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A Novel Approach for Siderophore Production by *Pseudomonas mendocina* under Solid State Fermentation using Inert Support

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Abstract: ***Many researchers around the globe have reported siderophore production using microorganisms like bacteria and fungi and most of them employed submerged fermentation. In this study we developed strategies to produce bacterial siderophore using solid state fermentation using a nutritionally inert substrate. KRB1 (identified as Pseudomonas mendocina by MTCC) isolated from the paddy fields of Kuttanad, Kerala was selected and important fermentation parameters were optimized to enhance siderophore production. There was an increase in the quantity of siderophore produced after the optimization steps. Tap water was found to be the most suitable extraction medium. Fermentation conditions like temperature of 30^{o}C and 24 hours of incubation, medium pH – 7 and a moisture content of 0.6% turned out to be optimum for siderophore production through solid state fermentation. Sucrose and peptone were the preferred carbon and nitrogen source respectively which enhanced the production whereas, all the amino acids adversely affected siderophore production. Also an inoculum concentration of O.D 3 resulted in the production of highest quantity of siderophore. The siderophore produced by KRB1 was found to be of hydroxamate nature after characterization studies.***

Keywords: Siderophore, Solid State Fermentation, *Pseudomonas mendocina*, Polystyrene beads, Inert support

1. Introduction

All living organisms ranging from the simple microorganisms to the complex human beings require the continuous supply of nutrients to sustain its life activities. Nutrients can be variously categorized depending upon its availability and the concentration needed by the organisms. Iron is one such micronutrient which has a variety of functions in a living organism. When the availability of biologically needed iron decreases, it disturbs the physiological activities of all living organisms including plants, animals and even the microbes. During such situations most microbes and some plants secretes certain iron binding agents known as Siderophore[1]. The term siderophore was coined in 1973 and was described as low molecular weight molecules that bind ferric iron with an extremely high affinity[2]. Based on the chemical nature of the iron binding group, siderophores fall in to different classes irrespective of the organism producing it. They may be classified into hydroxamate, catecholate, carboxylate or even mixed types. Majority of the siderophores are found to be of either hydroxamate or catecholate variety[3]. In the present study siderophore production was carried out using bacteria isolated from the paddy field of Kuttanad, Kerala which is assigned as a *Globally Important Agriculture Heritage Site*, by FAO. Also important process parameters were optimized to enhance siderophore production using nutritionally inert support under solid state fermentation.

2. Materials and Methods

Bacterial strain used in the present study

KRB1, isolated from the paddy fields of Kuttanad, Kerala during an earlier investigation was used for siderophore production under solid state fermentation[4]. Partial characterization of the bacterial strain was done in our laboratory which was later sent to MTCC, Chandigarh for its identification up to the species level.

Siderophore production by the selected strain

Tests using both Chrome Azurol Sulphonate (CAS) agar and Succinate medium (pH - 7) were carried out to confirm siderophore production by the selected strain. CAS agar was a selective medium which was used for the identification of siderophore producing bacteria. Formation of orange halos around the bacterial colonies when grown on CAS agar was a positive indication of siderophore production[5]. CAS agar was prepared as per the method of Alexander and Zuberer [6]. Also, obtaining a peak at or near 404 nm during the spectral analysis of the cell free supernatant of succinate medium grown bacterial culture, further confirms siderophore production[7].

Substrate for siderophore production under Solid State Fermentation (SSF)

Solid-state fermentation (SSF) is the technique of growing micro-organisms on moist solid material in the absence or near-absence of free water by making use of a natural substrate or an inert substrate as solid support[8]. For the present study, Polystyrene, a nutritionally inert substrate was selected for the fermentation process. Polystyrene (poly (1-phenylethene-1, 2-diyl)), is a polymerization product of the monomer styrene. It is a long-chain hydrocarbon with the chemical formula $(C_8H_8)_n$ and a molecular weight of 1,00,000 – 4,00,000. Polystyrene beads which are available in the markets are found in its expanded form, which makes them lighter in weight and consume more space than the unexpanded polystyrene beads. Hence the expanded polystyrene beads were pre-treated with temperature and pressure which resulted in solid beads of smaller size (**Photograph - 1**). 10 g pre-treated polystyrene beads, in 250 ml Erlenmeyer flasks, was selected as

substrate for siderophore production under solid state fermentation throughout this study.

