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ORIGINAL PAPER

Production of a potentially novel antimicrobial compound by a biofilm-forming marine *Streptomyces* sp. in a niche-mimic rotating disk bioreactor

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Abstract After initial small-scale experiments, a 25.0 l rotating disk bioreactor (RDBR) was investigated for the cultivation of a biofilm-forming salt-tolerant *Streptomyces* sp. MS1/7, producing an antimicrobial compound. Peak activity attainment rate, PAAR (ratio of the peak antimicrobial activity, PAMA and the time taken to attain PAMA) was determined. Of the three pH values examined (8.0, 9.0 and 10.0) maximum PAAR (1.82 mm/h) was attained at pH 9.0. Three aeration rates (9.0, 6.0 and 3.0 l/min) were considered at three levels (25, 50 and 75%) of disk submergence. At the highest aeration rate and 50% submergence level, PAMA (41 mm), PAAR (1.86 mm/h) and biofilm density (BD, 0.91 g/ml) attained their highest values. At any given aeration rate, PAMA was always higher at 50% submergence level. This supports our earlier premise that ideal intertidal conditions, 12 h periods of immersion and emersion, promote maximum BD and antimicrobial production in the niche-mimic RDBR.

Keywords Marine · Actinobacterium · Biofilm · Niche-mimic · Rotating disk bioreactor

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Introduction

Research into bioreactor engineering and fermentation protocol design in the field of marine bacterial products has commenced in the recent years as reviewed by Lang et al. [1]. Batch cultivations on a scale ranging from 3 to 1,000-l bioreactors have been reported for the production of the antimicrobial compounds thiomarinol, hydroxyakalone, macrolactins, and chalcomycin B by *Alteromonas* sp., *Agrobacterium aurantiacum*, *Bacillus* sp. and *Streptomyces* sp., respectively. The time course of bioxalomycin production by a *Streptomyces* sp. was studied in 30 and 300-L bioreactors. A comparison of three automated feeding strategies for peptone and yeast extract solutions was performed in 2 l fed-batch cultures for the production of eicosapentaenoic acid by *Alteromonas putrefaciens*. Using a 75-l bioreactor the cultivation of *Agrobacterium* sp. producing agrochelin was carried out. Large scale production of bioactive compounds from marine sponge associated bacteria were the subject of recent investigations such as the cultivation of a *Microbacterium* sp., isolated from *Halichondria panicea* producing glycoglycerolipids in a 10-l bioreactor and a 30-l cultivation of a yellow-pigmented bacterium producing antimicrobial fatty acids isolated from *Crella rosea* [1]. Recently Muffler and Ulber [2] applied different fed-batch strategies to circumvent catabolite repression during the production of a soluble AMP-independent sulfite oxidase by *Sulfitobacter pontiacus*.

Yan et al. [3], [4] were critical of the use of conventional shake flasks and bioreactor systems for the cultivation of marine microorganisms and established that well agitated suspension cultures in closed flasks represent artificial growth conditions that are very different from the natural environment from where the microorganism has been isolated. The successful operation of their novel bioreactors

(the modified roller bottle and air-membrane surface bioreactors) led the research group to propose the new concept of "niche-mimic bioreactors", which essentially is the cultivation of the producing microbe in reactor conditions that mimic its ecological niche [4]. In our previous study [5], we introduced the rotating disk bioreactor (RDBR), operated at a rotational speed of 1.0 revolution per day with 50% submergence of discs and an aeration rate of 1.0 vvm in a sucrose-containing medium as a niche-mimicking bioreactor for the cultivation of three salt-tolerant estuarine actinobacteria: MS310 (99% similar in its 16S rRNA gene sequence to *Streptomyces parvallus*), MS3/20 and MS1/7 isolated from the intertidal regions off the Bay of Bengal, India. Peak antimicrobial activity with the marine isolate MS310 cultivated in the RDBR was attained much earlier than that reported with a terrestrial isolate of *S. parvallus* grown in a STBR. The same operating conditions allowed sustained production of an antimicrobial compound by MS1/7. However, identical results were not obtained with the isolate MS3/20, which did not form a biofilm as extensively as the other two isolates. In the present study we further investigate the application of the RDBR for the cultivation of MS1/7 (MS310 to be reported elsewhere) by first studying the effect of media constituents, medium pH, temperature and aeration on biofilm formation and antimicrobial production in a beaker and a shake flask. Next, we used the data obtained in the small-scale level to study the effect of medium pH, degree of submergence of the disks in the tank and aeration rate in the previously designed RDBR on biofilm formation and the production of antimicrobial compounds by the selected isolate.

MS1/7 was isolated from the intertidal regions of the *Sundarbans* delta off the Bay of Bengal and described in Saha et al. [6]. It produces an antimicrobial compound having antileukemic property as well (MW 300.2, predicted molecular formula $C_{20}H_{28}O_2$). The compound inhibits three Gram-positive and three Gram-negative multiple drug resistant (MDR) bacteria (defined as that disease-causing bacterium which can survive in the presence of structurally unrelated antimicrobial compounds targeted at distinct bacterial physiological mechanisms to eradicate the disease-causing bacterium), seven non-clinical Gram-positive, four Gram-negative bacteria and five fungi.

Materials and methods

Microorganism

The strain was maintained on an enrichment medium (EM) (all units in g/l, K_2HPO_4 0.5, casein 3.0, starch 10.0, peptone 1.0, yeast extract 1.0, malt extract 10.0 and agar 15.0, distilled water 500 ml and natural seawater 500 ml, pH 7.4) slant, stored at 4 °C, and subcultured every month.

