

Identification and characterization of two amylase producing bacteria *Cellulosimicrobium* sp. and *Demequina* sp. isolated from marine organisms

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تعريف وتحديد صفات نوعين من البكتريا (*Cellulosimicrobium* sp) و (*Demequina* sp) المفترزة للأميليز تم عزلهما من كائنات بحرية

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ABSTRACT. Marine sources have been known to yield novel compounds with a wide range of bioactivity with various commercial applications. In this study, the abilities of bacteria isolated from eight marine organisms to produce α -amylase were examined. All eight organisms were found to harbor amylase producing bacteria. Two bacterial species isolated from the green alga *Ulva rigida* and the sponge *Mycale* sp. were further identified and their α -amylases were purified and characterized. The bacterial species isolated from *U. rigida* and *Mycale* sp. were identified by DNA sequencing as *Cellulosimicrobium* sp. and *Demequina* sp., respectively. *Cellulosimicrobium* sp. obtained maximum cell growth and amylase production at 29°C and in the presence of lactose as a carbon source. Optimal cell growth and amylase production by *Demequina* sp. was observed at 35°C. While lactose enhanced cell growth of *Demequina* sp., maximum amylase production was found when fructose and glycerol were the available sources of carbon. Both strains grew better in the presence of tryptone, whilst peptone stimulated amylase production. Maximal cell growth and amylase production by both of the strains was found at a medium salinity of 3% NaCl.

KEYWORDS: Natural products, enzymes, amylase, bacteria.

المستخلص: تُعزف المصادر البحرية بتوفيرها لمركبات جديدة لها تأثيرات حيوية واسعة النطاق وتطبيقات تجارية متعددة. تم فحص القدرة على إفراز الأميليز ألفا من قبل بكتريات تم عزلها من ثمانية كائنات بحرية. وجد أن جميع الكائنات البحرية الثمان تحتوي على بكتريات منتجة للأميليز. كذلك تم عزل نوعين من البكتريا من الطحلب الأخضر *Ulva rigida* ومن الأسفنجية *Mycale* sp. وتم تعريفهما، وتنقية إنزيمتهما الأميليزية وتحديد صفاتهما. تم تعريف النوعين البكتريين اللذين تم عزلهما من *Ulva rigida* ومن *Mycale* sp. على أنهما *Cellulosimicrobium* sp. و *Demequina* sp. بالتوالي، وذلك من خلال تقنية الترتيب التسلسلي للحمض النووي DNA. حققت البكتريا *Cellulosimicrobium* sp. أقصى نمو للخلايا وأقصى إنتاج للأميليز عند 29 درجة مئوية في وجود اللاكتوز كمصدر كربوني، بينما كان أفضل نمو للخلايا وأفضل إنتاج للأميليز لنوع *Demequina* sp عند 35 درجة مئوية. ورغم أن اللاكتوز قد حسّن النمو الخلوي ل *Demequina* sp، إلا أن أقصى إنتاج للأميليز في هذا النوع قد وُجد عندما كان الفركتوز والجليسرول هما المصدرين المتاحين للكربون. ولقد نما النوعان بشكل أفضل في وجود التربتون، بينما حفز الببتون إنتاج الأميليز. وُجد أيضا أن أقصى نمو للخلايا وإنتاج للأميليز كان في كلي النوعين عند مستوى الملوحة المتوسط 3 % NaCl.

الكلمات المفتاحية: المنتجات الطبيعية، الأنزيمات، الأميليز

Introduction

The sea is a unique resource with a large diversity of natural products isolated mainly from invertebrates, such as sponges, tunicates, bryozoans and molluscs, as well as from bacteria (Proksch *et al.*, 2002). Symbiotic microorganisms are often proposed as the true producers of natural products isolated from marine invertebrates. Numerous examples exist in

which structurally related or identical compounds have been reported from taxonomically distinct invertebrates and cultured microorganisms (Moore, 1999; Proksch *et al.*, 2002). Recently, research has focused on marine microorganisms as sources of many metabolites that have a potential to be used as antibiotics, antimicrobials, or antitumor agents and pharmaceuticals.

Marine microorganisms are a valuable source of novel enzymes with ideal characteristics because of the halophilic nature of the marine bacteria (Mohapatra *et al.*, 1998). Halophiles are microorganisms that live, grow, and multiply in highly saline environments. Moderately halophilic bacteria are able to grow over a wide range of salt concentrations from 0.4 to 3.5M. Exoenzymes from

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these microorganisms with polymer degrading ability at low water activities are of interest in many harsh industrial processes where concentrated salt solutions would inhibit many enzymatic conversions. Furthermore, most halobacterial enzymes are known to be thermotolerant and remain stable at room temperature for a long time (Chakraborty *et al.*, 2008(b); Mohapatra *et al.*, 1998).