Photograph 1: Pre-treated polystyrene beads

Quantification of siderophore produced

Siderophore produced after the fermentation was quantitatively estimated as per the method of Schwyn & Neilands (1987)[5]. 0.5 ml of the cell-free culture supernatant was treated with 0.5 ml of CAS assay solution and 10 µl of 0.2 M 5-sulphosalicylic acid (shuttle solution). A colour change from blue to orange was noted depending on the concentration of siderophore produced. After an incubation period of about 20 minutes, the absorbance was noted at 620 nm using a spectrophotometer. Siderophore production was calculated as Percentage siderophore units (PSU) = $[(A_r - A_s)/A_r]$ 100 where, A_r = Absorbance of reference at 630 nm and A_s = Absorbance of sample at 630 nm.

Optimization of fermentation conditions

Fermentation conditions were optimized so as to increase the yield of siderophore. All the experiments were carried out as batch cultures and in duplicate with adequate controls. The strategy followed for optimization experiments was to optimize one parameter at a time and include it at the optimized value in the next experiment. The following parameters were optimized for the present study:

Buffer for the extraction of siderophore

An essential requirement in solid state fermentation is that the metabolites produced must be extracted with a suitable medium or buffer in order to quantify the amount of metabolites produced. Hence this was the first step to be optimized. Tap water, distilled water, succinate medium, succinate buffer, phosphate buffer and acetate buffer were used as extraction medium. The extraction was carried out using 10 ml of the above solutions with a contact time of about 30 minutes, at room temperature with mechanical agitation at a speed of about 150 rpm using an orbital shaker.

Temperature of incubation

The effect of incubation temperature on siderophore production by *Pseudomonas mendocina* was determined by incubating the inoculated flasks containing polystyrene beads at different temperatures like 25°C, 30°C, 35°C, 40°C and 45°C.

pH of medium and hours of incubation

The effect of the pH of the succinate medium on siderophore production was studied from acidic to alkaline range i.e. from pH 5 to pH 9. 1 N NaOH and 1 N HCl were used to alter the pH of the succinate medium, which was used to moisten the surface of the polystyrene beads. Siderophore production was examined every 24, 48, 72 and 96 hours.

Inoculum concentration

Batch cultures containing polystyrene beads moistened with succinate medium were incubated with inoculum of the selected bacteria of increasing range from 0.5 to 3.0 optical densities at 620 nm ($O.D_{620}$), to check the impact of inoculum concentration on siderophore production under solid state fermentation.

Carbon and Nitrogen sources

Glucose, glycerol, sodium acetate and sucrose were the additional carbon sources tested. They were incorporated at 0.5%, 1%, 1.5%, 2%, 2.5% and 3% (w/w) to the polystyrene beads using appropriate controls. The nitrogen sources studied included ammonium sulphate, urea, sodium nitrate and peptone separately at the concentrations of 0.5%, 1%, 1.5%, 2%, 2.5% and 3% (w/w) of polystyrene beads during the fermentation process.

Amino acid sources

To study the effect of the various amino acids on siderophore production, flasks containing polystyrene beads were treated with asparagine, glutamic acid, glycine and histidine separately at the concentrations of 0.5%, 1%, 1.5%, 2%, 2.5% and 3% (w/w). The amino acids were filter sterilized through 0.4 µ sized syringe driven filters to avoid denaturation during autoclaving.

Effect of moisture content

The effect of initial moisture content of the polystyrene beads on siderophore production was found out by preparing the solid substrate with varying initial moisture contents in the range of 1:0 to 1:1 (w/v) ratios. This was achieved by altering the amount of succinate medium used to moisten the substrate.

Chemical characterization of the siderophore

Various tests were carried out to determine the chemical nature of the iron binding group of the siderophore produced by the selected bacterial strain. All tests were carried out using the cell free supernatant of the culture. Hydroxamate nature of the siderophore was tested by Csaky assay (1948)[9] and Tetrazolium salt test[10] whereas Arnow's assay (1937)[11] was used to determine the catecholate nature and Vogel's test (1987)[12] was the basis for the determination of carboxylate siderophores.

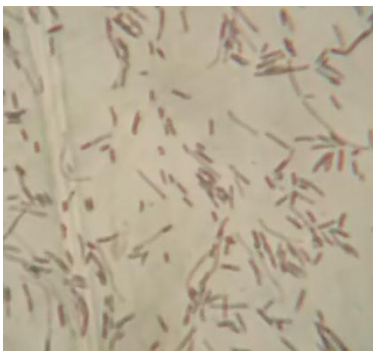
Scanning Electron Microscopic analysis

SEM analysis was carried out to see bacterial adsorption, growth and bio-film formation on to the surface of polystyrene beads. Beads inoculated with KRB1 (*Pseudomonas mendocina*) was mounted on brass stub which was then coated with a thin layer of gold using a JOEL JFC-1600 Auto Fine Coater. The gold coated beads were later viewed with a scanning electron microscope (JOEL JSM-6390 LA Analytical Scanning Electron Microscope) at an accelerating voltage of 20kV.

