

Kinetic Studies on Planktonic Cell Growth, Biofilm Formation and Antimicrobials Production by a Salt-Tolerant Estuarine Actinobacterial Strain *Streptomyces Sundarbansensis* Sp Nov.

Sreyashi Sarkar¹

¹Department of Chemical Engineering

¹Jadavpur University, Kolkata 700 032, India, Tel. 0091 33 2418 2866

Abstract---In this study, effect of initial cultivation temperature on antimicrobials production, planktonic cell growth and biofilm formation by a salt-tolerant estuarine actinobacterial strain *Streptomyces sundarbansensis* sp nov. was investigated in sucrose based medium at shaken flask level for three different temperatures (25 °C, 30 °C and 35 °C). Peak antimicrobial activity (35 mm) was found at 30°C compared to the peak values of 29.5 mm, 24 mm attained at 25 °C and 35 °C respectively. From the growth curves obtained for both planktonic and biofilm growth, $\mu_{max,B}$ (0.043 h⁻¹) and $\mu_{max,P}$ (0.063 h⁻¹) were also maximum at 30°C. Product (antimicrobials) concentration increases linearly with total cell growth that comprised of both the planktonic cell growth and the biofilm growth. Luedeking – Piret model satisfactorily described the dependence of antimicrobials productions on total cell growth. Antimicrobials production was found to be strongly growth associated.

Keywords: *Streptomyces*, antimicrobial, planktonic, biofilm, kinetic, Luedeking-Piret

I. INTRODUCTION

Surface attachment and biofilm formation are known to influence metabolite production by microorganisms. Marine surface associated microorganisms may require conditions that resemble their native environment in order to produce the maximum amount of bioactive metabolites. For example, several studies have shown an increased production of antimicrobial compounds when the surface associated bacteria were grown, in vitro, to form surface attached biofilms [1].

The Class Actinobacteria and, more specifically, bacteria belonging to the order Actinomycetales (commonly called Actinomycetes) account for approximately 7,000 of the compounds reported in the Dictionary of Natural Products [2]. The genus *Streptomyces* alone accounts for a remarkable 80% of the actinomycete natural products reported to date. As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have characteristics different from those of terrestrial actinomycetes and therefore may produce different types of bioactive compounds.

The Bay of Bengal has recently been targeted as a potential source of marine-derived bacterial bioactive compounds by Indian investigators. The Sundarbans, the world's largest tidal mangrove forest, lies on the delta of the Ganges, Brahmaputra, and Meghna rivers off the Bay of Bengal. The biotechnological applications of the microbial biodiversity of the Sundarbans remained unexplored until Mukherjee and co-workers [3, 4, 5, 6, 7] reported the isolation of three antimicrobial compounds, an unidentified

protease [8], an esterase [9], a ribonuclease [10] and 2-allyloxyphenol [11, 12].

There is, however, a lack of research into bioreactor engineering and fermentation protocol design in the field of marine bacterial antibiotic production. Most production strategies are carried out at the shake-flask level and lack a mechanistic understanding of the antibiotic production process, offering poor prospects for successful scale-up [13].

What is more, knowledge of the kinetics of antimicrobial production is important for the evaluation of the biofilm processes coupled with the antimicrobial production and the design of special type of biofilm reactors for the microorganisms producing their desired products at highest rates when surface attached. The study of kinetics provides insight into the dynamics of cell culture systems, as well as the interactions of organisms with their surrounding environments [14]. Better understanding of these dynamics, as well as the determination of important kinetic parameter values, will facilitate both the optimization and scaling-up of bioprocesses. Mathematical modeling can be helpful for understanding the behaviour of biological processes and predicting the overall performance of the reactor system. However, to best of our knowledge, the growth kinetics of these types of marine isolates have not yet been investigated.

