Preliminary Studies on Siderophore Production and Probiotic Effect of Bacteria Associated with the Guppy, *Poecilia reticulata* Peters, 1859

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Abstract

To study the possible use of probiotics in aquaculture, we evaluated the *in vitro* antagonism of antibacterial strains isolated from the guppy, *Poecilia reticulata* Peters, 1859 against the fish-pathogenic bacteria such as *Aeromonas hydrophila*, *Vibrio cholerae*, *Flavobacterium*, *Acinetobacter* and *Alcaligenes*. As iron is important in virulence and bacterial interactions, the effect of four isolates, namely, MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4 was studied under iron-rich and iron-limited conditions which established the presence of a siderophore-based iron-sequestration mechanism in the isolates from guppy. All the four isolates produced siderophores in the presence of an iron-chelating compound, 2, 2' dipyridyl. The ability to synthesize siderophores would undoubtedly be an advantage for survival, growth and pathogenicity of the isolates. All the investigated pathogenic strains displayed growth along each of the four selected strains under both iron-sufficient and iron-limited conditions. Both $FeCl_3$ and tetrazolium tests produced positive results for all the four selected strains indicating the presence of hydroxamate siderophores. On basis of quantitative assay, the isolate MBTU_PB2 produces maximum siderophore (2.71 mg$^.$ mL^{-1}) followed by MBTU_PB3 (2.23 mg$^.$mL^{-1}), MBTU_PB4 (2.20 mg$^.$mL^{-1}) and MBTU_PB1 (1.26 mg$^.$mL^{-1}). These siderophore producing isolates could find potential applications, such as in the medical, fish farming and food microbiology industries.

Introduction

Aquaculture is one of the fastest developing growth sectors in the world. Disease control in aquaculture industry has been achieved by following different methods using traditional ways, antibiotics and synthetic chemicals. However, the use of such expensive chemotherapeutants for controlling diseases has been widely criticised for their negative impacts such as accumulation of residues and development of drug resistance. There is a reduced consumer preference for aqua products treated with antibiotics and traditional methods are ineffective against controlling new diseases in large aquaculture systems (Raaska and Mattila-Sandholm, 1995; Sahu et al. 2008). Therefore, alternative methods need to be developed to maintain a healthy microbial environment in the aquaculture systems thereby to maintain the health of the cultured organisms. Earlier studies have used siderophore producing bacteria as antagonistic biocontrol agents (probiotics) to displace and inhibit the proliferation of pathogens by the iron competition process, which was found to be a better remedy than administering antibiotics. Probiotics are usually live microorganisms which

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when administered in adequate amounts confer a healthy effect on the host, and are emerging as significant microbial food supplements in the field of prophylaxis (Geovanny et al. 2007). The bacteria inhabiting the aquaculture environment compete for available nutrients and this is suggested as a mode of action for probiotic bacteria (Balcazar et al. 2006). The most described competition for nutrients is the competition for iron by production of siderophores. Siderophores are low molecular weight ferric ion specific chelating agents. The role of these compounds is to scavenge iron from the environment and to make the mineral, which is almost always essential, available to the microbial cell. Fgaier and Eberl (2010) were able to model competition between an iron chelator microorganism and a non-chelator microorganism. The former is a beneficial bacterium that can inhibit the growth of the pathogenic non-chelator through its iron-chelating capability. This phenomenon of iron chelation was shown to be able to prevent the pathogen from proliferating to numbers capable of causing disease.

The high density of fish in hatchery tanks and ponds is conducive to the spread of pathogens and the aquaculture industry has recognised that the use of siderophore producing bacteria to displace pathogenic bacteria through iron competition is a better remedy than the use of antibiotics (Havenaar et al. 1992; Gatesoupe, 1999). Siderophore producing bacteria is also used extensively as food safety agents in the food industry, where they act as natural biocontrol additives against spoilage. Their ability to produce the iron-chelating siderophores gives them an advantage against other bacteria that compete for the same substrate, iron and this lead to the regulation and preservation of the food. Additionally, in agricultural microbiology they have been used as plant growth promoting bacteria that control root pathogens. We recently reported the isolation and selection of four potential probiotic bacteria from the natural flora of guppy based on their production of antimicrobial substances (such as, bacterocin-like inhibitory substances), antibiotic sensitivity tests, plasmid profiles and *in vitro* growth characteristics like lag period and doubling time (Balakrishna and Keerthi, 2011). The present study was undertaken to characterise siderophores produced by these four isolated strains (MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4) from the ornamental fish guppy**.**

Materials and Methods

Bacterial strains

Four isolated strains of potential probiotic bacteria from *Poecilia reticulata* , namely, MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4 showed moderate to strong antagonistic activities against the six tested indicator strains (*Aeromonas hydrophila* 1739, *Vibrio cholerae* 3906, *Flavobacterium* 2495, *Acinetobacter* 1271, *Alcaligenes* 1424 and *E. coli* K 12). These isolates were selected and identified using 16S rDNA gene sequence analysis (NCBI GenBank accession numbers JN247799, JN247800, JN247801 and JN247802 for MBTU_PB4, MBTU_PB3, MBTU_PB1 and MBTU_PB2, respectively) and were used for the production and characterisation of siderophores.