Effect of sugars on antimicrobial compound production and biofilm formation

Based on the report of Mc Courtie and Douglas [7] the effect of five sugars (Table 1) were tested for biofilm formation and antimicrobial compound production in a basal medium (PM_3) used in our earlier investigation [5] (all units in g/l): starch 2.0, soybean meal 2.0, yeast extract 0.5, $CaCO_3$ 0.32, $CuSO_4$ 0.005, $MnCl_2$ 0.005, $ZnSO_4$ 0.005, seawater 500 ml, distilled water 500 ml, pH 7.2, where 500 mM of one of the above-mentioned carbon sources were tested. Medium in a 250-ml beaker was aseptically sealed with aluminium foil and autoclavable tape. After autoclaving at 121 °C for 20 min at 10 psig, the beaker was cooled to room temperature (25 °C). A polymethyl methacrylate (the same material used for constructing the bioreactor) block (6.0 cm × 4.0 cm × 2.5 cm), whose one surface was roughened and another surface was kept smooth, was used for the study. It was washed thoroughly with sodium hypochlorite, exposed to ultra violet light for 1 h and then placed into the beaker containing sterile medium keeping its roughened surface in upward direction. The block was carefully fixed with autoclavable tape to the

Table 1 Effect of different sugars on biofilm formation and antimicrobial compound production by MS1/7 in a 250-ml shaken beaker containing PMMA block

Sugar	Surface density of biofilm formed on PMMA block (g/cm^2)	Surface density of biofilm formed on glass wall (g/cm^2)	Total weight of biofilm formed on PMMA block and glass (g)	Peak antimicrobial activity–diameter of zone of inhibition (mm)
Sucrose	0.058 ± 0.001	0.058 ± 0.001	1.39 ± 0.02	30 ± 1
Maltose	0.059 ± 0.002	0.058 ± 0.002	1.40 ± 0.03	28 ± 2
Galactose	0.029 ± 0.003	0.041 ± 0.003	0.84 ± 0.02	25 ± 1
Glucose	0.053 ± 0.003	0.046 ± 0.002	1.19 ± 0.03	22 ± 2
Fructose[a]	0.052 ± 0.002	0.043 ± 0.002	1.14 ± 0.03	7 ± 0

[a] No antimicrobial production. Diameter of the hole is 7 mm. Zone of inhibition data include the hole diameter

inside of the beaker so that it does not move during incubation and again exposed to ultra violet light for another 1 h (See Supplementary File 1). The beaker was then inoculated with 5.0 ml of 4×10^9 CFU/ml spore and substrate mycelium suspension and incubated at 30 °C (180 rot/mim) for 5 days. Antimicrobial compound production was measured at 48 h, 72, 96 and 120 h and biofilm formation on the polymethyl methacrylate surface as well as glass surface were measured at the end of the experiment.

Determination of antimicrobial activity was carried out following the method described in [5] by centrifuging the liquid medium containing the suspended cells and plating 150–200 µl of the supernatant against *Staphylococcus aureus* MTCC 96 as the test microorganism. Maximum antimicrobial activity at a defined time interval is described as peak antimicrobial activity (PAMA) corresponding to the diameter of zone of inhibition. Approximately 12 cm^2 area of the total biofilm surface was scraped off at the end of the experiment and previously weighed on a digital electronic balance measuring grams up to four decimal places (AFCOSET, Model ER-180A, Mumbai, India). Planktonic cell growth, defined as growth measured from samples withdrawn from suspension and not from the biofilm and expressed as colony-forming units (CFU) per ml at 48, 72, 96 and 120 h was determined by plating 100 µl of a suitably diluted suspension culture broth on an Enrichment Medium (EM, see "Microorganism") agar plate followed by incubation at 28–30 °C for 48–72 h and counting the CFUs.

Effect of initial medium pH on antimicrobial compound and biofilm formation and density

MS1/7 was grown in a shaken beaker (250 ml) in the production medium (PM_3) at varying initial pHs (4.0, 7.0 and 9.0). Six beakers were incubated at 30 °C (180 rot/min) for 6 days. Antimicrobial compound production, biofilm formation (as weight) and planktonic cell growth were determined as described before ("Effect of sugars on antimicrobial compound production and biofilm formation"). The pH was measured at the end of the experiment.

As it is well known that the performance of a biofilm reactor is governed by the biofilm density and thickness because the overall reaction rate critically depends on these parameters [8], we thought it would be more appropriate to measure the biofilm density rather than biofilm weight in experiments prior to the RDBR cultivation. However, the amount of biofilm formed on the polymethyl methacrylate block in the shaken beaker was not enough for unequivocal measurement of biofilm density in a 144-h study. As it was observed that the weight of the biofilm (per unit area) on the polymethyl methacrylate surface was almost the same as on the glass surface (see Table 1), measurements of biofilm density were carried out in 500-ml shaken flasks.

As alkaline pH of the medium favoured antimicrobial compound production, experiments on the effect of initial medium pH were next performed by varying initial pH in the alkaline range (8.0, 9.0 and 10.0) in 500-ml shaken flasks containing 100 ml of PM_3. Six flasks were incubated at 30 °C on a rotary shaker (180 rot/min) for 6 days. Effect of pH on activity of the isolate was studied by measuring the antimicrobial compound production determined at 12 h interval and the biofilm density at the time when antimicrobial compound production was maximum. The biofilm density was determined upon termination of the cultivation when the production of antimicrobial compounds was maximum. This termination time was determined by cultivating MS1/7 for 6 days and sampling 0.5 ml of the extracellular culture broth every 12 h for determining the antimicrobial activity. If the highest production of the antimicrobial compound occurred at the same reproducible time for the two consecutive cultivations, then the experiment was stopped at this predetermined time in the third cultivation. The antimicrobial activity was again determined for comparison with the earlier two cultivations. The pH was determined at the end of the experiment.

Biofilm density (BD) was measured by the method described by Sakurai et al. [9]. Of the biofilm formed on the wall surface of shaken flask, a portion measuring 12 cm^2 was scraped out using a small sterile spatula at the time when peak antimicrobial activity was attained. The weight of the scraped biomass was measured by the difference method and then dipped into a 10-ml measuring cylinder with 0.1 ml graduations containing 5 ml water. The displacement of water measured and biofilm density was calculated. The biofilm thickness was calculated by dividing the biofilm volume by the biofilm surface area [9].

