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## Carboxymethyl-cellulase and Filter-paperase Activity of New Strains Isolated from Persian Gulf

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### ABSTRACT

The aim of this research was the study of carbon cycle in the sea and isolation of new industrial strains. Samples of sediment and water were collected from some parts of Persian Gulf. First, the samples were enriched in Marine Broth medium. Then screening of cellulolytic isolates by using a congo red test were made on carboxymethyl cellulose (CMC) agar plates. The bacterial cultures were incubated in a shaking incubator at 37°C for 5 days. FPase and CMCase activity of cellulolytic strains was assayed and their enzymatic activity was drawn as a graph. The assay for the enzymatic activity was based on the release of glucose that was detected using dinitrosalicylic acid (DNS). In this study, 70 isolates of bacteria were isolated from sediments and waters collected from Persian Gulf. It was found that 43 isolates showed positive results with clear zone around the cultures. Therefore, It was demonstrated isolates of S-G<sub>20</sub>, S-G<sub>19</sub> and W-G<sub>15</sub> displayed the highest enzyme activity of 0.091, 0.089 and 0.084 U mL<sup>-1</sup> for CMCase and highest enzyme activity of 0.079, 0.074 and 0.072 U mL<sup>-1</sup> for FPase respectively. The phylogenetic analysis of these strains using its 16S rDNA sequence data showed that strain S-G<sub>20</sub> had highest homology (98%) with *Streptomyces variabilis*, S-G<sub>19</sub> showed 97% similarity with *Kocuria rosea* and W-G<sub>15</sub> showed 99% similarity with *Stenotrophomonas maltophilia*. Although cellulose-producing organisms are rarely found in the sea, cellulose-degrading bacteria are commonly found in the sea.

**Key words:** Carboxymethyl-cellulase, filter-paperase, *Streptomyces*, *Kocuria*, *Stenotrophomonas*, DNS, Persian Gulf

### INTRODUCTION

Among the three major habitats of biosphere, the marine realm which covers 70% of the earth's surface provides the largest inhabitable space for living organisms, particularly microbes. Marine microbes represent a potential source for commercially important bioactive compounds. They also play a crucial role in decomposition of organic matter such as; chitin, pectin, cellulose and cycling of nutrients (Das *et al.*, 2006). The homopolymeric cellulose made of anhydro-D-glucose linked by  $\beta$ -1,4 bonds constitutes the most abundant biopolymer on earth. Cellulose is commonly degraded by an enzyme called cellulase (Kotchoni *et al.*, 2006; Abou-Taleb *et al.*, 2009). Cellulases are inducible enzymes (Ibrahim and El-diwany, 2007) that can be produced by fungi, bacteria or actinomycetes during their growth on cellulosic materials, but the most common producer is fungi (Lee and Koo, 2001; Ariffin *et al.*, 2006). High cost of cellulase is mainly due to the substrates used in production and also the slow growth rate of fungi. Bacteria, which has high growth rate as

compared to fungi has good potential to be used in cellulase production. However, the application of bacteria in producing cellulase is not widely used. However, cellulases produced by bacteria are often more effective catalysts. They may also be less inhibited by the presence of material that has already been hydrolyzed (feedback inhibition). The greatest potential importance is the ease with which bacteria can be genetically engineered. This is needed especially in order to enhance cellulase production (Ariffin *et al.*, 2006). The complete enzymatic hydrolysis of cellulosic materials needs at least three different types of cellulase; endoglucanase (1,4- $\beta$ -D-glucan-4-glucanohydrolase, carboxymethylcellulase or CMCase; EC 3.2.1.4), exocellobiohydrolase (1,4- $\beta$ -D-glucan glucohydrolase; EC 3.2.1.74) and  $\beta$ -glucosidase ( $\beta$ -D-glucosideglucohydrolase; EC 3.2.1.21) (Yi *et al.*, 1999; Wood, 1989). The endoglucanase randomly hydrolyzes the  $\beta$ -1,4 bonds in the cellulose molecule, and the exocellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Lastly, the cellobiose is converted to glucose by  $\beta$ -glucosidase (Ibrahim and El-diwany, 2007). Cellulases have attracted much interest because of the diversity of their application. The major industrial applications of cellulases are in textile industry (Kasana *et al.*, 2008) for bio-polishing of fabrics and producing stonewashed look of denims, as well as in household laundry detergents (Kasana *et al.*, 2008) for improving fabric softness, brightness and anti-deposition (Ibrahim and El-diwany, 2007). Besides, they are used in animal feeds (Kasana *et al.*, 2008) for improving the nutritional quality and digestibility, in processing of fruit juices and in baking, while de-inking of paper is yet another emerging application. A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals (Gong *et al.*, 1999; Himmel *et al.*, 1999).

Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to the wide range of environments (Kim *et al.*, 2009). Due to the vast usefulness of cellulase, this project aim to isolate cellulase producing bacteria from Persian Gulf in order to apply for isolation of significant industrial strains.

## **MATERIALS AND METHODS**

**Sources of media and analytical chemicals:** All chemicals used were of analytical grade. Media and chemicals used in this study were purchased from HiMedia, India, Sigma- Aldrich, America and Merck, Germany.

**Collection of samples:** The surface seawater was collected with sterile glass bottles, whereas deep water samples were taken with a horizontal water sampler from depths of 5, 10 and 15 m. Sediment samples were obtained with grab from depths of 2, 5 and 10 m. In this study, The sediment and water samples were collected in spring and winter seasons from some parts of Persian gulf. The study was conducted from April 2009 to May 2010. Aliquots of water and sediment samples were transferred to the laboratory and were stored at 4°C and subjected to bacteriological examinations within several hours after collection (Herbert, 1988).

**Preparation of collected samples:** For preparation of collected sediment samples, 10 g of sediment samples were weighted aseptically and strewed within 250 mL Erlenmeyer flasks which contains 100 mL sterile distilled water and they were shaken for 24 h in constant impeller speed of 140 rpm. Then after settling insoluble ingredients, supernatant of mentioned suspension which contains bacteria, utilized. Water samples for 30 sec or 20 times were shaken extremely. (Herbert, 1988).

**Enrichment of cellulase producers:** For enrichment of the water and sediment samples for cellulases producing bacteria, 5 mL of water samples and 2 mL of supernatants of sediments suspension were inoculated in the Erlenmeyer flasks which contains marine broth medium. The cultures were incubated for 48-72 h at 30°C on rotary shaker in 140 rpm (Kim *et al.*, 2009).

**Screening of cellulase producers:** A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The bacteria were grown on CMC agar containing (g L<sup>-1</sup>) KH<sub>2</sub>PO<sub>4</sub> 1.0, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5, NaCl 0.5, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.01, MnSO<sub>4</sub>·H<sub>2</sub>O 0.01, NH<sub>4</sub>NO<sub>3</sub> 0.3, CMC 10.0, Agar 12.0. The pH was adjusted to 7.0 with 1 M NaOH. The CMC agar plates were incubated at 37°C for 5 days to allow for the secretion of cellulase. At the end of the incubation, to visualize the hydrolysis zone, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M NaCl for 15 min. To indicate the cellulase activity of the organisms, diameters of clear zone around colonies on CMC agar were measured. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulase activity producer. The largest ratio was assumed to contain the highest activity. Besides, a more quantitative assay method was used to determine the cellulase activity of the selected bacterial isolate in liquid medium (Ariffin *et al.*, 2006). The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a dinitrosalicylic acid (DNS) method (Miller, 1959).