Siderophore production test

The above four isolated bacteria strains from *P. reticulata*, were grown separately under iron limited condition to secrete siderophore (Payne, 1994). The iron limited condition was made by the addition of an iron-chelating compound 2, 2' dipyridyl to Brain Heart Infusion (BHI) agar (HiMedia). The isolated strains were separately grown on a 0.45 µm standard nitrocellulose filter placed on a BHI agar plate containing 2, 2' dipyridyl at a concentration of 0.3 mM. Plates were incubated at 37 ºC for 48 hr. After the incubation, filters containing the bacterial isolates were removed. The five indicator pathogenic strains separately grown in BHI media containing   2, 2' dipyridyl were added to liquid soft agar medium and then poured over the corresponding plates on which the selected bacterial strains had been grown. The 20 resulting plates were then incubated at 37 ºC for 24 hr. A control was made by using *E.coli* K 12 (indicator strain) instead of pathogenic strains. All experiments were done in duplicates.

Inhibitory activity of isolates cultured with and without Fe

The four bacterial strains isolated from the guppy were grown separately in BHI broth with and without iron (0.3 mM 2,2'-dipyridyl) at 37 ºC for 48 hr (Spanggaard et al. 2001; Payne, 1994). After 2 days of incubation, sterile filtered culture supernatants were prepared as previously described above. The six indicator strains (pre-cultured at 37 ºC in BHI broth) were then individually inoculated in each mixture of 50% BHI broth and 50% sterile filtered supernatant prepared from the isolates MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4 cultured with and without iron. Then the growth (as determined by OD_{450}) of each indicator strain at 37 ºC for 24, 48 and 72 hr incubations was compared in BHI with and without iron.

The chrome azurol sulfonate (CAS) assay for common siderophores

Production of siderophores by the isolates was determined using the CAS universal chemical assay (Schwyn and Neilands, 1987). Water used for these experiments was passed through a Millipore filtration system and had a resistance of at least 15 mega ohm·cm^{-1}. All glass wares were soaked overnight in 6 M HCl and rinsed with distilled water several times to remove traces of iron. CAS agar diffusion assay was performed as follows: 60.5 mg of CAS was dissolved in 50 mL deionised water and mixed with 10 mL iron (III) solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl). Under stirring, this solution was slowly mixed with 72.9 mg hexadecyltrimethylammonium bromide (HDTMA) dissolved in 40 mL water. The resultant dark blue solution was autoclaved and mixed with an autoclaved mixture of 900 mL water, 15 g agar, 30.24 g piperazine-N,N' –bis (2-ethanesulfonic acid) (PIPES) and 12 g of a solution of 50% (w/v) NaOH to raise the pH to the pK_a of PIPES (6.8). The CAS agar plates were punched with 5 mm diameter holes by using a gel puncher. The isolates were grown in iron deficient minimal medium M9 (Maniatis et al. 1982) for 24 hr and cell free supernatant were obtained by centrifugation of the cultures at 10,000 rpm at 4 ºC for 15 min, followed by filtration using 0.45 µm pore size. Seventy microlitres of this cell free

culture supernatant was filled in the CAS agar plate. After incubation at 37 °C for 48 hr, the size of the orange, purple, or purplish-red halo formed around each well was measured.

Detection of chemical nature of siderophores

The culture filtrates obtained as described above, were subjected to chemical assay for detecting the chemical nature of siderophores. The types of siderophores were determined by using specific assays: $FeCl_3$ test, tetrazolium test and ferric perchlorate assay for hydroxamates (Neilands, 1981; Snow, 1954; Atkin et al. 1970), $FeCl_3$ test and Arnow's test for catecholates (Neilands, 1981; Arnow, 1937) and spectrophotometric test for carboxylates (Shenker et al.1992).