In this paper, the effect of temperature on antimicrobials production, planktonic growth and biofilm formation by a salt-tolerant estuarine actinobacterial strain, *Streptomyces sundarbansensis* sp nov., designated as MS1/7^T (=MTCC 5272^T), a novel actinomycete that produces a cytotoxic antimicrobial of molecular weight 300.2 and predicted molecular formula C₂₀H₂₈O₂ [4] and also 2-allyloxyphenol [11, 12] grown in sucrose based medium in a 500 mL glass Erlenmeyer flask at different temperatures (25, 30, 35 °C) is reported. The objectives of this study were to characterize the suspended growth, biofilm formation and antimicrobials production patterns of *Streptomyces sundarbansensis* sp nov. cultivated in shake flasks to get maximum antimicrobials production.

II. MATERIALS AND METHODS

A. Microorganism

The strain MS1/7^T is described as *Streptomyces sundarbansensis* sp nov. (=MTCC 5272^T) [12] was isolated from the intertidal estuarine sediments of the Sundarbans delta off the Bay of Bengal. It produces an antimicrobial compound having antileukemic property as well (MW 300.2, predicted molecular formula C₂₀H₂₈O₂) [4]. 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. 2-allyloxyphenol was also obtained as a natural product (in any living system) for the first time from this strain [11, 12].

The strain was maintained on an enrichment medium (EM) (all units in g/L, K₂HPO₄ 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. Sucrose based medium (SBM) (all units in g/L, starch 2.0, soybean meal 2.0, yeast extract 0.5, CaCO₃ 0.32, CuSO₄ 0.005, MnCl₂ 0.005, ZnSO₄ 0.005, sucrose 170 g/L, seawater 500 ml, distilled water 500 ml, pH 9 was used as the production medium and 5.0 ml of 6 x 10⁹ CFU/ml spore and substrate mycelium suspension was used as inoculum.

B. Effect of initial cultivation temperature on antimicrobials production, planktonic cell growth and biofilm formation

Shaken flasks i.e. 500 mL Erlenmeyer flasks containing 100 ml of Sucrose based medium (PM₃ medium with 170 g/L sucrose with initial pH 9) [7] were incubated at various temperatures (25, 30, 35 °C) and the shaker was run at 180 rpm for 72 hours.

Antimicrobial activity was expressed as diameter of zone of inhibition and determined following earlier worker [5, 6, 7]. Maximum antimicrobial activity at a defined time interval is described as peak antimicrobial activity (PAMA) corresponding to the diameter of zone of inhibition.

Planktonic cell growth, defined as growth measured from samples withdrawn from suspension and not from the biofilm was determined by recording wet weight of the suspended cell mass as reported earlier [6].

Dynamic monitoring of biofilm growth provides an opportunity to examine the dependence of antimicrobials production by the estuarine actinobacterial strain *Streptomyces sundarbansensis* sp nov., on overall biomass growth, i.e. the sum total of biomass growth in the planktonic (i.e. suspended) and biofilm (i.e. surface attached) phases. Different existing methods for biofilm growth monitoring e.g. CLSM and fluorescent reading are inapplicable to the shaken flask system due to the problem associated with the sampling of biofilm, thus, biofilm formation was measured dynamically by the method described earlier [15]. Of the biofilm formed on the wall surface of shaken flask, small circular patches of biofilms, measuring approximately 5 mm in diameter (i.e. surface area = π/16 cm²) was scrapped off using a sterile circular loop and a sterile spatula at every 12 h, and wet mass of the biofilm was measured by the difference method by weighing on a digital electronic balance measuring grams up to four decimal places (AFCOSET, Model ER-180A, Mumbai, India). Thus, the biofilm surface density, σ was determined. Biofilm samples were taken in triplicate and all the experiments described were conducted thrice and averages of the values with less than 10% deviation are reported.

If A= surface area of the circular strip on the shaken flask wall on which biofilm formation occurs (at the

liquid-air interface) and V= volume of culture medium, then the volumetric concentration of biofilm-phase biomass may be obtained from the relation

$$X_B(t) = \sigma(t) \times (A/V) \dots\dots\dots (1)$$

where (A/V), which is actually the specific surface area of the biofilm, may be considered constant. Thus, by virtue of eqn. 1, X_B(t) varies directly with σ(t).