Effect of aeration and agitation on antimicrobial compound production and biofilm density

MS1/7 was cultivated in eight shake flasks (500 ml) containing the sucrose based production medium (PM_3) at various shaker speeds (0, 140, 180 and 220 rot/min). The pH of the medium and temperature were kept constant at 9.0 (the pH where maximum activity was obtained) and 30 °C and the shaker run for 6 days. Antimicrobial compound production and biofilm density were measured as described in ("Effect of initial medium pH on antimicrobial compound and biofilm formation and density"). Next, the isolate was cultivated in a shake flask (500 ml) containing different volumes (100, 150, 200 ml, corresponding to 80, 70 and 60% voidage respectively) of PM_3 at 180 rot/min (gave best biofilm formation and antimicrobial

compound production in the earlier experiments). The volume of air above the culture medium in shaken flasks serves to quantify the initial aeration in terms of the "voidage", which is the void volume occupied by air expressed as % of the flask volume. Biofilm density and planktonic cell growth were recorded.

Effect of initial cultivation temperature on antimicrobial compound production and biofilm density

Flasks (500 ml) containing 100 ml of PM_3 medium were incubated at various temperatures (25, 30, 35 °C). The pH of the medium was kept at its optimum level of 9.0 and the shaker was run at 180 rot/min for 6 days. Antimicrobial compound production, biofilm density and planktonic cell growth were measured as explained before. All experiments described in ("Effect of sugars on antimicrobial compound production and biofilm formation", "Effect of initial medium pH on antimicrobial compound and biofilm formation and density", "Effect of aeration and agitation on antimicrobial compound production and biofilm density", "Effect of initial cultivation temperature on antimicrobial compound production and biofilm density") were conducted thrice and averages of the values with less than 10% deviation are reported.

Construction of the niche-mimic rotating disk bioreactor (RDBR) and cultivation conditions

The niche-mimic reactor (Fig. 1 and Supplementary File 2) made of polymethyl methacrylate, volume of 25 l was designed by our group along with engineers from M/s Plastic Abhiyanta, Kolkata, India, as reported earlier [5]. The disk surfaces were roughened to facilitate surface attachment by the biofilm-forming marine actinobacterium. The reactor contains ten disks that are coaxially mounted on a shaft, which is rotated at an ultra-low speed of one revolution per day (rev/day). At this rotational speed, when operated with half the volume of the tank filled with liquid medium (i.e., at 50% submergence level), the disks would remain exposed to air and submerged in the medium alternatively for 12 h each, thus mimicking the niche intertidal conditions of the location from where the marine isolate was collected. The level of submergence of the disks was varied to study the effect of the deviation from the optimal niche environmental conditions on the antimicrobial compound production and biofilm formation.

The reactor was disinfected by repeated washing with sodium hypochlorite and exposure to ultra violet light [5]. Sterile air was supplied into the RDBR using an air compressor and passage through an air filter. The supplied air was uniformly dispersed into the medium using a rectangular sparger having uniformly spaced holes in the downward direction. Ports on the top lid of the reactor were available for sampling, addition of medium/inoculum/antifoam, pH, dissolved oxygen and temperature sensors and air exhaust. 0.5 g of substrate mycelium mass per 100 ml medium ($4.0–5.0 \times 10^9$ CFU/ml) was used as inoculum and the RDBR was operated in the batch mode. The bioprocess medium composition was identical to the production medium (PM_3) used earlier [5] and described in the earlier "Effect of sugars on antimicrobial compound production and biofilm formation".

Effect of initial medium pH on peak activity attainment rate (PAAR) and biofilm density in the RDBR

For a proper evaluation of process productivity in a batch bioreactor system, the level of peak antimicrobial activity

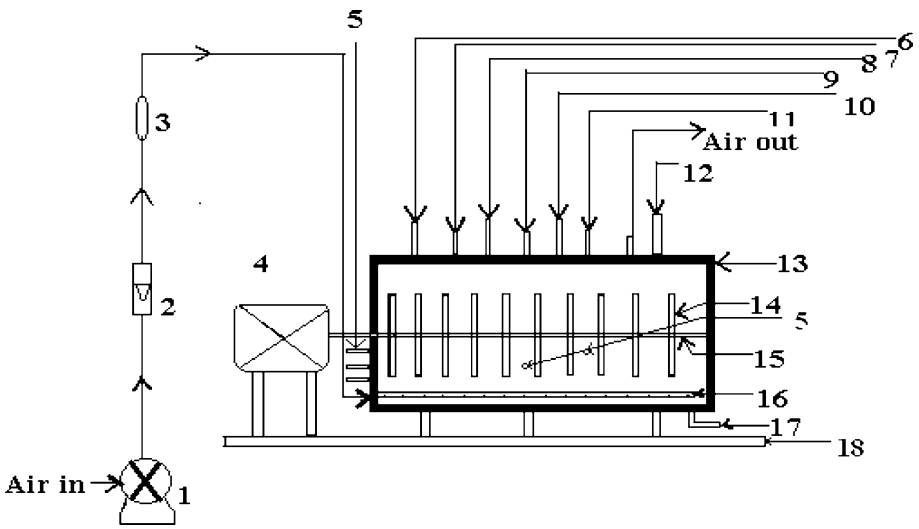

Fig. 1 Schematic of the ultra-low-speed rotating disk bioreactor (RDBR) (*1-Air pump,2-rotameter,3-air filter,4-electrical motor and reducing gear train,5-sampling port,6-temperature sensor,7-antifoam port,8-inoculation and medium addition port,9-acid port,10-pH sensor,11-alkali port,12-DO sensor,13-reactor vessel,14-rotating coaxial disks,15-shaft,16-sparger, 17-drain,18-base plate*)