3. Results and Discussion

Bacterial strain used

KRB1, used in the present study was identified as *Pseudomonas mendocina* by MTCC, Chandigarh. Tests carried out at our lab showed it to be positive for catalase, oxidase and citrate utilization whereas negative results were obtained for indole test and H_2S production. KRB1 was found to be motile and the result of Gram staining showed it to be Gram negative short rods (**Photograph - 2**).



Photograph– 2 Gram stained image of KRB1

Siderophore production by the selected strain

KRB1 was identified to be a siderophore producer using Chrome Azurol Sulphonate (CAS) agar plate assay. After 24 hours of incubation on CAS agar, KRB1 showed orange halos around its colony whereas a non-siderophore producer failed to produce such a halo (**Photograph 3**).

Photograph – 3 A - non siderophore producer, B - siderophore producer (KRB1)

Further, spectral scan analysis using a UV- Visible spectrophotometer gave peak at 401 nm confirming that KRB1 produce siderophore in Succinate medium (**Figure - 1**).

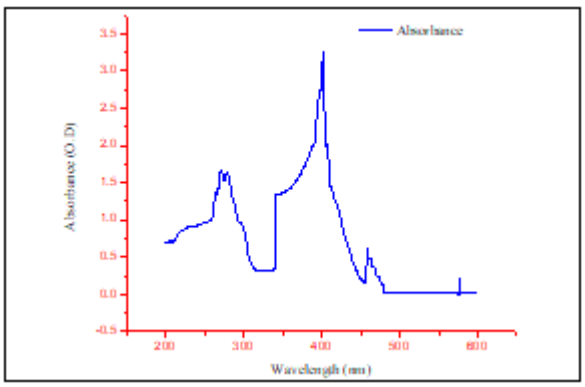


Figure 1: Absorbance spectrum of *P. mendocina* showing peak at 401nm

Optimization of fermentation conditions

Buffer for the extraction of siderophore

Of the various media/buffer tested for their efficiency to extract the siderophore, distilled water, tap water, succinate medium and succinate buffer gave positive results whereas phosphate buffer and acetate buffer completely failed to extract the siderophore. Tap water (TW) proved to be the most suitable medium for the extraction of siderophore produced by KRB1 which resulted in 56.98 PSU. The percentage siderophore units (PSU) obtained for the various media/buffers is as follows: Distilled water – 52.41, Tap water (56.98), Succinate medium (21.72), Succinate Buffer (41.05), Phosphate buffer (–3.92) and Acetate buffer (–10.68).

Temperature of incubation

During the optimization of temperature of incubation, it was found that *P. mendocina* preferred lower temperatures for siderophore production. 30°C was found to be the ideal temperature of incubation for maximum siderophore production by *P. mendocina* which yielded 50.72 PSU and a significant decrease in the yield was recorded at 40°C.PSU obtained for the corresponding temperatures is as given: 25°C (48.54), 30°C (50.72), 35°C (46.22), 40°C (32.56), 45°C (40.4).

pH of medium & hours of incubation

For P. mendocina, siderophore production was found to be maximum at the neutral pH of 7 and then it showed a declining trend towards both increasing acidity and alkalinity. The following are the PSU obtained for the various pH tested – 5(32.28), 6 (44.37), 7(51.87), 8(44.5), 9(38.08). P. mendocina produced maximum amount of siderophore i.e. 55.54 PSU within 24 hours of incubation, there after decreasing the production as the incubation time continued. 46.92 PSU was obtained for 48 hours whereas 35.47 PSU for 72 hours. Lowest quantity of 30.95 PSU was recorded for 96 hours of incubation.

Inoculum concentration

Inoculum concentration showed a mixed effect on siderophore production by *P. mendocina*. There was an increase in siderophore production towards increasing concentrations of inoculum until 1.5 O.D where 49.27 PSU was obtained. But the production dropped to 32.81 PSU at 2 O.D and later, again showed an increase in yield towards higher inoculum concentrations and finally gave a maximum production of 53.84 PSU at O.D 3 (**Figure - 2**).