III. RESULTS AND DISCUSSION

Based on the time-series data (t_B, X_B) (where X_B is the biofilm phase volumetric biomass concentration, similar to the planktonic biomass concentration, X_P), biofilm growth curves at 25 °C, 30 °C and 35 °C, are plotted in Fig. 1. All the curves are seen to ascend steadily up to a peak before declining to some extent. Maximum specific growth rate, μ_{max,B} is highest at 30°C – 0.043 h⁻¹, compared to that at 25°C – 0.038 h⁻¹ and 35°C – 0.031 h⁻¹.

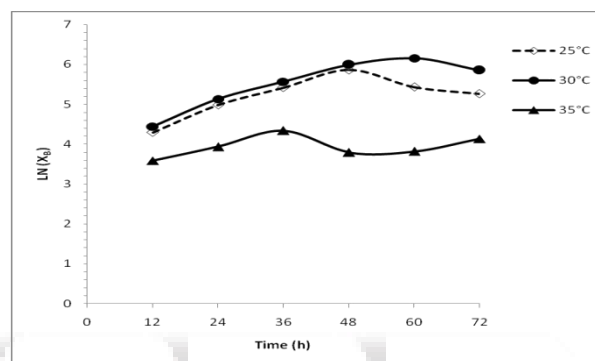


Fig. 1: Growth curve of biofilm phase

For the planktonic phase growth curves (Fig. 2), the observations are qualitatively similar. Maximum specific growth rate for the planktonic phase μ_{max,P} is also highest at 30°C – 0.063 h⁻¹, followed by that at 25°C– 0.058 h⁻¹ and 35°C– 0.046 h⁻¹. However, μ_{max,P} values (i.e. for planktonic growth) were always higher than the corresponding μ_{max,B} values (i.e. for biofilm growth) (at all the cultivation temperatures) by 44 % - 52 % , i.e. on an average by almost one and half times. Thus the optimum temperature for biofilm growth as well as for planktonic growth is the same i.e. 30°C.

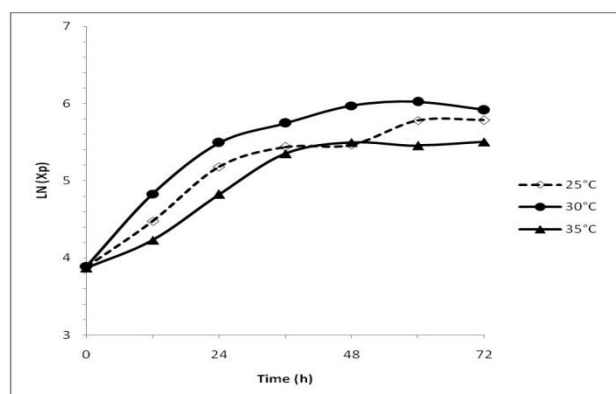


Fig. 2: Growth curve of planktonic phase

The growth-dependence of product formation (i.e. antimicrobials synthesis) by *Streptomyces sundarbansensis* sp nov. at all the cultivation temperatures considered, is evident from Fig. 3(A)-(C). Product concentration, P and total biomass concentration, X_T are seen to rise steadily

attaining their respective peak values almost simultaneously (before gradually plateauing off) at all the three temperatures. Thus, confirming the growth-associated antimicrobials production by *Streptomyces sundarbansensis* sp nov. Maximum antimicrobial activity P_{max} was highest at 30°C – 37 mm and compared to that at 25°C – 29.5 mm and 35°C – 16.5 mm and so was $X_{T,max}$, also highest for 30°C – 778.3 g/L compared to 25°C – 586.6 g/L and 35°C – 332.9 g/L. Thus, product formation being growth-associated is also favoured at 30°C, similar to cell growth. It may be noted here, that the optimum temperature for growth as well as for antimicrobials synthesis thus determined, i.e. 30 °C is close to the mean seawater temperature (February–November) of the sampling site from where the isolate was sourced.