Free-living bacteria and bacteria attached to marine sediments usually excrete large amounts of extracellular enzymes for the hydrolysis of intractable macromolecules (Mohapatra *et al.*, 2003). The enzymes secreted by the bacteria remain associated with the cell and are released slowly to the environment to aid in the nutrition of their host. Until now, researchers have isolated several enzymes such as acetylcholinesterase, amylase, urethanase, cellulase, and alginate and pectin lyases from bacteria and fungi associated with marine sponges and algae (Mohapatra *et al.*, 2003). Although many microorganisms produce amylase, the ones most commonly used for industrial production are *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquifaciens* and *Aspergillus niger* (Vidyalakshmi, 2009).

The sea around Oman is rich in marine organisms that could be a potential source of natural products that are not yet exploited. The overall objectives of this study was to isolate bacteria associated with Omani marine organisms, study their abilities to produce amylase and to investigate the optimal conditions (temperature, pH and salinity, carbon and nitrogen sources) for bacterial growth and enzyme production.

Materials and methods

Isolation of pure cultures from marine environments

Samples from eight marine organisms: the red sponge *Mycale* sp., the tunicate red *Ascidia* sp., the tunicate black *Ascidia nigra*, the whitesponge *Chalina* sp., the yellow sponge *Hemiasterella* sp., the sea anemone *Heteractis crispa* and the green alga *Ulva rigida* and sea water were collected from Bandar Al Khairan (depth 1-5 m), Arabian Gulf, Sultanate of Oman. The marine organisms were transferred into plastic bags immediately after collection and kept in ice until transferred to the laboratory. In the laboratory, the samples were transferred to conical flasks containing marine broth and placed in a rotary shaker at 180 rpm at 29°C for about 24 to 48 hours. After incubation, a loop full of each sample mixture was streaked onto nutrient agar and incubated for another 24 hours. The bacterial colonies were separated and sub-cultured several times until pure bacterial cultures were obtained.

Screening of amylolytic enzymes production

To check the amylolytic activity of the isolated bacterial strains, each pure bacterial culture was cultured in



Figure 1. Starch hydrolysis by amylase producing bacteria visualized by Lugol's-iodine coloration.

marine broth and incubated for about 24 hours. Subsequently, about 20 µl of each bacterial culture was dropped on Starch agar and incubated for 24 to 48 hours. After incubation, amylase production was determined by the development of a colourless halo zone surrounding the bacterial colonies when Lugol's iodine solution (1% iodine in 2% potassium iodide w/v) was added to the plate (Fig.1). The diameters of the digestion zones were measured to check the intensity of the enzyme activity of the bacteria. Two of the fastest growing bacteria with largest starch digestion zones were chosen for further identification and analysis.

Bacterial identification

The two bacterial strains were first identified by gram staining, API Coryne and negative staining (TEM test). Then, further identification was done by DNA sequencing and fatty acid analysis profile.

DNA sequencing

The bacterial strains were identified by comparative analysis of their 16S rRNA gene sequences as described in Lau *et al.*, 2002. Fragments of DNA sequences (about 700 bp) obtained from individual primers were assembled using the Sequencher® software package (Gene Codes, USA). The closest match to the 16S rDNA gene sequence of the respective bacteria was retrieved by comparison with data from the GenBank (www.ncbi.nlm.nih.gov).

Bacterial fatty acids analysis

The sample preparation and analysis for bacterial fatty acid determinations were performed as described by Sasser, 1990 using hexane mixed with methyl tert butyl ether for the extraction of the fatty acids from the bacterial strains.