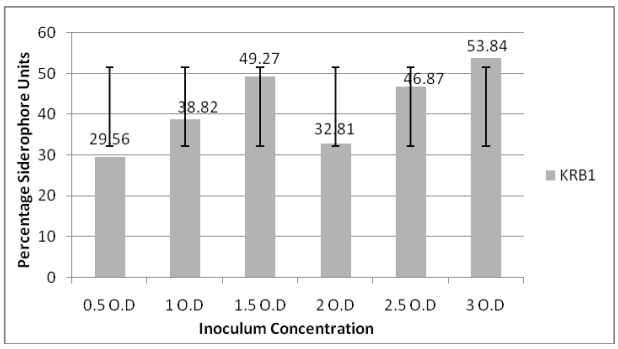


Figure 2: Effect of inoculum concentration on siderophore production by *Pseudomonas mendocina* under SSF

Carbon and Nitrogen sources

P. mendocina produced a maximum of 65.26 PSU at 1% (w/w) concentration of sucrose thus making it as the most preferred carbon source. The remaining three carbon sources stimulated siderophore production only to a negligible extent. Glucose did not support siderophore production at higher concentrations. When compared to the control, it was clear that both glycerol and sodium acetate did not have any notable stimulating effect on siderophore production (**Figure - 3**). From the various nitrogen sources tested, 3% w/w concentration of peptone alone supported siderophore production by *Pseudomonas mendocina* which produced 56.36 PSU. Whereas ammonium chloride, urea and sodium nitrate turned out to be poor nitrogen sources for siderophore production by *P. mendocina* (**Figure - 4**).

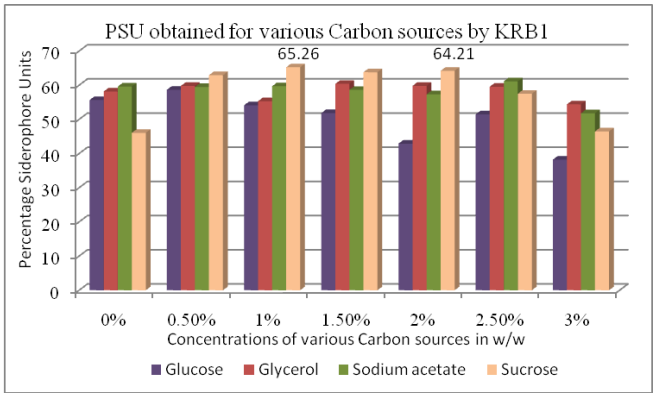


Figure 3: Effect of various carbon sources on siderophore production

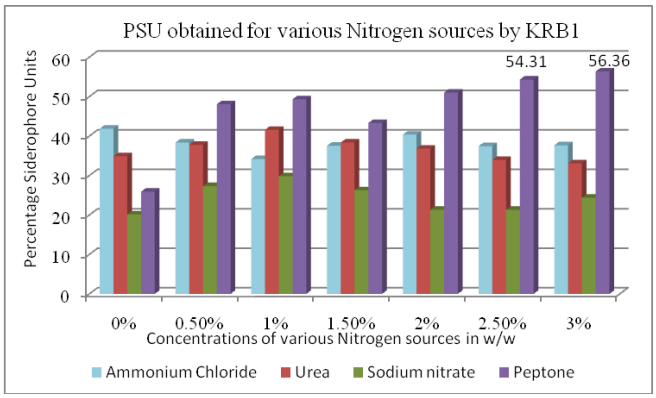


Figure 4: Effect of various nitrogen sources on siderophore production by *Pseudomonas mendocina* under Solid state fermentation

Amino acid sources

All the amino acids tested adversely affected siderophore production by *Pseudomonas mendocina* during solid state fermentation. In case of all four amino acids, maximum unit of siderophore was recorded for control where the medium was devoid of any amino acids (**Figure - 5**).

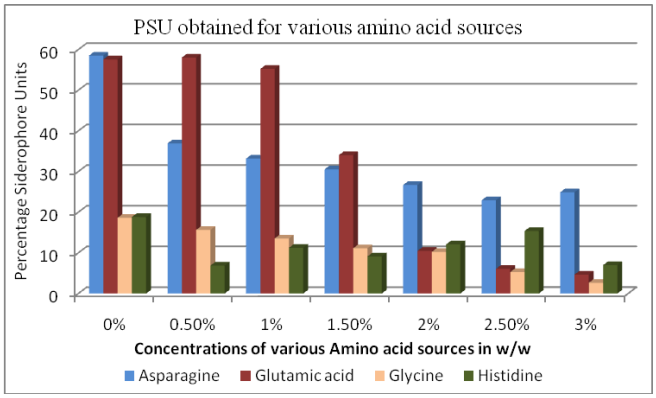


Figure 5: Effect of various amino acid sources on siderophore production by *Pseudomonas mendocina* under Solid state fermentation