(PAMA) obtained should be considered together with the time taken to attain that peak level. This is because the latter is a measure of the batch process time, which ultimately determines the number of batches processed in a given time interval. We, therefore define the peak activity attainment rate (PAAR) as:

$$\text{PAAR} = \frac{\text{peak antimicrobial activity (mm)}}{\text{time taken to attain peak (h)}}$$

Initial pH of the production medium was varied (8.0, 9.0, 10.0) and the process was operated at room temperature (27–30 °C). The rotational speed was maintained constant at 1.0 rev/day and the aeration rate at 9.0 l/min during the course of the investigations. The present design of the RDBR does not facilitate dynamic monitoring of biofilm density during cell cultivation due to obvious constraints on aseptic removal of biofilm. Therefore, the biofilm density was determined upon termination of the process when the peak activity attainment rate is achieved. This termination time was determined by running the reactor twice for 4 days and sampling 1.0 ml of the extracellular culture broth every hour for determining the antimicrobial activity. If the highest production of the antimicrobial compound occurred at the same reproducible time for the two consecutive runs, then the reactor was stopped at this predetermined time in the third run. The antimicrobial activity was again determined for comparison with the earlier two runs. Planktonic cell growth with respect to time was determined as described before ("Effect of sugars on antimicrobial compound production and biofilm formation").

Effect of level of submergence of the disks and aeration rate on peak activity attainment rate (PAAR) and biofilm formation in the RDBR

To examine the simultaneous effect of aeration rate and level of submergence, a 3^n experimental programme was designed, with each of the two ($n = 2$) parameters (i.e. aeration rate and % submergence) considered at three levels viz. high (9.0 l/min), medium (6.0 l/min) and low (3.0 l/min). The level of disk submergence was varied above and below the 50% level, i.e. 75 and 25%. The rotational speed was maintained constant at 1.0 rev/day, the pH at 9.0, while the medium temperature varied between 26 and 28 °C during the course of the investigations. The biofilm density and planktonic cell growth were determined as described before. Each process in the RDBR was performed at least twice and averages of the values (PAMA, PAAR, biofilm density and cell growth) with less than 10% deviation are reported.

Results

Effect of sugars on antimicrobial compound production and biofilm formation in shaken beakers

From Table 1 it is evident that for the sucrose and maltose based media, both biofilm formation as well as antimicrobial production were much higher than the other sugars. In fact, the surface densities and total weights of biofilm formed as well as the peak antimicrobial activity obtained with sucrose and maltose are almost identical with antimicrobial production being just about 10% higher for sucrose, which, being a cheaper and a readily available carbon source, was thus selected.

Effect of initial medium pH on antimicrobial compound production and biofilm formation in shaken beakers

Whereas no antimicrobial activity was detectable when initial medium pH was acidic (pH 4), for neutral (27 ± 1 mm) and alkaline pH (33 ± 1 mm), significant antimicrobial activity was observed. Biofilm formation (after the end of the cultivation) on the polymethyl methacrylate and glass surfaces too was maximum (0.064 ± 0.001 g/cm^2) at pH 9.0 and lower at pH 7.0 (0.058 ± 0.002 g/cm^2). The pH at the end of the experiment was 9.78 (initial pH 9.0) and 8.01 (initial pH 7.0). In all the small-scale cultivations using the polymethyl methacrylate block, it was observed that biofilm formation was more on the rough surface than the smooth surface (Supplementary File 3). Planktonic cell growth recorded with respect to time was almost similar when MS1/7 was cultivated at pH 7.0 and 9.0, while scanty growth was observed at pH 4.0. This suggests that the effect of pH on antimicrobial production should be more closely examined in the alkaline range to identify the optimum pH.

Effect of initial alkaline pH on antimicrobial compound production and biofilm density in shaken flasks

From these experiments, it was confirmed that pH 9 is indeed the optimum with regard to antimicrobial production, with a higher peak at 48 h (the time when antimicrobial activity is maximum) antimicrobial activity (i.e. 32 ± 1 mm) than that (29.5 ± 1 mm) for both pH 8 and pH 10. Again, the corresponding biofilm densities for pH 8 and pH 10 are comparable, i.e. 0.56 ± 0.02 g/ml for pH 8 and 0.51 ± 0.01 g/ml respectively, however, that for pH 9 is higher, at 0.76 ± 0.01 g/ml. The pH at the end of the cultivation was 8.45 (initial pH 8.0), 9.56 (initial pH 9.0) and 9.88 (initial pH 10.0). The trends in BD and

PAMA values are thus similar—closely comparable values at pH 8 and pH 10 and a much higher value at pH 9—such trends are indicative of an association between biofilm formation and antimicrobial synthesis.

Effect of aeration and agitation on antimicrobial compound production and biofilm density in shaken flasks

The time profiles of antimicrobial activity at different levels of aeration (Fig. 2) demonstrate that the activity values are consistently higher for higher aeration. In fact, the peak activity (attained incidentally at 48 h for all the aeration levels examined), increases by 24% on increasing the aeration level from 60 to 70% voidage and on further increasing the aeration level from 70 to 80% voidage, the peak activity increases further by 48%. Biofilm density at the time of peak antimicrobial compound production is also highest at the highest aeration level (0.82 ± 0.01 g/ml) and decreases with decreasing aeration level (0.76 ± 0.01 g/ml) at 70% voidage and (0.40 ± 0.02 g/ml) at 60% voidage. The association between biofilm formation and antimicrobial production is again evident. Adequate aeration is thus critical for biofilm formation and antimicrobial production.

Effect of initial cultivation temperature on antimicrobial compound production and biofilm density in shaken flasks

The peak antimicrobial activity, 33 ± 1 mm (after 48 h, for all temperatures studied) at 30 °C was clearly the highest, being about 10% higher vis-à-vis the peak at 25 °C, but 33% higher than that corresponding to 35 °C. The biofilm density corresponding to the peak antimicrobial activity was also found to be highest at 30 °C (0.83 ± 0.01 g/ml), being about 1.41 times higher than that at 25 °C, but as much as four times higher than that at 35 °C. The optimum temperature thus determined is close to the mean seawater temperature (February–November) of the sampling site from where the isolate was sourced. Once again, it is noted that biofilm density and peak antimicrobial activity values rise and fall in tandem, thus indicating an association between biofilm formation and antimicrobial production.

