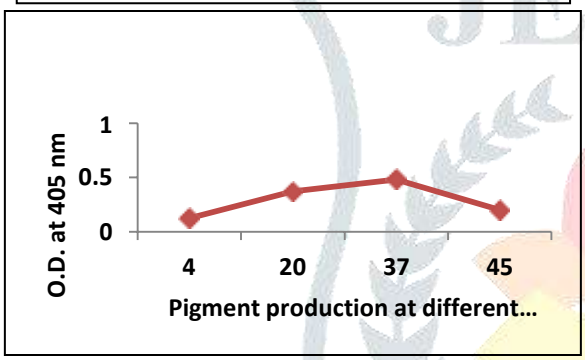


Graph 2

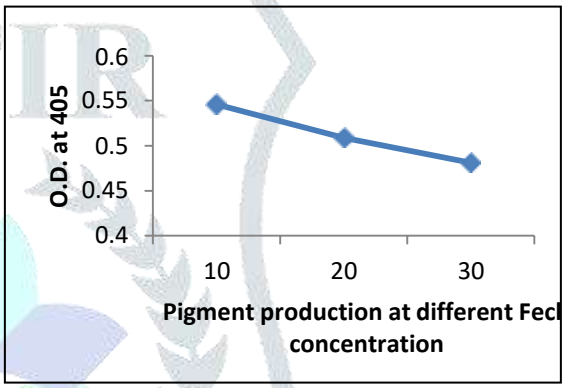


Graph 3

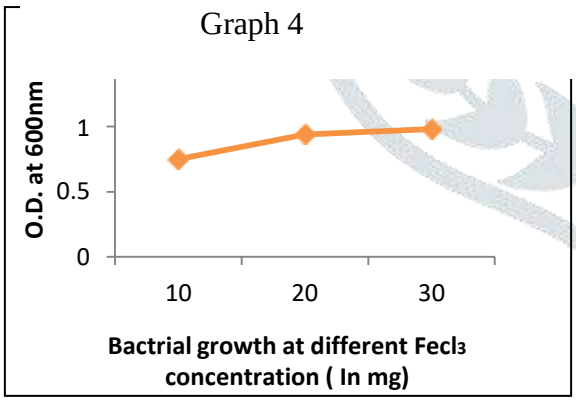
as observed that the growth of bacteria at600 nm was maximum in King's B Medium Nutrient broth shows lowest bacterial and its pigment production at 405 nm was intermediate as compared with succinate broth growth and pigmentation(in graph7,8).



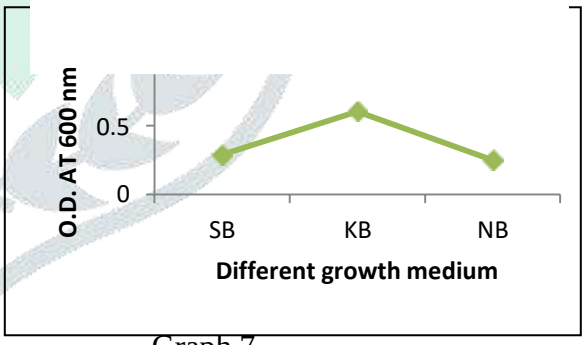
Graph 4



Graph 6



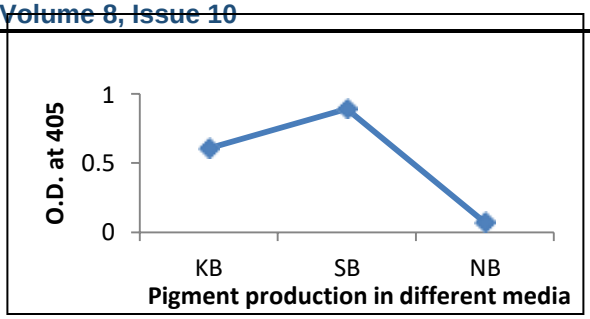
Graph 5



Graph 7

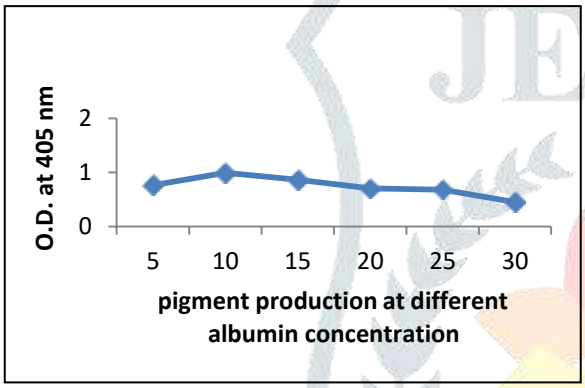
The comparative analysis of conventional media :

