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Studies on the Antagonistic Effect of *Serretia rubidaea* for Biofilm Development Capability

Jabun Naher[1], Shahidul A. Chowdhury[1], Abdullah A. Mamun[2], Nuruddin Mahmud[1], Whahida Shumi[1*], Ruhul A. Khan[2]

[1]Department of Microbiology, University of Chittagong, Chittagong, Bangladesh
[2]Institute of Radiation and Polymer Technology, Bangladesh Atomic Energy Commission, Dhaka, Bangladesh
Email: [*]wshumi@gmail.com

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Abstract

This study was performed to compare the growth dynamics of *Serretia* in single and co-culture biofilms condition and also evaluated its antagonistic effect against pathogenic bacteria. One preserved isolated subculture was identified on the basis of their morphological, cultural and biochemical characteristics. The isolate was belonged to the genus *Serretia* and provisionally identified as *Serretia rubidaea.* This isolate was exposed to different environmental condition. The experiments were carried out in Luria Bertani (LB) broth. The growth rate in different environmental parameter was measured by crystal violet staining of bacteria in Spectrophotometer. It was studied the ability of selected microorganism to generate biofilm on the test tube surfaces at different temperatures, pH values, NaCl concentrations and medium content. It was found that *Serretia* grew well at 27˚C after 48 h and co-culture grew well at 37˚C after 24 h. The 0.5% NaCl concentration was optimum for both. The results indicated that high concentration of NaCl clearly inhibited the adherence of the cells to the tube surfaces. Other analysis suggested that pH 7 was required for *Serretia* but pH 5 was suitable for co-culture. An attempt was also made to detect the inhibitory effects of Ciprofloxacin on the selected isolate and co-culture. It was found that, at 10 µg/mlantibiotic concentration, the *Serretia* single culture was more resistant than co-culture. The antagonistic effect of *Serretia* against pathogen showed that *Serretia* produced a small zone of inhibition.

Keywords

Antagonistic Effect, Biofilm, Biosurfactant, Inhibitory Effect, Antimicrobial Properties,

[*]Corresponding author.

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Co-Culture

1. Introduction

Biofilms are a collection of microorganisms which are encased within a matrix of organic materials generated by the microbes themselves and attached onto surfaces like plastics, stainless steel, glasses and initiating a growth process [1] [2]. It can be considered as a deposit where microorganisms are highly adhered onto a surface by means of appendages of either protein or polysaccharide nature, referred to as glycocalyx [3] [4]. Such appendage protrudes externally from the outer membrane of gram-negative cells or from the peptideoglycan of gram-positive ones. Under a hydrated state, it contains from 98% - 99% of water, thus protecting the cells against dehydration [5]. It has been found to contain water channels that help distribute nutrients and signaling molecules. It has an increased resistance to detergents and antibiotics, and this resistance to antibiotics in both stationary phase cells and biofilms may be due to the presence of persister cells [6]. Lateral gene transfer is greatly facilitated in biofilms and leads to a more stable biofilm structure [7]. Biofilms also provide an ideal niche for the exchange of extra chromosomal DNA (plasmid). Dispersal of cells from the biofilm colony is an essential stage of the biofilm life cycle. Enzymes that degrade the extracellular matrix, such as dispersin B and deoxyribonuclease, may play a role in biofilm dispersal [8] [9]. Biofilm matrix degrading enzymes may be useful as anti-biofilm agents [6] [7]. Like most soft materials biofilms are viscoelastic [10] [11]. A considerable number of both spoilage and pathogenic microorganisms are able to participate at a higher or lower intensity in both adhesion processes and biofilm formation. *Pseudomonas aeruginosa* [12], *Pseudomonas fragi*, *Micrococcus* spp and *Enterococcus faecium* [3] [4], are some of the spoilage microorganisms while *Listeria monocytogenes Yersinia enterocolitica*, *Salmonella* sp, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* [1] [5] are pathogenic ones. Biofilms play an important role in the ecology of the earth and the sustainability of life. They are capable of solving a huge problem in the cleaning of wastewater and used successfully in water and wastewater treatment for over the century. Other beneficial applications of biofilms are in cleaning up oil and gasoline spills by bioremediation. Biofilm retards water flow and prevents the immediate loss of water from the soil where water scarcity is tremendous. Due to its water retention properties it has a potential use as thickener, expander or viscosity enhancer in a variety of materials including paints, stains, dyes, oils, greases, among many others. Bacterial biofilms are also responsible for several chronic diseases that are difficult to treat. It has been implicated in a variety of human infections such as endocarditis, osteomyelitis, chronic otitis media, gastrointestinal ulcers, urinary tract infections, chronic lung infection, cystic fibrosis in patients, caries, periodontitis, formation of dental plaque, gingivitis [7] and coating on contact lenses [13]. More recently it has been noted that bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in healing or treating infected skin wounds. Biofouling is the detrimental development of biofilms which decrease heat transfer in heat exchangers, increase the pressure drop in pipelines and enhance corrosion. The standard assay for measuring biofilm formation is the crystal violet (CV) assay, which involves quantification of dye bound to cells within a biofilm. Scanning electron microscopy (SEM) has also been used to examine biofilm formation. *Serretia* is a genus of Gram negative, facultatively anaerobic, rod shaped bacteria of the Enterobacteriaceae. This genus is responsible for about 2% of nosocomial infections of the bloodstream, lower respiratory tract, urinary tract, surgical wounds, skin and soft tissues in adult patients [14]. They are unique by their production of three enzymes such as DNase, lipase and gelatinase [15].