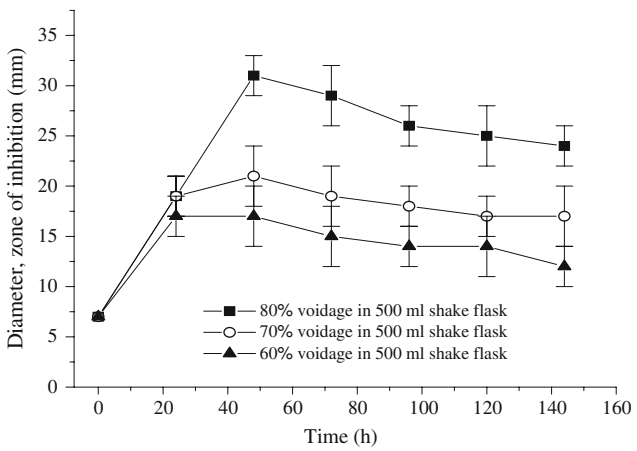

Fig. 2 Effect of aeration on antimicrobial compound production by MS1/7 in a 500-ml shaken flask at pH 9.0 and 30 °C in the sucrose based medium (PM_3). Values of diameter of zone of inhibition, include cup diameter of 7 mm. (Voidage, measure of aeration, is the void volume occupied by air expressed as % of the flask volume)

Figure 3 shows the correlation between biofilm density and thickness for all the culture conditions tested ("Effect of initial medium pH on antimicrobial compound production and biofilm formation in shaken beakers", "Effect of initial alkaline pH on antimicrobial compound production and biofilm density in shaken flasks", "Effect of aeration and agitation on antimicrobial compound production and biofilm density in shaken flasks", "Effect of initial cultivation temperature on antimicrobial compound production and biofilm density in shaken flasks"). It is seen that a high biofilm density is related to a low film thickness and at the best conditions (pH 9.0, temperature 30 °C and voidage 80%), when film thickness was the lowest, the density was the highest. The reported values of biofilm thickness varied within 1.0% of the replicate values.

Effect of initial medium pH on the peak activity attainment rate and biofilm density in the RDBR

It is seen that at pH 9.0 the maximum value of peak activity attainment rate (1.82 mm/h) was attained compared to the values 0.83 and 0.70 mm/h attained at pH 8.0 and 10.0, respectively. Therefore, all successive RDBR experiments were performed with the medium pH (pre-sterilization) at 9.0. The final pHs were 6.40 for pH 8, 6.83 for pH 9 and 7.42 for pH 10. Biofilm density after 22 h (the time to attain peak antimicrobial activity) is maximum (0.91 ± 0.01 g/ml) at pH 9.0, lower at pH 8.0 after 25 h (0.73 ± 0.02 g/ml) and pH 10.0 after 29 h (0.66 ± 0.01 g/ml). Planktonic cell growth at the time when peak antimicrobial activity was reached was about 30% higher at pH 9.0 compared to 8.0 and 10.0.

Effect of aeration rate and disk submergence on antimicrobial compound production and biofilm formation in the RDBR

Both of the process operating parameters, i.e. disk submergence level and aeration rate had a very significant effect on the peak antimicrobial compound production and biofilm formation. The aeration rate was increased from 3.0 to 9.0 l/min at three submergence levels, meaning

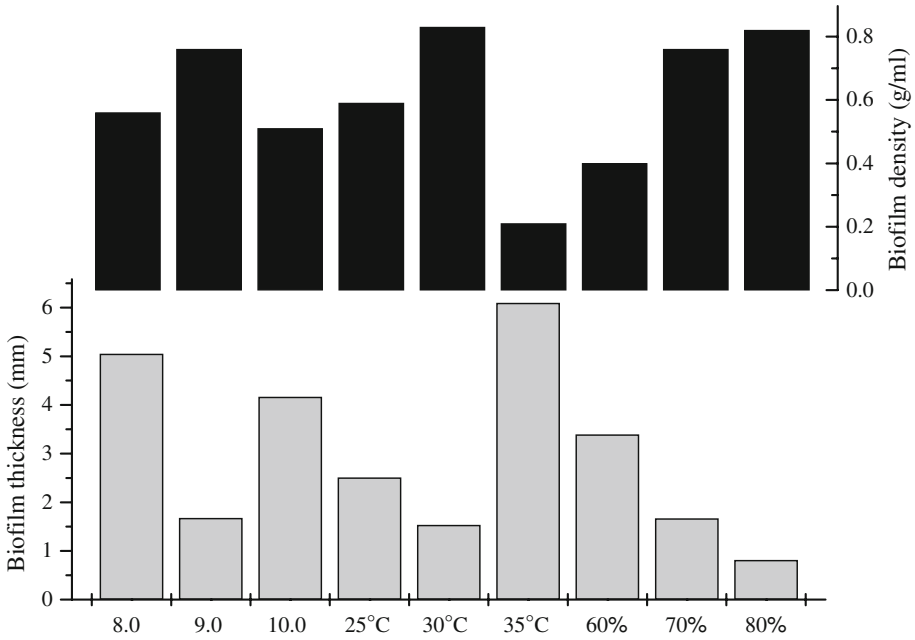

Fig. 3 Relationship between biofilm thickness and density during cultivation of MS1/7 in shake flasks under various cultural conditions.*From left to right*, the *first three columns* represent pHs, the middle three temperatures and the final three voidages. Biofilm thickness varied within 1.0% of the replicate values

a threefold increase in air supply at each submergence level. PAAR (at 9.0 l/min relative to 3.0 l/min) doubled at 25% submergence level, but increased only by about one-third at 50 and 75% submergence level of the disks (Table 2). The small increase in PAAR at 75 and 50% submergence levels despite the larger volume of medium could not be attributed to the biofilm densities obtained at the time corresponding to maximum PAMA. It appears that a high DO level (near 100% saturation for the initial 14 h) at 25% submergence in contrast to the rapid utilization of DO at 50 and 75% submergence may override the effect of biofilm density (Fig. 4). Although this experiment highlights the pivotal importance of DO as a factor influencing antimicrobial compound production by the estuarine isolate, the low PAAR values obtained at 25% submergence does not qualify this level of submergence to be the ideal. Figure 4 shows the time course of planktonic cell growth and variation of the dissolved oxygen levels for the three levels of disk submergence examined.