### *Quantification of siderophores*

A standard curve was obtained with aqueous dilutions of deferoxamine mesylate (standard hydroxamine was purchased from Sigma-Aldrich, USA) by noting its absorbance at 630 nm after 1hr of incubation with the CAS assay solution, prepared as described by Schwyn and Neilands (1987). The absorbances of the samples (culture supernatants incubated for 1 hr with the CAS assay solution) at 630 nm were measured at room temperature. The quantity of siderophores in the samples was then extrapolated from the standard curve and denoted in $\mu g^. mL^{-1}$.

A reference was prepared with exactly the same medium (M9) used for growing isolates, but uninoculated. The percentages of iron-binding compounds of the siderophore type were calculated by subtracting the sample absorbance (A_s) values from the reference (A_r). Siderophore units are defined as $[(A_r - A_s)/ A_r] 100 = \%$ siderophore units.

Results

Siderophore production

Siderophore production by each selected strain from *P. reticulata* was primarily monitored by the appearance of clear zone in an iron deficient media containing 2,2'dipyridyl which is an iron chelator against standard indicator strain *E. coli* K12. The negative result (absence of growth) with *E. coli* indicates the absence of a corresponding siderophore receptor and its inability to grow in low iron concentration. The results (see Fig. 1) indicated that the four strains produced a diffusible compound which inhibited the growth of the indicator strain and this compound was preferentially produced during incubation in low iron condition. Similar results were obtained using the other five indicator strains.
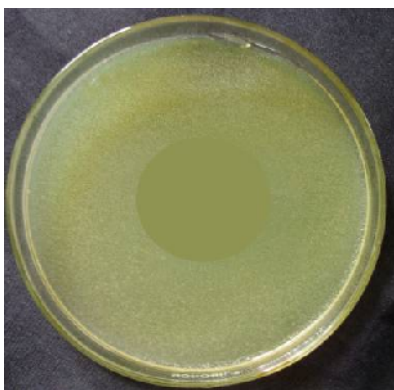
Fig 1. Siderophore production of isolated strain MBTU_PB2 on iron deficient medium using *E.coli* K12 as indicator strain.

Inhibitory activity of isolates cultured with and without Fe

The inhibitory activity of the isolates cultured with and without iron is compared in Fig. 2. All the indicator strains showed growth under both iron-sufficient and iron-limited conditions. Iron-sufficient case caused more-rapid growth of the indicator strains for all the studied strains. In this case, after 2 days of incubation, the OD was nearly 1.1 in all the mixtures. At these conditions, MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4 showed maximum inhibition against *Alcaligenes*, *E. coli* K12, *A. hydrophila* and *V. cholera,* respectively. In contrast, the growth of the indicator strains was reduced when grown in mixtures of 50% BHI broth and 50% sterile filtered supernatant from the isolated strains cultured without iron. In this case, after 2 days of incubation, the OD was nearly 0.9 in all the mixtures. At these conditions, MBTU_PB1 and MBTU_PB4 showed maximum inhibitions against *Acinetobacter* while MBTU_PB2 and MBTU_PB3 showed maximum inhibitions against *Flavobacterium.*