The objective of the present research was to evaluate the antagonistic activity of single microbial community due to their extracellular protein and secreted enzyme and to observe the effect of environmental parameters for the formation of single and co-culture biofilm and the inhibitory effects of Ciprofloxacin against both culture condition.

2. Materials and Methods

2.1. Materials and the Characteristics of Bacteria *Serretia rubidaea*

2.1.1. Materials

For the present research work, subcultures of previously isolated bacteria were used. These cultures were kept

preserved in the laboratory of Microbiology Department, Chittagong University. The preserved culture was then enriched in nutrient broth for 4 hrs at 37˚C temperature. To confirm the purity of the isolate the enriched culture was seeded onto Nutrient Agar (NA) medium following streak plate method and ensure the presence of similar types of colonies in the medium. To facilitate the handling of the isolate it was designated as NW. One set of purified bacterial subculture was preserved as stock culture in polyethylene bag at 4˚C temperature. Occasional sub-culturing (after 3 to 4 weeks) was maintained to keep the culture in active condition and used for further studies.

2.1.2. The Characteristics of Bacteria *Serretia rubidaea*

Serratia is a genus of Gram-negative, facultatively anaerobic, rod-shaped bacteria of the Enterobacteriaceae family. The most common species in the genus, *S. marcescens*, is normally the only pathogen and usually causes nosocomial infections. However, rare strains of *S. plymuthica*, *S. liquefaciens*, *S. rubidaea*, and *S. odoriferae* have caused diseases through infection. The *S. rubidaea* was described for the first time in 1940 as *Bacterium rubidaea* and was later reclassified as *S. rubidaea*. It has been isolated from coconuts and from vegetable salads, but it has not been reported from water, insects, small mammals, or animal territories.

2.2. Identification

With an aim to characterize the selected bacterial isolate the renowned morphological and biochemical methods were followed. Based on the results or characteristics obtained from following experiments, the isolate was identified.