**Analyses of 16S rDNA gene sequences:** For the sequence analysis, bacterial genomic DNA was extracted and purified using DNA Extraction Kit (BIONEER). Two primers annealing at the 5' and 3' end of the 16S rDNA were 5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3' (F primer) and 5'-CCCGGGATCCAAGCTTACGGTTACCTTGGTACGACTT-3' (R primer), respectively. PCR amplification was performed in a final reaction volume of 50 µL and the reaction mixture contained each primer in a quantity of 0.5 µL, each deoxynucleoside triphosphate in a quantity of 1 and 5 µL PCR buffer, 1.5 µL MgCL<sub>2</sub>, 1.5 µL Genom, 2 µL Taq DNA polymerase and 37.5 µL distilled water. The PCR reaction was run for 35 cycles in a DNA thermal cycler (Model C1000™, BioRAD, USA). The following thermal profile was used for the PCR: denaturation at 94°C for 1 min, primer annealing at 50°C for 40 sec and extension at 72°C for 1 min. The final cycle included extension for 20 min at 72°C to ensure full extension of the products. The amplified PCR products were then analyzed in a 1.0% (w/v) agarose gel, excised from the gel and purified. Purified PCR products for sequencing dispatched to Genfanavar company. Then the 16S-rDNA sequences of the isolates obtained were compared directly with sequences in the NCBI (National Centre for Biotechnology information) database using Basic Local Alignment Search Tool (BLAST) in internet.

**Cellulase production medium:** The medium used for production of the carboxymethyl cellulase (CMCase) contained the following components: 0.25% (w/v) yeast extract, 0.5% K<sub>2</sub>HPO<sub>4</sub>, 0.1% NaCl, 0.02% MgSO<sub>4</sub>·7H<sub>2</sub>O and 0.06% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 2.0% carboxymethyl cellulose (CMC) was used as carbon source. The pH was adjusted to initial pH 7.0 by 1M NaOH. Fermentation was carried out in 250 mL plugged Erlenmeyer flasks each containing 150 mL sterile production medium. The inoculated flasks were incubated at the temperature of 37°C on rotary shaker at 140 rpm under aerobic condition (Kim *et al.*, 2009).

**Preparation of crude enzyme:** After incubation, 1 mm of culture broth were withdrawn at intervals and centrifuged at 5000 rpm for 20 min at 4°C and the supernatant served as the source of crude enzymes. The crude enzyme solution was utilized for determination of enzyme activities (Immanuel *et al.*, 2006).

### Enzyme assay procedure

**Determination of Carboxymethyl-cellulase (CMCase) (Endoglucanase) activity:** CMCase activity was assayed using a modified method described by Wood and Bhat (1998) with some modifications. The CMCase activity was measured by mixing 0.1 mL of enzyme solution with 0.1 mL of 1.0% (w/v) CMC in 10 mM sodium phosphate buffer, pH 7.0 at 37°C for 60 min. The reaction was stopped by adding 1.0 mL 3,5-dinitro salicylic acid (DNS) reagent. The mixture was boiled for 10 min cooled in ice and its optical density at 546 nm was determined. The CMCase activity was measure by using a calibration curve for glucose. One unit of CMCase was defined as the amount of enzyme that released 1  $\mu$ mol of glucose per min.

**Determination of Filter-paperae (FPase) activity:** The activity of FPase was assayed according to the method explained by Wood and Bhat with some modifications. Briefly, the methods are similar to the CMCase assay method , but the substrate used was Whatman No. 1 filter paper (FP) strip (1 $\times$ 6 cm) soaked in 1.0 mL and 10 mM sodium phosphate buffer pH 7.0 at 37°C for 60 min. The reaction was stopped by adding 1.0 mL 3,5-dinitro salicylic acid (DNS) reagent. The mixture was boiled for 10 min cooled in ice and its optical density at 546 nm was determined. One unit of FPase was defined as the amount of enzyme that released 1  $\mu$ mol of glucose per min.