The time courses of antimicrobial compound production at the highest aeration rate (9.0 l/min) were followed at three submergence levels of the disks (25, 50 and 75%). The corresponding biofilm densities were also recorded and are summarized in Table 2. It is seen that at the highest aeration rate (9.0 l/min) and at 50% disk submergence level, the PAMA as well as PAAR and biofilm density have assumed their highest values (shown in boldface). Planktonic cell growth was higher when MS1/7 was cultivated at 50% submergence level compared to the other two submergence levels.

Clearly, PAMA values corresponding to a given level of disk submergence increase with increased aeration, for all the disk submergence levels examined. Further, for any given aeration rate, the PAMA value is always seen to be highest at 50% submergence, i.e. at the niche-mimic condition. Of the PAMA values at 50% disk submergence, increase in aeration from 3 to 6 l/min causes an increase of 1.12 times whereas that for 6–9 l/min results in a further increase of 1.26 times, giving an overall increase in PAMA of 40% in increasing aeration from 3 to 9 l/min. Thus, exactly as in case of the small-scale shaken cultures (Fig. 2), antimicrobial production increases drastically with increase in aeration rate in the RDBR.

The trends in BD values (corresponding to PAMA) are similar to those in PAMA. At all the aeration rates, whereas BD was comparable for 25 and 75% disk submergence levels, the values at 50% level were significantly higher. Expectedly, the highest BD value occurs at the 50% level with highest aeration, i.e. 9 l/min. The overall trends in PAAR, which is a measure of RDBR productivity, are similar too. Evidently, there is a tremendous increase in productivity when the RDBR is run at 50% disk submergence with 9 l/min aeration. From the data presented in Table 2, it may be concluded that antimicrobial production by MS 1/7 appears to be growth-associated. Thus, it is clearly established that the niche-mimic RDBR operated at 1.0 rev/day and 50% submergence of disks with high aeration rate results in maximum production of antimicrobial compound by the estuarine isolate MS1/7.

Figure 5 shows the relationship between PAAR, biofilm thickness and density for the processes operated at different pHs and three aeration levels with 50% disk submergence. It is seen that a high biofilm density is associated with a low film thickness and at the best conditions (pH 9.0, aeration 9.0 l/min), film thickness was the lowest and

Table 2 Effect of disk submergence level and aeration rate on peak antimicrobial activity (PAMA, reported as diameter of zone of inhibition, mm), time at which peak activity was attained (h), peak activity attainment rate (PAAR, mm/h) and volumetric biofilm density (BD, g/ml) during cultivation of MS1/7 in the RDBR (BD values correspond to the time at which PAMA was attained)

Aeration rate (l/min)	3.0				6.0				9.0			
Disk Submergence level (%)	PAMA (mm)	Time (h)	PAAR (mm/h)	BD (g/ml)	PAMA (mm)	Time (h)	PAAR (mm/h)	BD (g/ml)	PAMA (mm)	Time (h)	PAAR (mm/h)	BD (g/ml)
25	14 ± 1	31	0.45 ± 0.02	0.45 ± 0.02	20 ± 1	27	0.74 ± 0.02	0.48 ± 0.03	22 ± 1	25	0.88 ± 0.01	0.51 ± 0.03
50	26 ± 2	19	1.37 ± 0.03	0.65 ± 0.01	29 ± 2	23	1.26 ± 0.02	0.72 ± 0.02	41 ± 2	22	1.86 ± 0.02	0.91 ± 0.03
75	18 ± 1	23	0.79 ± 0.03	0.40 ± 0.02	24 ± 1	23	1.04 ± 0.01	0.50 ± 0.01	26 ± 1	25	1.04 ± 0.01	0.55 ± 0.02

Boldface indicates the highest values

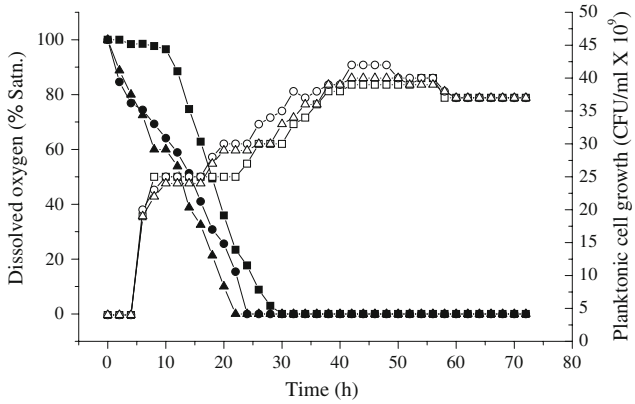

Fig. 4 Variation of dissolved oxygen levels and planktonic cell growth of MS1/7 RDBR (Conditions: 1 rev/day, pH 9.0, temperature 30 °C, aeration rate 9.0 l/min). DO levels indicated by:*filled squares*-25% submergence,*circles*-50% submergence,*triangles*-75% submergence. Planktonic cell growth indicated by:*open squares*-25% submergence,*circles*-50% submergence,*triangles*-75% submergence. The replicate data varied within 10% of the values shown

density the highest. The reported values of biofilm thickness varied within 1.0% of the replicate values.