(a)   (b)
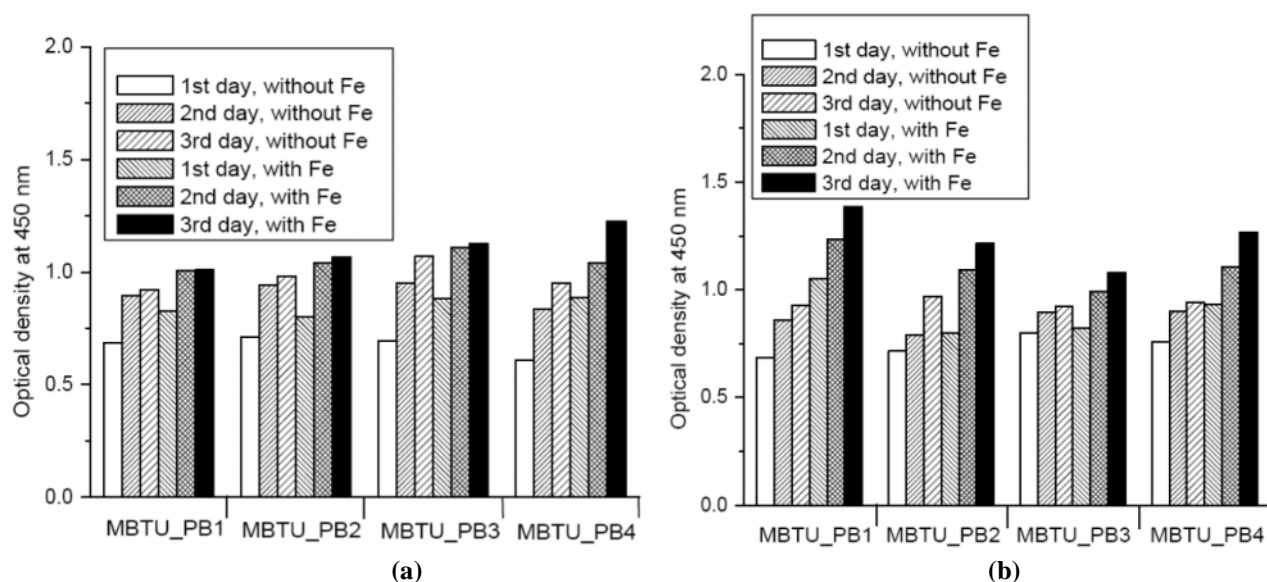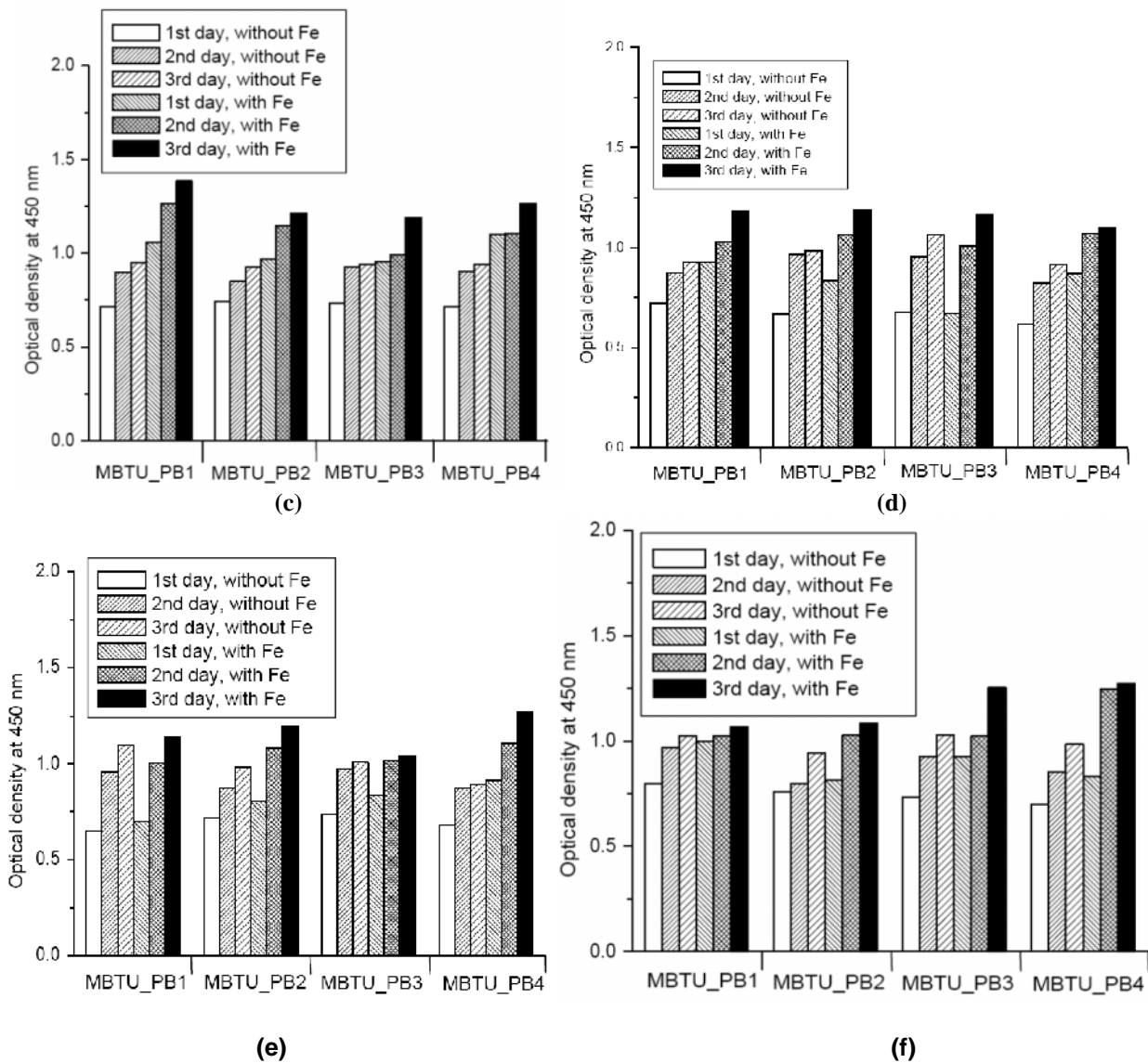
Fig 2. Inhibitory activity of the four isolates cultured with and without Fe against indicator strains (a) *V.cholerae*, (b) *Flavobacterium*, (c) *A.hydrophila,* (d) *Acinetobacter,* (e) *Alcaligenes* and (f) *E. coli* .Error bars are less than 5%.