## RESULTS

**Screening of cellulase producer:** In this study, Screening of bacteria was conducted by using the Congo red test as a preliminary study for identifying cellulase producers. The results indicated After 5 days of incubation, from 70 bacterial isolates, just 43 isolates showed positive results with ratio of clear zone diameter to colony diameter ranging from 1 to 5.6 mm (data not shown). Since the sole carbon source in CMC agar was CMC(cellulose), therefore the result of the test was a strong evident that cellulase was produced in order to degrade cellulose. isolates of S-G<sub>20</sub>, S-G<sub>19</sub> and W-G<sub>15</sub> gave the highest ratio of clear zone diameter to colony diameter (Table 1). This indicated more cellulose degradation in CMC agar plate cultured with S-G<sub>20</sub>, S-G<sub>19</sub> and W-G<sub>15</sub> as compared to plates cultured with the other strains (Fig. 1a, b).

**Identification of the cellulases producing bacteria:** Three strains showing clear zone after staining with Congo red, designed as S-G<sub>20</sub>, S-G<sub>19</sub> and W-G<sub>15</sub>. phylogenetic analysis of these strains using its 16S rDNA sequence data showed that strain S-G<sub>20</sub> had highest homology (98%) with *Streptomyces variabilis*, S-G<sub>19</sub> showed 97% similarity with *Kocuria rosea* and W-G<sub>15</sub> showed 99% similarities with *Stenotrophomonas maltophilia*.

Table 1: The ratio of clear zone diameter to colony diameter in *Streptomyces* S-G<sub>20</sub>, *Kocuria* S-G<sub>19</sub> and *Stenotrophomonas* W-G<sub>15</sub>

Strain	The ratio of clear zone diameter to colony diameter
<i>Streptomyces</i> S-G <sub>20</sub>	5.6
<i>Kocuria</i> S-G <sub>19</sub>	5.0
<i>Stenotrophomonas</i> W-G <sub>15</sub>	4.5

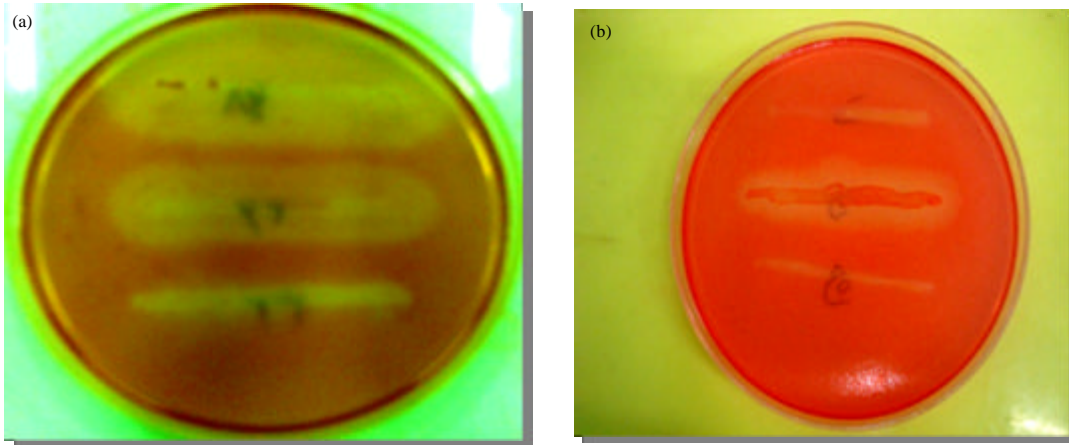


Fig. 1: Effect of Congo red and NaCl on the cellulolytic zone in CMC agar plates. (a) S-G<sub>20</sub> and S-G<sub>19</sub> and (b) W-G<sub>15</sub>