2.3. Biofilm Production and Screening Procedure

Biofilm production was done by the modified method of Christensen *et al*. (1982) [16]. In this method, one loopful from the subculture of isolate was inoculated in 5 ml LB broth containing test tubes and incubated for 4 hours at 37˚C temperature for enrichment. Then the suspension was dispensed in different test tube at inoculum to medium ratio of 1:20 and incubated at 37˚C temperature for different periods (24, 48, 72, 96 and 120 hours respectively). After incubation biofilm was seen by staining procedure. The dye used for this purpose was ammonium oxalate crystal violet. Here the assay was done by the modified method [17]. In this assay, after the respective incubation times, the culture medium was discarded from the tubes carefully. Then the tubes washed with sterile distilled water to remove loosely associated bacteria and air dried for 30 minutes. The tubes were stained with 1% ammonium oxalate crystal violet solution and left for 30 minutes at room temperature. Tubes were then inverted to remove the crystal violet and rinsed twice with sterile distilled water to remove excess crystal violet (CV). Five ml of 95% ethanol solution was added in each tube which can act as a distaining agent and incubated for 30 minute at room temperature. This could dissociate the bioflim forming cells and solubilized the remaining crystal violet attached to the cell. The absorbance of the retained dye was measured by spectrophotometer at 600 nm.

2.4. Different Parameters for Biofilm Development

For the detection of optimum growth parameter the single and co culture organisms were inoculated in different media parameters at inoculums to medium ratio of 1:20. Then the tubes were incubated at different temperatures (10˚C, 27˚C, 37˚C and 45˚C) for different incubation time. After incubation the biofilm was assayed by the modified method [17].

2.5. Antibiotic Sensitivity Pattern of Single and Co-Culture Microorganisms

The disc diffusion method [18] was used to determine the antibacterial activity of antibiotics against the isolate. The test was done for NW single and co-culture on Mueller-Hinton agar plates. For this purpose, 25 ml of medium was poured into 90 mm diameter sterile Petri dishes to a depth of 4 mm on a level surface to make the depth of the medium uniform and left at 37˚C temperature overnight to check sterility. The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) was measured only for Ciprofloxacin which is a broad spectrum antibiotic.

2.5.1. Inoculum Preparation

For inoculum preparation 5 ml LB broth was dispensed in screw cap tubes and sterilized by autoclaving at 121˚C for 20 minutes. The tubes were cooled and kept in an incubator for 24 hours at 37˚C to check sterility. Then few tubes were inoculated with purified isolate NW and other with 1:1 ratio of NW and pathogenic bacteria. All tubes were placed in an incubator at 37˚C for 4 hrs. After incubation bacterial suspension was saturated with a sterile cotton bud swab and excess inoculum was removed by turning the swab against the side of the tube. Inoculums were spread evenly over the entire surface of the Mueller-Hinton agar plates by swabbing back and forth across the agar in three directions to give uniform inoculums to the entire surface. Different cotton swab were used for single co-culture bacterial suspension in different Petri plates. These plates were allowed to dry before applying discs. For test discs sterile filter paper discs (Whatman No. 1, 6 mm) were impregnated with (10 µg/disc, 25 µg/disc, 50 µg/disc) antibiotic solution and left to dry under the laminar flow cabinet for 40 mins. These discs were applied on the inoculated plates with the help of a sterile forceps. The forceps was dipped in alcohol and flamed for sterilization. These plates were then placed in a refrigerator at 4˚C for 3 hours. This temperature could inhibit the growth of the organisms and within this time the antibiotics could diffuse in to the media. Then the plates were placed in an incubator at 37˚C for 18 hrs in inverted position. After 18 hrs of incubation, plates were examined and the diameters of zone of inhibition were measured in mm.

2.5.2. MIC and MBC Value Determination

The minimum inhibitory concentration (MIC) of Ciprofloxacin was determined by using the serial dilution method with LB broth and the final concentrations of antibiotic were (10 µg/ml, 25 µg/ml and 50 µg/ml). Then 4.75 ml of different concentrated antibiotic solution were placed in different tubes and autoclaved. The diluted purified and co-culture bacterial suspensions (10^{-1} to 10^{-5}) were added in each tube at a ratio of 1:20. Each antibiotic concentration was assayed in triplicate. The MIC values were taken as the lowest concentration of the antibiotic that showed no turbidity after 24 hours of incubation at 37˚C. The turbidity of the tubes was interpreted as visible growth of the microorganisms. The minimum bactericidal concentration (MBC) was determined by culturing the suspension of the tube showing no apparent growth in a sterile LB agar plate. The least concentration showing no visible growth of isolate and co culture microorganisms was taken as MBC for that antibiotic. In this study MBC was done by drop plate method.