CAS assay for common siderophores

All the four isolates produced siderophores, as indicated by the formation of purple-like halos around the well in the CAS agar plates (see Fig. 3), further confirming their ability to survive and grow in the iron deficient conditions. As expected, no colour change was observed in the un-inoculated plates after incubation for 48 hr. The colour change in the CAS agar suggested the production of siderophores by the microorganisms (Neilands, 1984; Payne, 1994).
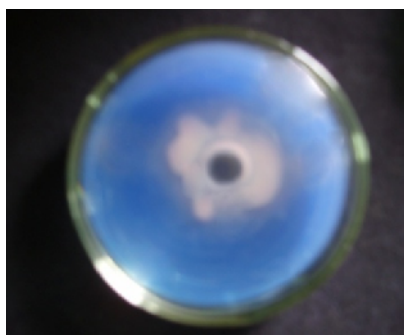
Fig 3. CAS agar diffusion assay for bacterial isolate MBTU_PB2.

Chemical nature of siderophores

Detection of chemical nature of the siderophores indicated that all the four isolated strains produced hydroxamate type siderophores. Both $FeCl_3$ test (for detecting catecholates and hydroxamates) and tetrazolium test (for detecting only hydroxamates) produced positive results. However, both Arnow's test (for catecholates) and Shenker's test (for carboxylates) harboured negative results indicating that the four bacterial strains did not produce catecholate and carboxylate type siderophores. The spectral scan for each isolate using $FeCl_3$ showed a peak between 420 and 450 nm. Tetrazolium test is based on the capacity of hydroxamic acids to reduce tetrazolium salt by hydrolysis of hydroxamate group in the presence of strong alkali. Instant appearance of a deep red colour on addition of tetrazolium salt and NaOH to the test sample indicated the presence of a hydroxamate siderophore.

Spectral analyses are helpful to confirm the hydroxamate nature and determine the precise structure of the hydroxamate siderophores (Payne, 1994). The spectral scans using siderophores and iron-perchlorate assay revealed that the siderophore produced by each isolate showed a peak between 425 and 520 nm (see Fig. 4), depending on the iron-hydroxamate coordination structure (Atkin et al. 1970). This result further confirmed the production of hydroxamate siderophores by all the four isolated strains.
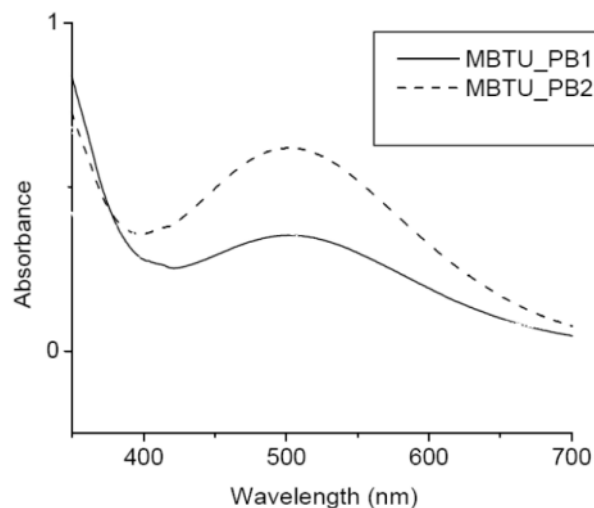
Fig 4. UV-visible absorption spectra of hydroxamate siderophores.

Quantification of siderophores

The concentrations (in µg·mL^{-1}) of hydroxamate siderophores (and the corresponding percentage values) are given in Table 1**.** Of the four isolates under identical conditions, MBTU_PB2 produced maximum amount of siderophore, while MBTU_PB1 produced the least amount. Difference in the quantity of siderophore production (38- 80%) is a logical observation and several earlier reports have indicated variations in siderophore production with time, space and environment (Sayyed et al. 2005).

Table 1. Quantification of hydroxamate siderophore.