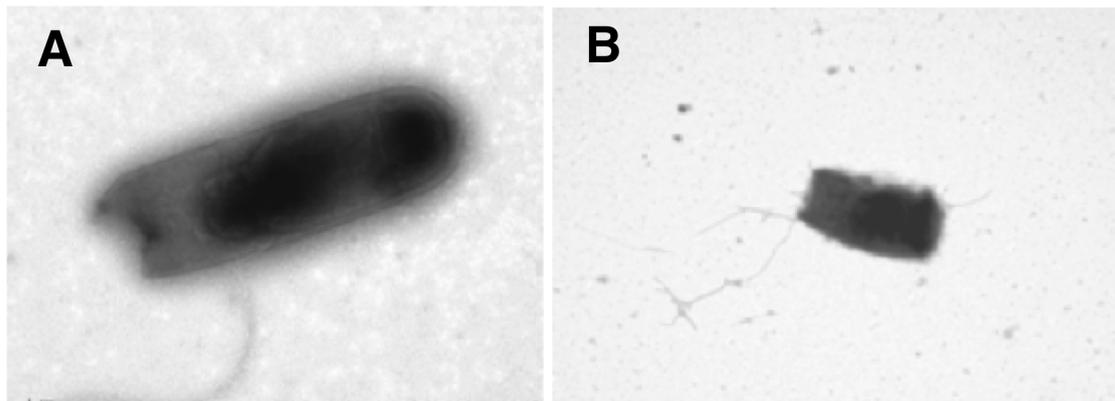


Figure 2. (a) *Cellulosimicrobium sp.* isolated from the green alga *Ulva rigida* after negative staining (TEM test) under electron microscope at 25000x magnifications. (b) *Demequina sp.* isolated from sponge *Mycale sp.* after negative staining (TEM test) under electron microscope at 15000x magnification.

GC analysis

One μl of each extract of bacterial fatty acids in hexane- methyl tert butyl ether mixture was injected into a Gas Chromatograph (Agilent 6890 N) instrument equipped with Ultra-1 (crosslinked methyl silicone gum) column, HP catalogue No. 19091A-102 (25m long x 0.2mm i.d x 0.33 μl film thickness).

Analytical conditions

The injector and flame ionization detector (FID) temperatures were set at 250°C and 260°C, respectively. The oven temperature was programmed from 150°C (2 minutes) to 250°C (5 minutes) at 4°C/min. Helium was used as the carrier gas with a linear velocity of 20 cm/sec. The duration of the reaction was set for 40 minutes.

Data analysis

Retention times of the unknown bacterial fatty acids were compared to a mixture of standard bacterial fatty acid methyl esters (BAME) (Supelco, Catalogue No. 47080).

Sensitivity of bacterial strains to antibiotics

The bacterial strains were tested for their susceptibility to the following antibiotics: Ampicillin, Streptomycin, Chloramphenicol, and Penicillin G. Concentrations of 1 mg/ml and 10 mg/ml were used for each antibiotic. The test was performed using a disc diffusion bioassay.

The bacterial colonies of the two strains were evenly distributed on nutrient agar using sterile swabs. Small discs (diameter = 5.5 mm and surface area = 1 cm²) containing each type of antibiotic were placed in the media inoculated with bacteria and incubated for about 24 hours. Presence of clear zones around the discs correspondent to the bacterial growth inhibition was checked and their diameters were measured in millimetres with a ruler.

Optimization of bacterial yield and amylase Production

The effect of temperature, salinity and available carbon and nitrogen sources on bacterial growth was tested by measuring the optical density of the samples after incubation. Amylase production was determined by the amount of maltose produced as an end product of starch hydrolysis by amylase.

Bacterial yield test

Marine broth containing different NaCl concentration (0%, 3% 5%, 10% and 15% (w/v)) were prepared to test the effect of salinity. Solutions containing 1% of a specific carbon source namely starch, glucose, fructose, lactose, glycerol, sucrose or maltose along with 0.1% yeast extract were prepared in sea water. Solutions containing 1% peptone or tryptone as nitrogen sources were also prepared and supplemented with 0.1% yeast extract mixed with sea water. Test tubes containing 10 ml of each solution was inoculated with the bacterial cultures except the blank and incubated at 29°C for 24h. To test the effect of temperature, test tubes containing 10ml marine broth inoculated with the bacterial cultures were incubated at different temperatures (5°C, 29°C, 35°C and 60°C) for 24h. After incubation, the blank control was used to zero the spectrophotometer and the absorbance at 600 nm was measured for all the samples.

Determination of bacterial amylase production

The culture solutions prepared in the bacterial yield test, as above, were centrifuged at 10,000 rpm for 5 minutes at 15°C to separate the bacterial cells from the broth. 1 ml of cell free supernatant of each sample was removed and added to tubes containing 1 ml of 1% starch solution. 1 ml of distilled water with 1 ml of 1% starch solution was added to the blank tube. The tubes were incubated at 37°C for 12 minutes. After incubation, 1 ml of maltose colour reagent (1% 3,5-dinitrosalicylic acid,

Table 1. Results of gas chromatography mass spectrometry (GC-MS) analysis.