2.6. Crude Enzyme Preparation and Assay of Selected Isolate

To obtain crude enzyme 48 hours old culture of isolate was transferred to micro centrifuge tubes and centrifuged at 12,000 rpm for 15 min. Cells were discarded and resultant supernatant was used as the crude enzyme for various enzyme assay. Enzyme assay was done by spectrophotometrically.

2.6.1. Alkaline Protease Assay

A 3 ml of the supernatant including 3 ml of alkaline citrate phosphate buffer (pH 8) was mixed with 3 ml of alkaline casein substrate. The mixture was incubated at 40˚C in water bath for 60 minutes. The solution was mixed with 5 ml trichloro acetic acid (0.4 M). It blocks enzyme activity and precipitates the intact casein. The solution stands for 1 hour at room temperature and was centrifuged in a high speed Hitachi refrigerated centrifuge at 12,000 rpm for 10 min at 4˚C. Then, 1 ml of supernatant was mixed with 5 ml Na_2CO_3 (0.4 M) and 1 ml of Folin-ciocalteau phenol (0.1 M) reagent and was incubated at 40˚C in water bath for 20 minutes in a dark condition. The mixtures were shaken well and stand for 30 minutes. Six ml distilled water was mixed with this solution and vortexes. Finally, the OD of the mixed solution was measured at 650 nm wavelength using spectrophotometer [19]-[21].

2.6.2. Amylase Assay

This assay was done by using a reaction mixture consisting 1 ml of substrate solution (1.1% soluble starch in 50 mM citrate phosphate buffer pH 7.2) and 100 µl of the enzyme solution. The reaction mixture was incubated for 10 min at 30˚C. Reaction was stopped by adding 2 ml of dinitrosalicyclic acid (DNSA) reagent. The reaction mixture was heated to 100˚C for 10 min and cooled. Optical density of each sample with reaction mixture was taken at 650 nm in a spectrophotometer [22].

2.7. Evaluate the Antagonistic Effect of Selected Isolate

Antagonistic activity of purified isolate against pathogenic bacteria was determined by disc diffusion method. To evaluate antagonistic effect 24 hrs incubated culture broth of isolate was transferred to micro centrifuge tubes and centrifuged at 12,000 rpm for 15 min. Pilot were discarded and resultant cell free supernatant (CFS) was used to assays the antagonistic activity. Pure pathogenic bacteria colony was picked with a sterile cotton bud swab and was spread over the entire surface of different nutrient agar plates in a manner to give a uniform inoculum to the entire surface was allowed to dry. After drying Whatman No. 1 filter paper disc saturated with supernatant of NW was placed in seeded plate and incubate for 24 hours at 37˚C. A clear zone of inhibition around the disc was then measured.

2.8. Statistical Analysis

Each test was replicated at least five times. The values presented here is the average of five samples.

3. Results and Discussion

The morphological, cultural and biochemical characteristics of the preserved isolate was studied to identify it up to species. Morphological characteristics include size and shape, arrangement of the cells, presence or absence of spores, irregular forms, acid fast reaction, gram reaction. Cultural and physiological characteristics include temperature tolerance, salt tolerance, IMViC test, H_2S production, nitrate reduction test, fermentation of different carbohydrates. All these characteristics were then compared with the standard description of "Bergey's Manual of Determinative Bacteriology" and found that the isolates belong to the genus *Serretia*. Results are presented in **Table 1**. The optimum growth parameters for biofilm formation by single and coculture situation of *Serretia* showed that between 24 and 48 hours incubation period at different temperatures, the single culture was denser and more vigorous than their counterpart coculture condition as depicted in **Table 2**. Co-culture always showed less absorbance than *Serretia*. But *Serretia* showed high absorbance at 27˚C temperature for 48 hours. Here negative control was deducted from the test absorbance. These results are in accordance with the published values reported in the journal [23]. According to their study *Serretia* showed antimicrobial activity towards the pathogenic microorganisms and produced anti-biofilm potential glycolipid surfactant.