Bacteria	Absorbance (630nm)	Siderophore	
		%	mg ml^{-1}
MBTU_PB1	0.240	38	1.26
MBTU_PB2	0.515	80	2.71
MBTU_PB3	0.425	77	2.23
MBTU_PB4	0.419	66	2.20

Discussion

Production of antimicrobial agents and microbial antagonisms is important because these agents can play a significant role in reducing the use of so-called 'hard' agents e.g. antibiotics in various industrial fields such as medical industries, food industry and plant microbiology (Fgaier and Eberl, 2011). In the present study, we characterised the probiotic qualities of normal flora isolated from guppy and studied their antagonistic factors. Two possible explanations can be put forth for siderophore mediated antimicrobial activity: a) antibiotic-like activity and b) chelation of iron, resulting in an unavailability of iron to test organisms. Iron level is critical for siderophore production while iron plays an important role in the antimicrobial activity of siderophore-producing microorganisms (Neilands, 1984). Iron regulates siderophore production in several microorganisms, e.g. *Pseudomonas, Rhizobium* and pathogenic *Staphylococcus* strains (Cox, 1980; Meiwes et al. 1990; Reigh and O'Connell, 1993; Roy et al. 1994). Production of siderophore is also affected by growth rate and the degree to which iron is a growth-limiting nutrient for the siderophore producer (Evans et al. 1994). In addition to ferric iron, siderophores can bind manganese, copper and cobalt, while high concentrations of these elements can interfere with siderophore production and acquisition of iron (Guerinot, 1994). The present work established the presence of a siderophore-based iron-sequestration mechanism in the isolates from guppy. All the four isolates produced siderophores in the presence of an iron-chelating compound 2, 2' dipyridyl. The ability to synthesise siderophores would undoubtedly be an advantage for survival, growth and pathogenicity of the isolates. The nature of the siderophores differed with species. However, characterisation of siderophores based on the bacteria is not possible, because siderophores of varying nature have been produced by the same genus (Murugappan, 2011).

The role of iron on the antimicrobial activity of *Staphylococcus* siderophores was quite variable (Raaska and Mattila-Sandholm, 1995). At rather high levels (2 mM), antimicrobial activity could still be detected, although the degree of growth inhibition was clearly diminished. In these cases the possibility of other antimicrobial agents than siderophores should be kept in mind. The growth inhibitions achieved by siderophore containing culture medium was significant since the growth of moulds and yeasts is considered very difficult to control in food and feed products where they are important and common contaminants (Haikara and Niku-Paavola 1993; Mattila-Sandholm, 1994). In this study, we observed that all the investigated indicator strains displayed growth along each of the four selected strains under both iron-sufficient and iron-limited conditions.

Siderophore production in non-pathogenic *Staphylococcus* strains has been studied to a much lesser degree than other siderophore producers. Siderophores produced by *Staphylococcus* strains used as starter cultures in fermented meat products are generally recognised as safe (GRAS) status which opens new potential application possibilities for use of these strains and their antimicrobial agents in biocontrol of microbes in a wide spectrum of industrial fields (Raaska and Mattila-Sandholm 1995). The siderophore production of *Staphylococcus* strains was detected by the CAS assay (Schwyn and Neilands, 1987; Alexander and Zuberer, 1991). Two of the siderophore

producing strains, MBTU_PB2 and MBTU_PB3 belonged to the *Staphylococcus* sp. Studies of *in vitro* antagonism have often showed that substrate composition strongly affects the production of secondary metabolites and, hence, influences the inhibition patterns exhibited by the antagonistic bacteria (James and Gutterson, 1986; Shanahan et al. 1992; Fang et al. 1996). However, we found that BHI and M9 medium (data not shown) generally resulted in similar inhibition behaviour, while the lower nutrient content in M9 medium could stress the indicator strains and render them more susceptible to inhibition.

Both $FeCl_3$ and tetrazolium tests produced positive results for all the four selected strains indicating the presence of hydroxamate siderophores. *Staphylococcus* strains are known to accept a variety of hydroxamate siderophores (Sebulsky et al. 2000). These results are in line with Dave et al. (2006) in that the exogenous siderophore facilitates growth, promoting activity of other organisms under iron stress conditions. The majority of the species have been known to produce hydroxamate containing siderophores while very few contain catechol as the iron chelating group (Carson et al. 2000). Shift in $_{max}$ as a function of pH value was used to distinguish the ferric complexes of hydroxamate siderophores into mono-, di- or trihydroxamates (Payne, 1994). Rhodotorulic acid, which has two hydroxamate groups, displays an intermediate behaviour (Payne, 1994). Our results of spectral scans using siderophores and iron-perchlorate assay revealed that the siderophore produced by each isolate showed a peak in the range 425-520 nm, depending possibly on the iron-hydroxamate coordination structure. The amount of siderophores produced by the bacterial isolates varied and was found to be positively related to their growth (Murugappan, 2011). In the present study we observed a difference in quantity of siderophores produced by different organisms which is a logical happening. From a prophylactic point of view, non-pathogenic bacteria which produce siderophores are promising candidate probiotics against pathogens with low iron-uptake capability, e.g. *Vibrio parahaemolyticus.* Reports of strain-to-strain variation in the production of siderophores have been mentioned by Reigh and O'Connell (1988) and Roy et al. (1994). The current study could be combined with a modeling study and optimal control strategies for the efficient implementation of such probiotic bacteria (Fgaier and Eberl, 2010; Fgaier et al. 2008).