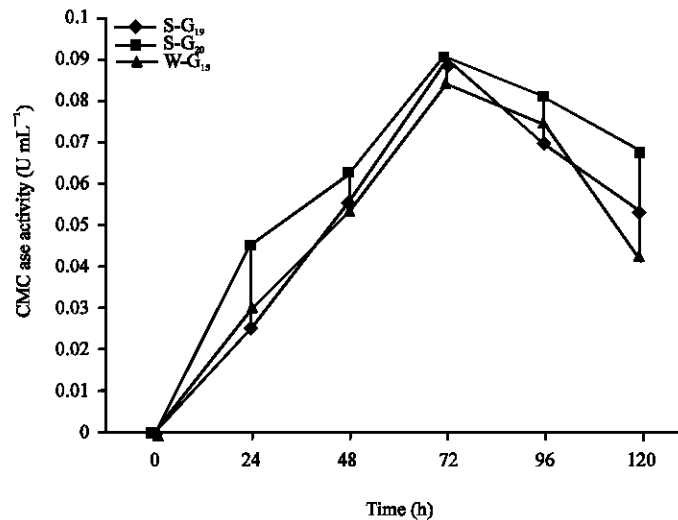


Fig. 2: Enzyme activities of *Streptomyces* S-G<sub>20</sub>, *Kocuria* S-G<sub>19</sub> and *Stenotrophomonas* W-G<sub>15</sub> grown on CMC as the only sources of carbon

### Enzyme assay

**The results of CMCase and FPase activity:** As it mentioned to assay CMCase and FPase activity, carboxymethyl cellulose and Whatman No. 1 filter paper as carbon sources used. The results indicated S-G<sub>20</sub>, S-G<sub>19</sub> and W-G<sub>15</sub> isolates were able to decompose carboxymethyl cellulose and Whatman No. 1 filter paper. The enzymatic hydrolysis produced sugars with reducing ends that reacted to dinitrosalicylic acid showing the high absorbance in 546 nm. In order to show CMCase and FPase activity, it is necessary to measure the glucose concentration which released by enzymatic hydrolysis. Therefore, calibration glucose curve was drawn. Then, enzymatic activity based on U mL<sup>-1</sup> stated.

Figure 2-5 illustrate cellulase activities and growth profile in the broth during fermentation for cellulase production in 250 mL Erlenmeyer flasks. Figure 2 illustrates Enzyme activities of

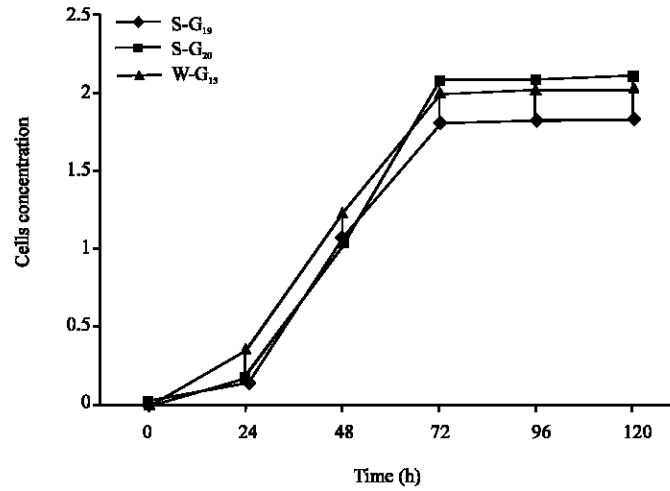


Fig. 3: Growth profile of *Streptomyces* S-G<sub>20</sub>, *Kocuria* S-G<sub>19</sub> and *Stenotrophomonas* W-G<sub>15</sub> during cellulase production on CMC