A large difference was observed in biofilm formation in various pH ranges. After adjusting the media pH such as pH 4, 5, 7, 8 and 9 (before autoclave) it was found that at pH 7, the growth of *Serretia* was increased while coculture showed extended growth in pH 5. In acidic pH level (pH 3), coculture interestingly produced biofilm (OD at 600 nm < 0.1). On the other hand, at a high alkaline pH level (pH 9), *Serretia* showed its potentiality in biofilm production. The results are presented in **Table 3**. It was postulated that molecular interactions between charged acidic groups in the biofilm slimeand the bacterial cell walls contracted the biofilm and permitted them to grow at acidic pH in the coculture condition.

The effects of salt concentration on the biofilm formation showed that the medium with 0.5% salt having a good growth for both *Serretia* and coculture. But when the salt concentration was doubled, they showed less absorbance. The findings are represented in **Table 4**.

Other parameter such as medium content showed that at 0.5% media content coculture grew well. But *Serretia* grew well at 2% media content (**Table 5**).

Minimum inhibitory concentration (MIC) is the lowest concentration of drugs at which no bacterial growth was visually observed after incubation at 37˚C for 24 hours. In this study, it was performed by micro dilution method at concentration of 10, 25 and 50 µg/ml. The MIC result was given in **Table 6**. The highest growth was found in 10 µg/ml at 10^{-1} dilution for *Serretia* single culture. Only pathogenic bacteria showed growth at increased concentration of antibiotic (50 µg/ml) but in coculture condition no growth was found at same concentration. Zone of inhibition by *Serretia* and coculture (10^{-1} and 10^{-2} dilution) at 10, 25 and 50 µg/disc antibiotic concentrations are presented in **Figure 1** and **Figure 2**. Figures illustrated that at 10^{-1} dilution *Serretia* showed higher inhibition zone than co-culture and at 10^{-2} dilution the growth was scattered in co-culture. Melphine *et al.* [24] demonstrated that co-cultures could cause an antibiotic susceptibility that differs from one of the pure cultures. The results found in this study were similar to the findings of Melphine *et al*. The co-culture biofilm showed lower growth as well as lower resistance than *Serretia* in 10 µg/ml at 10^{-1} and 10^{-2} dilution.

The antimicrobial property of the selected isolate was determined by the disc diffusion assay (DDA) method.

Table 1. Morphological, cultural, biochemical and physiological characteristics of the isolate NW.

Parameters	Observations
Agar colonies	Whitish, yellowish, circular, entire, convex, smooth colonies
Agar slant	Filiform
Broth culture	Turbidity found
Gram staining	Gram negative
Spore staining	Non spore-former
Shape	Short rod, cocci
Cell arrangement	Single, sometimes pair
Size	Length-1.54 µm, Width-0.98 µm
Motility test	Positive
Deep glucose agar test	Facultative anaerobes
Glucose fermentation	Dark greenish
Xylose fermentation	Bluish (alkaline)
Arabinose fermentation	Bluish (alkaline)
Lactose fermentation	Bluish (alkaline)
Sucrose fermentation	Bluish (alkaline)
Mannitol fermentation	Greenish (acidic)
Indole test	Positive
H_2S production	Negative
Urease test	Negative
Oxidase test	Positive
Citrate test	Positive
Catalase test	Highly positive
Methyl red reaction	Positive
Voges-proskaur	Positive
Nitrate reduction test	Positive
Inorganic salt	Negative (−)
Glucose hydrolysis	Greenish
Casein hydrolysis	Positive (+)
Starch hydrolysis	Positive (+)
Gelatin hydrolysis	Positive (+)

Identification: The morphology, cultural and biochemical characteristics of isolate NW was found to closely related with the genus *Serretia* while compared with the description given in "Bergey's Manual of Determinative Bacteriology" (8th ed. Buchanon and Gibbons 1974) and provisionally identified as *Serretia rubidaea*. But it differed with the standard description in oxidase test, H_2S production and Gelatin hydrolysis.