Effect of moisture content

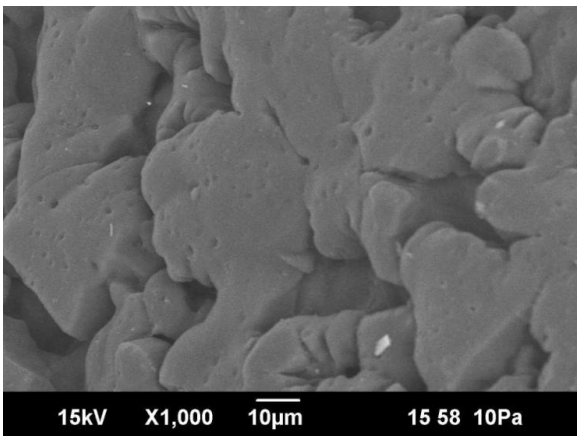
At 0% moisture level, the strain produced negligible amount of siderophore (9.36 PSU) and later, increased the production with increasing moisture content. *P. mendocina* produced maximum siderophore (89.31 PSU) at 60% (v/w) moisture level. It was found that the strain needed higher levels of moisture on an average, for siderophore production under solid state fermentation.

Chemical characterization of the siderophore

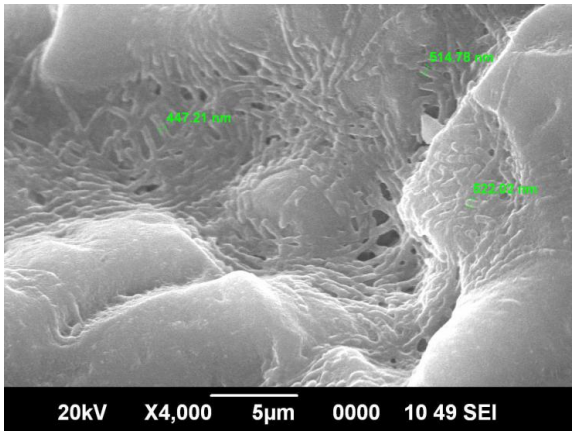
The cell free supernatant of *P. mendocina* culture was assayed to determine the chemical nature of the siderophore and it was found to be of hydroxamate type siderophore. Positive results were obtained for both tetrazolium salt test and Csaky assay. Hydroxamate nature of the siderophore was further confirmed when negative results were obtained for Arnow's assay and Vogel's test ruling out the possibility of catecholate and carboxylate type siderophore respectively.

Scanning Electron Microscopic analysis

SEM analysis clearly showed the adsorption of bacteria on to the surface of polystyrene beads and bio film formation after 24 hours of incubation (**Photograph - 5**) whereas un-inoculated bead surface did not show any microbial growth (**Photograph - 4**). This proves that the bacteria adhered to the surface of the beads during solid state fermentation.



Photograph 4: SEM of un-inoculated polystyrene beads



Photograph 5: SEM of *Pseudomonas mendocina* adsorbed on to the surface of polystyrene beads

Discussion

Siderophores have a wide range of applications that can be categorized as agricultural applications, ecological applications, medical applications, biotechnological applications etc. The present study was more focused towards the agricultural aspect of siderophore because the area from which the bacterium was isolated is well known for paddy cultivation in Kerala. Previous reports show that most of the siderophore producing bacteria isolated from such agrarian regions belong to *Pseudomonas* species. Noori & Saud (2012)[13] isolated 20 strains of *Pseudomonads* from the rhizosphere soils of paddy areas in Malaysia and these were tested positive for siderophore production. Siderophore producing *Pseudomonads* are very important in relation to plant growth and yield. They increase the availability of absorbable iron in the vicinity of plants thus making it available to them. This can reduce the input of chemical pesticides to an extent[14]. Siderophore producing bacteria are also known to act as bio-control agents who block the iron source to other pathogenic bacteria [15]. Also using nutritionally inert substrates in SSF is highly recommended because they can overcome most of the inherent problems of SSF. This is because the use of inert materials impregnated with a suitable media provides a homogenous aerobic condition throughout the fermenter and reduces the chances of impurities being added to the required microbial metabolite[16].

4. Conclusion

Many researchers have worked on siderophore production using both bacteria and fungi which are isolated from different environments. Almost all research on siderophore production have been carried out using submerged fermentation and to date, no work has been reported for the production of siderophores under solid state fermentation. Ours is a novel study on the isolation and characterisation of siderophore producing bacteria from Kuttanad region of Alleppey district in Kerala [4,17]. This is also the first known report on using polystyrene beads as inert supports for siderophore production through solid state fermentation using *Pseudomonas mendocina*.

5. Acknowledgement

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