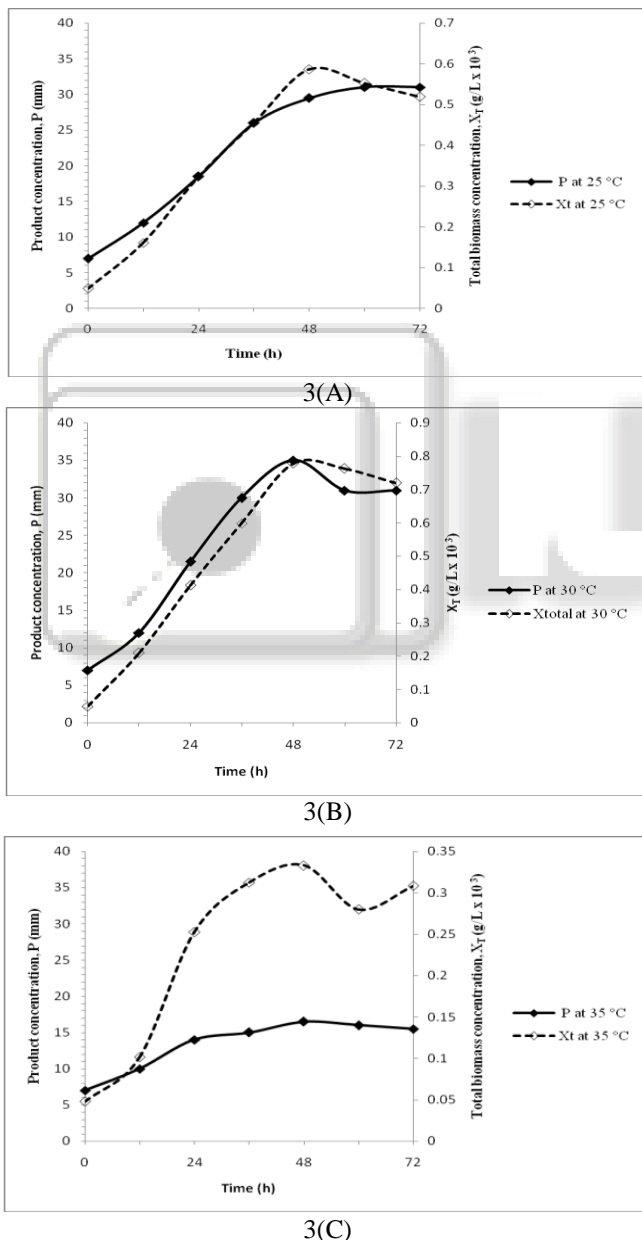


Fig. 3: Variation of product (antimicrobial) concentration, P (expressed as diameter of zone of inhibition, mm) and total biomass concentration, X_T (g/L) with cultivation time (h), at three temperatures viz. (A) 25°C, (B) 30°C and (C) 35°C

The rate of product formation is expressed by Leudeking and Piret rate model as follows:

$$\frac{dP}{dt} = \alpha X + \beta \frac{dX}{dt} \dots\dots\dots (2)$$

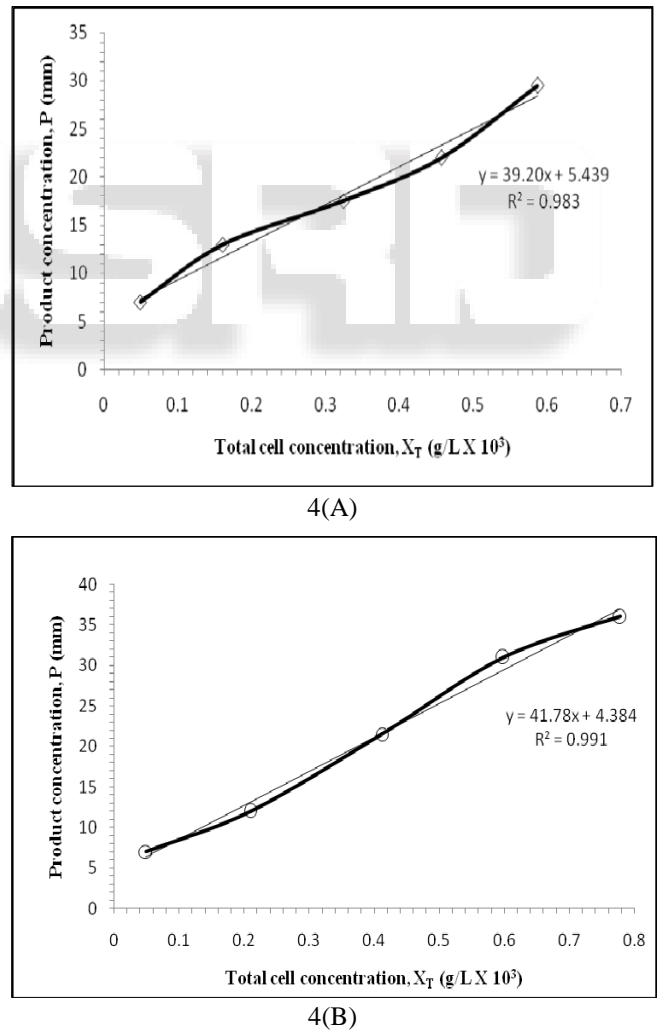
where α and β are the empirical constants.