Conclusion

Four selected siderophore producing bacteria from guppy were identified as possible candidates for use as probionts in fish aquaculture. The colour change in the CAS agar suggested the production of siderophores by the microorganisms. Both $FeCl_3$ and tetrazolium tests produced positive results for all the four selected strains indicating the presence of hydroxamate siderophores. The spectral scans based on the iron-perchlorate assay revealed that each siderophore showed a peak between 425 and 520 nm, depending possibly on the iron-hydroxamate coordination structure. Of the four isolates under identical conditions, MBTU_PB2 produced maximum amount of siderophore (80%), while MBTU_PB1 produced the least amount (38%). Siderophore producing bacteria find applications in many industries, such as in the medical, fish farming and food microbiology industries.

Acknowledgements

References

Alexander, D.B. and D.A. Zuberer. 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biology and Fertility of Soils 12:39–45.

Arnow, L.E. 1937. Colorimetric determination of the components of 3, 4-dihydroxyphenylalanine–tyrosine mixtures. Journal of Biological Chemistry 118:531–537.

Atkin, C.L., J.B. Neilands and H.J. Phaff. 1970. Rhodotorulic acid from species of *Leucosporidium, Rhodosporidium, Rhodotorula, Sporidiobolus,* and *Sporobolomyces,* and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*. Journal of Bacteriology 103:722 –733.

Balakrishna, A. and T.R. Keerthi. 2011. Screening of potential aquatic probiotic from major microflora of guppy (*Poecilia reticulata*). Frontiers of Chemical Science and Engineering (DOI 10.1007/s11705-012-1283-4).

Balcazar, J.L., I. de Blas., I. Ruiz-Zarazela., D.Vendrell, and J.L. Muzquiz. 2006. The role of probiotics in aquaculture. Veterinary Microbiology 114:173–186.

Carson, K.C., J. Meyer and M.J.Dilworth. 2000. Hydroxamate siderophores of root nodule bacteria. Soil Biology and Biochemistry 32:11 –21.

Cox, C.D. 1980. Iron uptake with ferripyochelin and ferric citrate by *Pseudomonas aeruginosa.* Journal of Bacteriology 142:581–587.

Dave, B.P., K. Anushman and P. Hajela. 2006. Siderophores of halophilic archaea and their chemical characterization. Indian Journal of Experimental Biology 44:340–344.

Evans, E., M.R.W. Brown and P. Gilbert. 1994. Iron chelator, exopolysaccharide and protease production in *Staphylococcus epidermidis:* a comparative study of the effects of specific growth rate in biofilm and planktonic culture. Microbiology 140:153-157.

Fang, W., M. Shi, L. Huang, J. Chen and Y.Wang. 1996. Antagonism of lactic acid bacteria towards *Staphylococcus aureus* and *Escherichia coli* on agar plates and in milk. Veterinary Research 27:3-12.

Fgaier, H. and H.J. Eberl 2010. A competition model between *Pseudomonas fluorescens* and pathogens via iron chelation. Journal of Theoretical Biology 263:566-578.

Fgaier, H. and H.J. Eberl. 2011. Antagonistic control of microbial pathogens under iron limitations by siderophore producing bacteria in a chemostat setup. Journal of Theoretical Biology 273:103-114.

Fgaier, H., B. Feher, R.C. McKellar and H.J. Eberl. 2008. Predictive modeling of siderphore production by *Pseudomonas fluorescens* under iron limitation. Journal of Theoretical Biology 251:348-362.

Gatesoupe, F.J. 1999. The use of probiotics in aquaculture. Aquaculture 180:147–165.

Geovanny, D.G.R., J.L. Balcázar and M.A. Shen. 2007. Probiotics as control agents in aquaculture. Journal of Ocean University of China 6 :76–79.

Guerinot, M.L. 1994. Microbial iron transport. Annual Review of Microbiology 48:743-772.

Haikara, A. and M.L. Niku-Paavola. 1993. Fungisidic substances produced by lactic acid bacteria. 4th Symposium on Lactic acid bacteria--genetics, metabolism and applications. FEMS Microbiology Reviews 12:120.