Discussion

Intertidal systems are a key interface of the ocean, atmosphere, and terrestrial environments, and as such, are characterized by frequent fluctuations in temperature, ion concentration, dessication, UV-irradiation, and wave action. The relative frequency of these fluctuations poses both physical and biochemical challenges to microorganisms which inhabit this environment. The characteristics and intensities of such stresses may vary substantially. When bacteria are associated with surfaces (e.g., sediment particles of intertidal sandflats, plant surfaces) they secrete a matrix of mucilaginous extracellular polymers (EPS) to form a microbial biofilm [10]. Investigations on the biofilm development process clearly showed that within seconds following the initial exposure of a surface to seawater, dissolved organic matter adheres to the surface and forms a thin (<100 nm) conditioning film. Consequently, the surface receives new physiochemical properties, which subsequently affect the type and kinetics of primary colonizers to be recruited to the surface. Among the primary colonizers bacteria are usually dominant components owing to their high abundance in seawater. Bacterial cells establish the first surface contact and interact with the conditioning films [11].

Biofilms and their associated mucilaginous secretions form a cohesive matrix surrounding the particles of intertidal sediments. The microbial biofilm is known to represent a common adaptation, perhaps even a life stage,

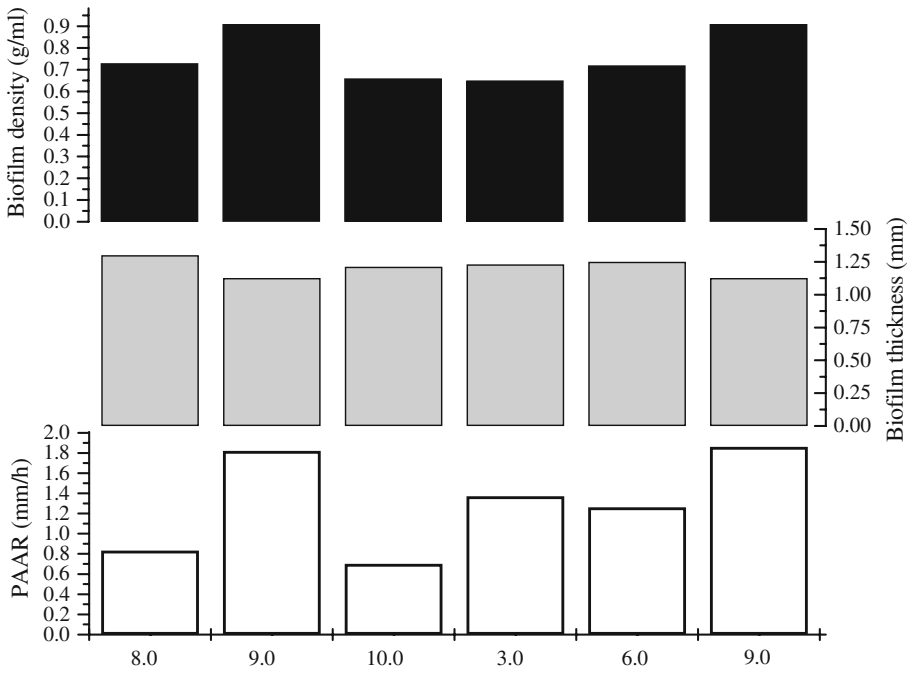

Fig. 5 Relationship between PAAR, biofilm thickness and density during cultivation of MS1/7 in the RDBR under various process conditions.*From left to right*, the*first three columns* represent pHs and the final three aeration rates in l/min. Biofilm thickness varied within 1.0% of the replicate values

of most bacteria in intertidal systems. The biofilm is a community of microbial cells whose activities may be structured or enhanced by the EPS matrix surrounding the cells. In general, the matrix forms a stabilizing and protective microenvironment, and may serve a variety of specific functions to cells. The physical state of exopolymers may vary considerably, and range from a tight, cohesive gel to a very loose slime to dissolved or colloidal states. EPS capsules, which surround cells and are in direct contact with cell membrane, will exert the most proximate protective effects [10].

Thus, the fundamental reason behind biofilm formation by MS1/7 is the inherent property of this intertidal bacterium. This natural phenomenon is the basis of the design of our ultra-low speed niche-mimicing RDBR. Another example where the intertidal environment has been exploited for technological advantage is the isolation of a *Microbacterium* sp. and a *Bacillus* sp. [12]. The EPS produced by these bacteria have potential applications in bioremediation as well as in biomedicine.

Among the five carbon sources studied for biofilm formation and antimicrobial compound production, sucrose produced the best effect. Yang et al. [13] also reported that sucrose among the other sugars (fructose, galactose, glucose and lactose) enhanced biofilm formation by a polymicrobial oral bacterial consortium. Sucrose was also the carbon source of choice of Thompson et al. [14] for their study on biofilm formation of *Enterobacter cloacae* Ecl and *Citrobacter freundii* Cf1 in both single-species and binary species biofilms. The authors also reported an optimal C:N ratio of 48:1 for biofilm formation by the above-mentioned bacteria, while we used a C:N ratio of 69:1, which is about 0.7 times more than the authors. Similarly, Hood and Zottola [15] observed that significantly higher numbers of *Pseudomonas fragi* cells attached to stainless steel surfaces when grown in a medium containing 1% reconstituted skim milk and sucrose, compared to tryptone soy broth. The pivotal effect of medium pH on antimicrobial production as well as biofilm formation on our isolate is supported by the investigations of Pongtharangkul and Demirci [16]. The authors concluded that fed-batch fermentation can be successfully used to enhance nisin production in biofilm reactors, with a constant pH as the most suitable pH profile.

We observed that the rough surface of the polymethyl methacrylate block allowed more biofilm formation than the smooth surface. This observation is similar to that of Morgan and Wilson [17] who observed that the type of polymethyl methacrylate used and its roughness, affect the early stages of biofilm formation by *Streptococcus oralis*. Maximum antimicrobial compound production and biofilm density were attained at 30 °C and decreased antimicrobial compound production as well as biofilm formation was observed at incubation temperatures above or below 30 °C. Incubation temperature plays a crucial role in biofilm formation by *Listeria monocytogenes* as observed by Di et al. [18], probably modifying cell surface hydrophobicity.