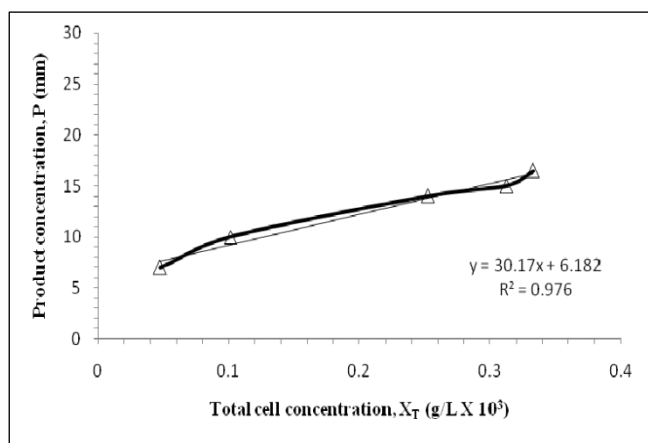
X = biomass concentration and $\frac{dX}{dt}$ = growth rate

This two-parameter kinetic expression was proven extremely useful and versatile in explaining product formation data for many different fermentation processes which is known as Leudeking and Piret kinetic expression [16]. For the growth associated product formation (i.e. when $\alpha = 0$, $\beta \neq 0$) Equation (2) can be simplified as follows:

$$P = K + \beta X \dots\dots\dots (3)$$

Product concentration increase linearly with cell growth (growth associated product formation) with β values of 0.03920 [mm/(g/L)] at 25°C, 0.04178 [mm/(g/L)] at 30°C and 0.03017 [mm/(g/L)] at 35°C [Fig. 4(A)-(C)]. The parameters of the Luedeking – Piret model evaluated indicate that the production of antimicrobials were mostly growth - associated. Thus product formation follows Leudeking Piret model





4(C)

Fig.4: Dependence of product (antimicrobial) concentration, P (expressed as diameter of zone of inhibition, mm) on total biomass concentration, X_T (g/L) at three temperatures viz. (A) 25°C, (B) 30°C and (C) 35°C

Kinetic studies of product (antimicrobials) formation based on dynamic evaluation of surface density of the growing biofilm is successfully demonstrated for *Streptomyces sundarbansensis sp nov.* grown in shaken cultures in sucrose-based media. Growth curves were obtained for both biofilm and planktonic phases for three different cultivation temperature, viz. 25°C, 30°C and 35°C – maximum specific growth rate was highest at 30°C for both biofilm and planktonic growth. Antimicrobials synthesis by this biofilm-forming marine isolate was found to be strictly growth-dependent with the peak in product (antimicrobials) and total biomass concentrations attained simultaneously for all the three temperatures. Product (antimicrobials) concentration increases linearly with total cell growth (growth associated product formation) following Luedeking – Piret model. Maximum concentration of product (antimicrobials) as well as of total biomass were both highest at 30°C; which is thus seem to be most favourable for cell growth and growth-dependent product formation. The results of this study would be of significance to the area of research involving growth associated bioactive compounds production both at shaken flask and large biofilm reactors.

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