Table 2. Effects of different temperature during 24 and 48 hours incubation period. Here biofilm formation is measured by crystal violet absorbance at 600 nm.

Microorganisms	Absorbance at different temperature for different incubation period					
	24 hours			48 hours		
	37˚C	27˚C	10˚C	37˚C	27˚C	10˚C
Serretia	0.292	0.397*	0.171*	0.354*	0.569*	0.128*
Co-culture	0.180	0.255	0.155	0.116	0.219	0.177

Note: *Indicate the highest absorbance.

Table 3. Absorbance at 600 nm with different media pH after optimum incubation period with optimum temperature.

Microorganisms	pH 4	pH 5	pH 7	pH 8	pH 9
Serretia	0.154	0.552	0.759*	0.491*	0.159*
Co culture	0.551*	0.638	0.397	0.224	0

Note: *Indicate the highest absorbance.

Table 4. Absorbance at 600 nm with different salt concentration after optimum incubation period.

Microorganism	Different Concentration			
	0%	0.5%	1%	2%
Serretia	0.311	0.808	0.397	0.199*
Co culture	0.137	1.105	0.180	0.135

Note: *Indicate the highest absorbance.

Table 5. Absorbance at 600 nm with different media content after optimum incubation period.

Microorganism	Medium Content		
	0.5%	1%	2%
Serretia	0.320	0.397	1.603*
Co culture	0.843	0.180	0.117

Note: *Indicate the highest absorbance.

Table 6. Growth pattern (absorbance) of selected microorganism and coculture (10^{-1} and 10^{-2} dilution) at different antibiotic concentration.

Selected microorganisms	Antibiotic at different concentration (µg/ml) Absorbance at 600 nm					
	10 µg		25 µg		50 µg	
	10^{-1} dilution	10^{-2} dilution	10^{-1} dilution	10^{-2} dilution	10^{-1} dilution	10^{-2} dilution
Pathogen	0.224	0.181	0.159	0.97	0.06	0.03
Serretia	0.254	0.126	0.071	0.02	0	0
Coculture	0.223	0	0.085	0.079	0	0

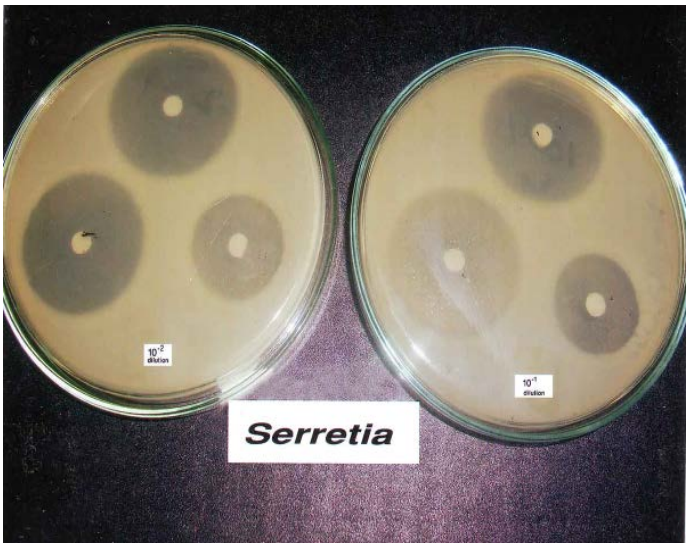

Figure 1. Zone of inhibition produced by NW (*Serretia rubidaea*) at different dilution.