Havenaar, R., B. Ten Brink and J.H.J.Huis in't Veld. 1992. Selection of strains for probiotic use. In: Probiotics the scientific basis. (ed. R. Fuller). pp. 209-229. Chapman and Hall London.

James D.W. and N.I.Gutterson. 1986. Multiple antibiotics produced by *Pseudomonas fluorescens* HV37a and their differential regulation by glucose. Applied and Environmental Microbiology 52:1183-1189

Maniatis, T., E.F. Fritsch and J. Sambrook 1982. Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York. 68 pp.

Mattila-Sandholm, T. 1994. Nature's preservation. In: Minimal Processing of Foods, VTT Symposium 142, Espoo, (eds. R. Ahvenainen, T. Mattila-Sandholm and T. Ohlsson), pp. 103-117. Technical Research Centre of Finland.

Meiwes. J., H.P.Fiedler, H. Haag, H. Zahner, S. Konetschny-Rapp and G. Jung. 1990. Isolation and characterization of staphyloferrin A, a compound with siderophore activity from *Staphylococcus hyicus* DSM 20459. FEMS Microbiology Letters 67:201-206.

Murugappan, R.M., A. Aravinth and M. Karthikeyan. 2011. Chemical and structural characterization of hydroxamate siderophore produced by marine *Vibrio harveyi*. Journal of Industrial Microbiology and Biotechnology 38:265–273.

Neilands, J.B. 1981. Microbial iron transport compounds (siderophores) as chelating agents. In: Development of Iron chelators for clinical use. (eds. A.E. Martell, W.F. Anderson and D.G. Badman), pp. 13-31. Elsevier Amsterdam.

Neilands, J.B. 1984. Methodology of siderophores. Structure and Bonding. 58:1–24.

Payne, S.M. 1994. Detection isolation and characterization of siderophores. Methods *in* Enzymology. 235:329–344.

Raaska, L. and T Mattila-Sandholm. 1995. Effects of iron level on the antagonistic action of siderophores from non-pathogenic *Staphylococcus* spp. Journal of Industrial Microbiology and Biotechnology 15:480-485.

Reigh G, O'Connell M. 1988. Siderophore production is strain specific in *Rhizobium*. In: Bothe, H, DeBruijin, FJ, Newto, WE (Eds), Nitrogen Fixation: Hundred Years After. Gustav Fischer, New York, pp 826-839.

Reigh, G. and O'Connell, M. 1993. Siderophore-mediated iron transport correlates with the presence of specific iron-regulated proteins in the outer membrane of *Rhizobium meliloti* Journal of Bacteriology 175:94-102.

Roy, N., P. Bhattacharyya and P.K. Chakrabartty. 1994. Iron acquisition during growth in an iron-deficient medium by *Rhizobium* sp. isolated from *Cicer arietinum.* Microbiology 140 :2811-2820.

Sahu, M.K., N.S. Swarnakumar, K. Sivakumar, T. Thangaradjou and L. Kannan. 2008. Probiotics in aquaculture: importance and future perspectives. Indian Journal of Microbiology 48:299–308.

Sayyed, R.Z., M.D. Badgujar, H.M. Sonawane, M.M. Mhaske and S.B. Chincholkar. 2005. Production of microbial iron chelators (siderophores) by fluorescent Pseudomonads. Indian Journal of Biotechnology 4:484–490.

Schwyn, B. and J.B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 160:47 –56.

Sebulsky, M.T., D. Hohnstein, M.D. Hunter and D.E. Heinrichs. 2000. Identification and characterization of membrane permease involved in iron-hydroxamate transport in *Staphylococcus aureus*. Journal of Bacteriology 182:4394–4400.

Shanahan, P., D.J. O'Sullivan, P.Simpson, J.D., Glennon and F. O'Gara. 1992. Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. Applied and Environmental Microbiology 58:353-358.

Shenker, M., I.Oliver, M. Helmann, Y. Hadar and Y. Chen. 1992. Utilization by tomatoes of iron mediated by a siderophore produced by *Rhizopus arrhizus.* Applied and Environmental Microbiology 15:2173-2182.

Snow, G.A. 1954. Mycobactin. A growth factor for *Mycobacterium johnei.* Part II. Degradation, and identification of fragments. Journal of the Chemical Society 49:2588-2596

Spanggaard, B., I. Huber, J. Neilson, E.B. Sick, C.B. Pipper, T. Martinussen, W.J.Slierendrecht and, L.Gram. 2001. The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. Environmental Microbiology 3: 755-765.