Aeration has a vital effect on the physiology and biofilm formation by the fish probiotic bacterium *Roseobacter* Strain 27-4. In the study by Bruhn et al. [19] *Roseobacter* strain 27-4 grown in marine broth (Difco 279110) under

static conditions grew as rosettes consisting of 9–10 cells, produced a brown pigment, and formed a biofilm at the air-liquid interface. Our observations are however, contrary to this report, where the highest aeration rate (Table 2) promoted biofilm formation and elaboration of the antimicrobial compounds.

In this RDBR study we sought to select the appropriate microenvironmetal conditions for maximizing the PAAR as the criterion of bioreactor productivity and at the said conditions, carry out a designed experimental program to examine the effects of aeration and disk submergence on RDBR productivity. Maximum value of peak activity attainment rate (1.86 mm/h) of antimicrobial compound production was attained with aeration rate of 9.0 l/min and pH 9.0 when RDBR was operated at an ultra-low rotational speed of 1.0 rev/day and 50% disk submergence. This investigation supports our assumption made in our earlier investigation that the ideal intertidal conditions of 12-h periods of inundation in water and exposure to air in a day promote maximum biofilm formation and antimicrobial compound production. Although the present study has resulted in only 27% increase in the PAAR compared to our earlier report [5], this investigation has allowed the identification of the appropriate bioprocess conditions, with confidence, as well as the positive relationship between antimicrobial compound production and biofilm formation. It was observed that for all the cultivation conditions examined in the shake flask level, namely, pH, aeration, agitation and temperature, maximum planktonic cell growth was associated with highest biofilm density. Similarly, for all the process conditions investigated in the RDBR namely, pH, level of submergence and aeration rate, maximum planktonic cell growth was again associated with highest biofilm density. Referring to Table 2 and Fig. 4, we find that biofilm density is highest when aeration rate is maximum (9.0 l/min). This situation is characterized by high planktonic growth along with rapid utilization of dissolved oxygen and is satisfied in the niche mimic condition (50% submergence). Conversely, in the 25% submergence state, planktonic growth is low as indicated by slow utilization of dissolved oxygen (DO level remains between 90 and 100% saturation for the first 14 h). It appears that a high planktonic growth (and therefore a high cell concentration) allows more cells to be drawn into biofilm formation after which a low oxygen concentration slows down biofilm growth giving the film more time to consolidate and hence attain a higher density [20].

It was generally observed that a higher biomass concentration leads to a higher product concentration. We attempted to calculate the specific production rate (quantity product/time/biomass) and note if this value varies or remains constant during the various culture conditions. Although the values have not been presented, it was observed that the rate was highest in the niche-mimic condition in the RDBR and differed in the various process conditions. This implies that higher the cell concentration in the planktonic state, higher is the rate of recruitment of the cells into biofilm formation and thus high antimicrobial production. This observation reiterates that a consolidated biofilm is linked to high antimicrobial compound production. This relationship, however, does not hold for every cultural condition in the small-scale level. In this case, the small volume dictated the withdrawal of small number of samples at long time intervals (12 h). As can be seen from Table 2 where hourly samples were taken, a variation of 3–4 h affected PAAR and hence specific production rate. Thus the highest values of activity and biofilm density in the small-scale studies which were observed, for example, at 48 h may actually be at 50 h or 46 h. Due to this inaccuracy, we did not estimate PAAR for the small-scale experiments.

Tanyonac and Beyenal [8] noted the relationship between biofilm thickness and density. While in literature it has been generally assumed that biofilm density is constant and independent of thickness, some investigators held that film density depended on film thickness and some others observed reduced film density as thickness increased. We observed (Figs. 3, 5) that maximum biofilm density was associated with minimum biofilm thickness. Nikolov et al. [21] while studying the physico-chemical properties of the *Thiobacillus ferroxidans* biofilm formed on the surface of disks in a rotating biological contactor observed that when the biofilm thickness was maximal, its density was minimal but failed to offer any explanation for their observation.

Four major drawbacks in our present design are as follows: the reactor does not have pH, temperature and dissolved oxygen level control as well as the rotational speed is not variable. Although we speculate that the disk rotational speed will have an effect on the biofilm formation and antimicrobial compound production, such experiments could not be performed in this low-cost equipment where the speed was fixed at 1.0 rev/day. The first drive motor operates at the maximum speed, which is subsequently reduced to 1.0 rev/day by the speed reduction gear train. Accepting this shortcoming, it should be appreciated that the present work embodies the first report on the further development of the novel concept of niche-mimic reactors offered by the research group of Prof. Grant Burgess [3, 4]. We would also like to emphasize that the current design simulates the niche intertidal conditions. Control of pH and temperature would have given us a better insight into the process; however, the limitation in our design did not allow experiments with regulated pH and temperature. Control of pH and temperature, though, may not be necessary if the productivity remains unaltered

despite changes of pH [22] and reasonable temperature variation of 2–3 °C [23] during the process. Nevertheless, experiments with controlled pH and temperature in the improved design of reactor are planned for the future.

Conclusion

The niche-mimic operating condition of the RDBR (i.e. 50% disk submergence at 1 rev/day) along with maximum possible aeration is established as that most conducive to biofilm growth and antimicrobial production by the marine isolate MS1/7. The results of this study—for small-scale cultures as well as for RDBR, emphatically highlight the critical importance of adequate aeration in antimicrobial production and biofilm formation by the marine isolate MS1/7. A clearly discernible association between planktonic growth, biofilm density, thickness and production of antimicrobial compounds is noted in small-scale cultures as well as in the RDBR. This study has established that a careful selection of the bioprocess conditions in a niche-mimic reactor can deliver maximum amount of potentially novel metabolites of marine estuarine bacteria required for chemical structure elucidation and biomedical studies.

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