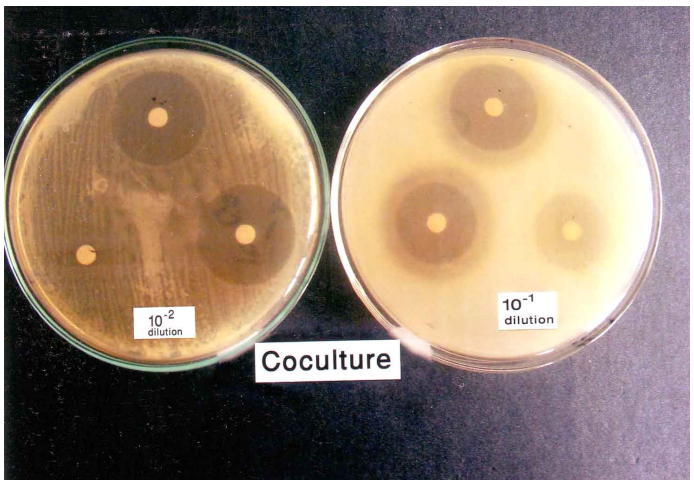

Figure 2. Zone of inhibition produced by coculture at different dilution.

The CFS (cell free supernatant) of the isolate (*Serretia rubidaea*) can reduce biofilm forming potential and antagonistic to other microorganisms. The zone diameters were found to be 11, 13 and 10 mm respectively, when 10 ml aliquots of the CFS were used. It was carried out the overall activity of the *Serretia* strain of various enzymatic screening to observe which type of enzyme had antagonistic effect. The overall activities were as follows: caseinase, amylase (starch), and lipolytic. Lipid hydrolysis was not shown by the strain. But the caseinase and amylase activities were shown by the strain. The result showed that *Serretia* produced starch and caseinase after 18, 24 and 48 hours of incubation (**Figure 3**). The absorbance of starch was 0.099, 0.41 and 0.002 but 0.111, 0 and 0 absorbances were found for caseinase. Finally, this is clear that after 24 hours of incubation, no caseinase activity was observed.

4. Conclusion

The growth dynamics of *Serretia* in single and co-culture biofilms condition was compared and evaluated the antagonistic effect against pathogenic bacteria. The cell free supernatant of the *Serretia rubidaea* can reduce biofilm forming potential and antagonistic to other microorganisms. It can be concluded that *Serretia* may produce

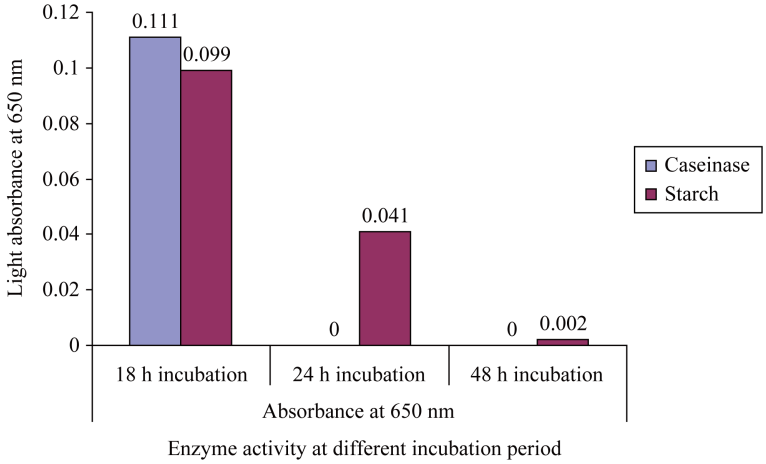

Figure 3. Enzyme activity showed that *Serretia* produce starch (0.099, 0.41 and 0.002) and caseinase (0.111, 0 and 0) after 18, 24 and 48 hours of incubation.

biosurfactant type product which may be amylase in nature. It was also revealed the inhibitory effect of Ciprofloxacin between *Serretia* single and co-culture condition. Further development of this bio-surfactant may be used as an antimicrobial agent against pathogen.

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