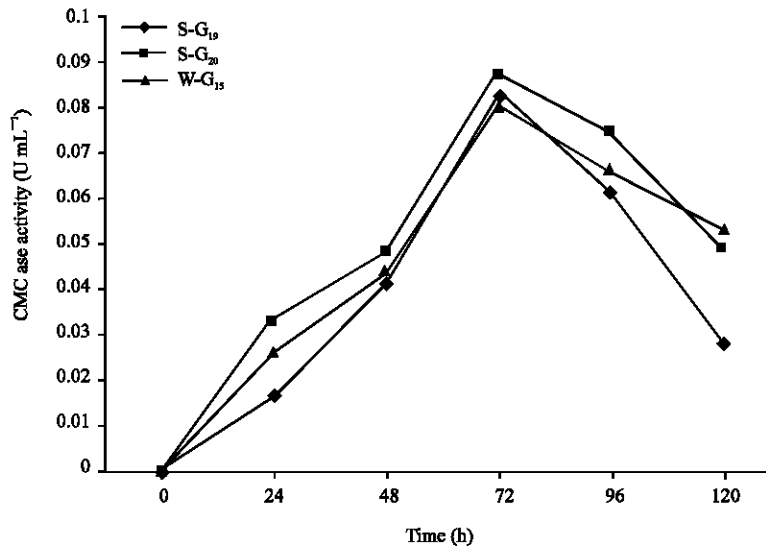


Fig. 4: Enzyme activities of *Streptomyces* S-G<sub>20</sub>, *Kocuria* S-G<sub>19</sub> and *Stenotrophomonas* W-G<sub>15</sub> grown on FP as the only sources of carbon

*Streptomyces* S-G<sub>20</sub>, *Kocuria* S-G<sub>19</sub> and *Stenotrophomonas* W-G<sub>15</sub> grown on CMC as the only sources of carbon and Fig. 4 illustrates Enzyme activities of *Streptomyces* S-G<sub>20</sub>, *Kocuria* S-G<sub>19</sub> and *Stenotrophomonas* W-G<sub>15</sub> grown on FP as the only sources of carbon. Figure 2 and 4 show that CMCcase and Fpase activity S-G<sub>20</sub>, S-G<sub>19</sub>, W-G<sub>15</sub> isolates during 24 and 48 h after cultivation increased and finally after 72 h reached to maximum. Then their anzyme activity reduced. Figure 3 and 5, respectively illustrate Growth profile of *Streptomyces* S-G<sub>20</sub>, *Kocuria* S-G<sub>19</sub> and *Stenotrophomonas* W-G<sub>15</sub> during cellulase production on CMC and FP. From the Fig. 3 and 5, it was seen that the cellulase was being produced during growth phase of S-G<sub>20</sub>, S-G<sub>19</sub>, W-G<sub>15</sub> isolates.

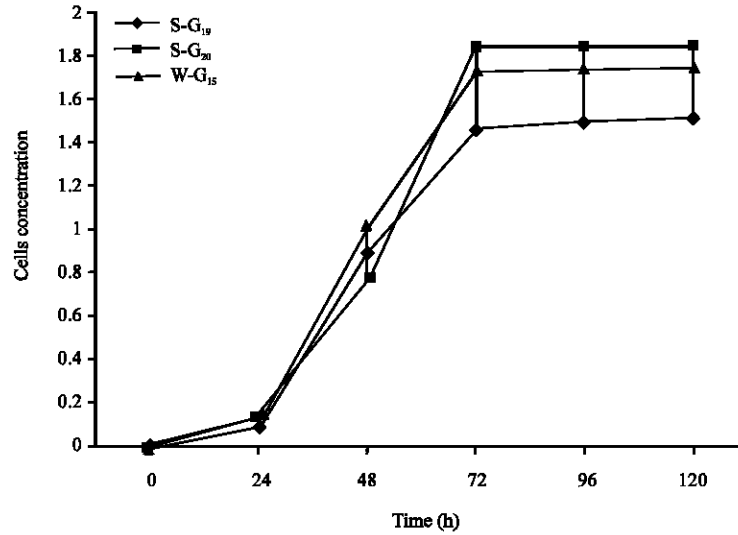


Fig. 5: Growth profile of *Streptomyces* S-G<sub>20</sub>, *Kocuria* S-G<sub>19</sub> and *Stenotrophomonas* W-G<sub>15</sub> during cellulase production on FP

Overall, maximum cellulase activity for *S. variabilis*, *K. rosea* and *S. maltophilia* was obtained after 72 h of fermentation with 0.091, 0.089 and 0.084 U mL<sup>-1</sup> for CMCase and 0.079, 0.074 and 0.072 U mL<sup>-1</sup> for FPase respectively. These results are in agreement with those of Narasimha *et al.* (2006, 2007) who found that carboxymethyl cellulose was the best carbon source followed by cellulose for cellulase production. A higher production of cellulase when CMC served as substrate may be as a result of induction of the enzyme since cellulose is known to be a universal inducer of cellulase synthesis. Paul and Varma (1993) had reported the induction of endocellulase by CMC. The growth profile of the bacterial isolates during fermentation shows that the cellulase was being produced during growth phase of the *S. variabilis*, *K. rosea* and *S. maltophilia*.