The nutrient broth, succinate citrate medium and king's B medium were taken for the analysis of bacterial growth and pigment production study. According to analysis it

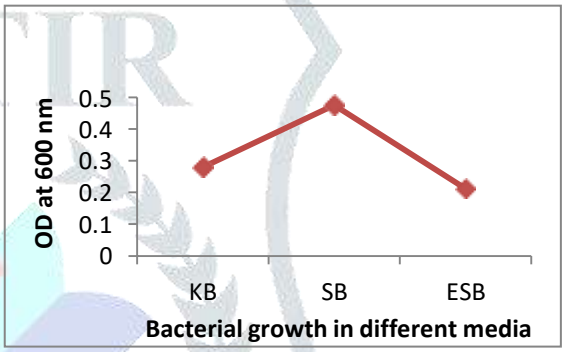


Graph 8 Diagnostic medium designing:

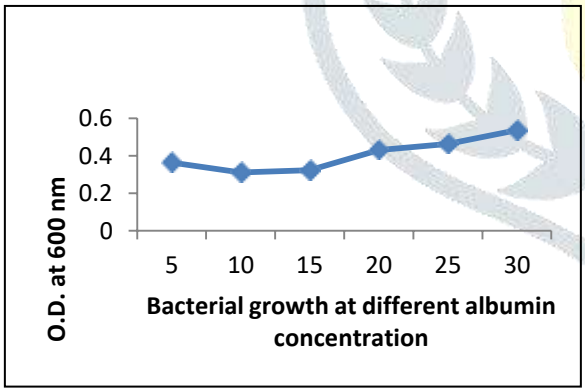
As per the results of conventional medium analysis Succinate medium shows intermediate bacterial growth with maximum amount of pigment production so this medium was taken for further medium designing purpose. Albumin was taken aschelating agent for FeIII ions so it was added in media to increase fluorescent pigment production as it made diagnostic easy. After 48 hrs of incubation it shows maximum growth and pigment production in10mg Albumin containing medium. As the data indicates it suggests that the Growth of bacteria was less in modified medium as compare to traditional Succinate medium but it eventually indicates the more amount of pigment production(in graph 9,10,11,12). Therefore it may be used as diagnostic medium.

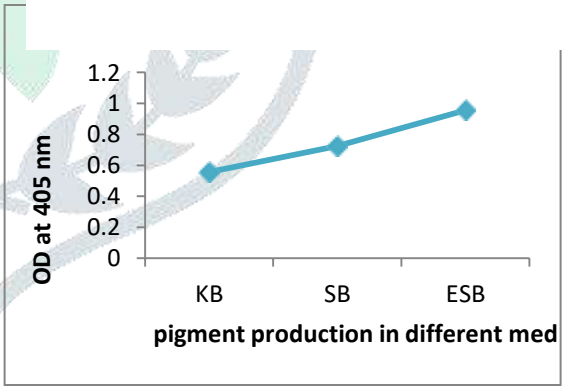


Graph 10



Graph 11





Graph 12 Graph 9

In figure left flask shows succinate medium and right flask shows succinate medium with egg white with increased

Conclusion:

The optimization parameters for the growth of bacteria obtained to be pH 7, Temperature 37°c and $FeCl_3$ concentration 30 mg for bacterial growth and 10 mg for pigment production.The present study suggests that , the simple modification in growth medium can make it easy diagnostic medium according to the values which were obtained and under the UV transilluminator observations it can be concluded thar the succinate citrate medium with Egg white can increases the pigment production capacity of bacteria and with the less growth and time .

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