## DISCUSSION

To our knowledge, this is the first report of cellulolytic bacteria in Persian Gulf. The screening method of cellulolytic in this study support Aboul-Enein *et al.* (2009) and Ariffin *et al.* (2006). To evaluate quality of cellulolytic isolates. They used congo red test. Although the congo red test was sensitive enough for primary isolation and screening of cellulolytic bacteria, but the clear zone width was not implied the amount of cellulase activity. In 2000, a report showed that among 77 thermotolerant bacterial isolates grown on CMC agar, an isolate CMU4-4 exhibited the highest enzyme activity whereas its clear zone was smaller than other isolates (Krootdilaganandh, 2000). The measurement of CMCase activity and FPase activity in this study also support Wood and Bhat (1998) and Miller (1959). The mentioned researchers, by using colorimetry method with DNS reagent and drawing calibration curve by D-glucose, investigated cellulolytic isolates activities. In this paper, three cellulolytic isolates *Streptomyces* S-G<sub>20</sub>, *Stenotrophomonas* W-G<sub>15</sub> and *Kocuria* S-G<sub>19</sub> were isolated. While, more studies about cellulose producing have made, we can rarely about, *Stenotrophomonas* W-G<sub>15</sub> and *Kocuria* S-G<sub>19</sub> producing cellulose something is done. Therefore, we can not compare these strains with other researches. In this study, it found *Streptomyces* S-G<sub>20</sub> indicated that maximum endoglucanase activity 0.079 U mL<sup>-1</sup> and maximum total cellulase activity 0.091 U mL<sup>-1</sup> after 72 h incubation. Chellapandi and Himanshu (2008) isolated 2 isolates of



*Streptomyces* BRC1 and BRC2. The researchers's observations showed that maximum endoglucanase activity was 6.4-6.6 U mL<sup>-1</sup> after 72-88 h incubation. Very much fluctuation of *Streptomyces* S-G<sub>20</sub> endoglucanase activity with *Streptomyces* BRC1 and BRC2 related to difference of isolates enzyme properties or lack of optimisational condition. Also, we can say that cellulose production is based on growth and it is done duration this phase and in Ariffin *et al.* (2006). Researches it clearly was seen. The results of CMCase and FPase activity in this research are in agreement with those of Narasimha *et al.* (2006, 2007) who found that carboxymethyl cellulose was the best carbon source followed by cellulose for cellulase production. A higher production of cellulase when CMC served as substrate may be as a result of induction of the enzyme since cellulose is known to be a universal inducer of cellulase synthesis. Paul and Varma (1993) had reported the induction of endocellulase by CMC. In other studies, there are many reports of cellulase production by bacterial species isolated from various natural sources. Optimum time for maximum enzyme production by different bacterial sp. has been reported to be quite variable. Maximum enzyme production stage of the organism largely depends upon the type of microbial strains and their genetic make up and on cultural and environmental conditions employed during growth of the organism (Bajaj *et al.*, 2009). The results of this work indicated that cellulolytic bacteria play a decisive role in the cycle of matter in marine environments, as they are able to breakdown cellulose into the components from which they have originated and Although cellulose-producing organisms are rarely found in the sea, cellulose-degrading bacteria are abundantly found in the sea. Several microorganisms capable of converting cellulose into simple carbohydrates had been discovered for decades. However, needs for newly isolated cellulolytic microbes were still remained. The *S. variabilis*, *K. rosea* and *S. maltophilia* showed a potential to convert cellulose into reducing sugars which could be readily used in many applications such as animal foods and a feed stock for production of valuable organic compounds.

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