	BAME name	Ret. (Min)	Cm	Dq
1	Methyl Dodecanoate (C12:0)	17.42	0.24	1.9
2	Methyl tridecanoate (C13:0)	20.05	0.31	0.0
3	Methyl 2-Hydroxydodecanoate (2-OH-C12:0)	20.38	0.05	0.0
4	Methyl 3-hydroxydodecanoate (3-OH-C12:0)	21.03	2.70	1.7
5	Methyl tetradecanoate (C14:0)	22.67	8.01	4.8
6	Methyl 13-methyltetradecanoate (i-C15:0)	24.32	1.22	3.2
7	Methyl 12-methyltetradecanoate (α -C15:0)	24.53	0.16	0.0
8	Methyl pentadecanoate (C15:0)	25.22	2.84	2.2
9	Methyl 3-hydroxytetradecanoate (3-OH-C14:0)	26.22	4.82	3.7
10	Methyl 14-methylpentadecanoate (i-C16:0)	26.81	1.94	0.7
11	Methyl cis-9-hexadecenoate (C16:19)	27.16	39.52	29.8
12	Methyl hexadecanoate (C16:0)	27.68	15.24	16.4
13	Methyl 15-methylhexadecanoate (i-C17:0)	29.22	1.58	6.6
14	Methyl heptadecanoate (C17:0)	30.1	1.27	1.3
15	Methyl trans-9-octadecenoate (C18:19)	32.17	19.85	26.7
16	Methyl octadecanoate (C18:0)	32.77	0.24	0.4

0.4 M NaOH, 1.06 M sodium potassium tartrate) was added to each tube and heated for 15 min at 100°C. The heated mixtures were cooled immediately in ice. Then, 9 ml distilled water was added to each tube. The blank was used to zero the spectrophotometer and the absorbance at 540 nm was measured for all the samples.

Results

Bacterial isolation and enzyme screening

All eight organisms tested were found to harbour enzyme producing bacteria. More than 150 species of marine bacteria were isolated and more than 50 species were screened for enzyme production (Figure 1). Pure colonies have been isolated and two species among the fastest growing strains with the highest ability to hydrolyze starch were selected from the green alga *Ulva rigida* (Strain I) and the red sponge *Mycale sp.* (Strain II).

Bacterial strain identification

The two strains showed characteristics of being Gram positive. According to API Coryne test, Strain I was identified from the ApiwebTM identification software as *Cellulosimicrobium cellulans* with 87.3% accuracy whereas Strain II was identified as *Cellulomonas sp.* with 99.9% accuracy. According to partial 16S RNA sequences, the two bacterial strains were identified as follows:

Strain I: *Cellulosimicrobium sp.* with 98% accuracy (Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococccineae; Promicromonosporaceae)

Strain II: *Demequina sp.* with 98% accuracy (Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococccineae; Cellulomonadaceae)

The TEM showed the structure of the two strains under different magnifications (Figure 2). Gas chromatographic analysis showed that the two bacterial strains had quite similar fatty acid compositions except for the absence of three types of bacterial fatty acids in *Demequina sp.* (Table 1). This can be explained by fact that genes *Cellulosimicrobium* and *Demequina* are highly similar. Previously *Demequina* was re-classified from *Cellulomonas* (Yi *et al.*, 2007; Ue *et al.* 2011). This fact supports our API Coryne test data that identified strain II as *Cellulomonas sp.*

Susceptibility to antibiotics

Both of the strains were highly susceptible to Chloramphenicol at concentrations of 1 mg/ml and 10 mg/ml with larger inhibition zones, specifically diameters of 30±2.8 mm and 32.5±3.5 mm for *Cellulosimicrobium sp.* and *Demequina sp.*, respectively, at a concentration of 10 mg/ml. The two bacterial isolates were inhibited by 10 mg/ml Streptomycin whereas they were not affected by the same antibiotic at the lower concentration of 1 mg/ml. No inhibitory effect on the growth of both isolates was observed by Ampicillin and Penicillin G.

The susceptibility of members of the genus *Cellulomonas* to different antibiotics was studied by Funke *et al.* (1995). All the clinical isolates of *Cellulomonas* species were found to be strongly susceptible to Ciprofloxacin, Clindamycin, Erythromycin and Gentamicin. However, they were less susceptible to Penicillin G, Rifampin, Tetracycline and Vancomycin.

It was found that the bacterium *Cellulosimicrobium cellulans* grew well on standard synthetic media and was susceptible to a variety of antibiotics, including Penicillin, Macrolides, and Glycopeptides (Heym, 2005). Yoon (2007) isolated *Cellulosimicrobium terreum* from soil and found it to be susceptible to Streptomycin (50 µg), Chloramphenicol (100 µg) and Penicillin G (20 U). Susceptibility to Ampicillin was observed by three clinical strains of *Cellulosimicrobium funkei* (Brown *et al.*, 2006). All these results are totally different from what we obtained from *Cellulosimicrobium sp.* in our study,

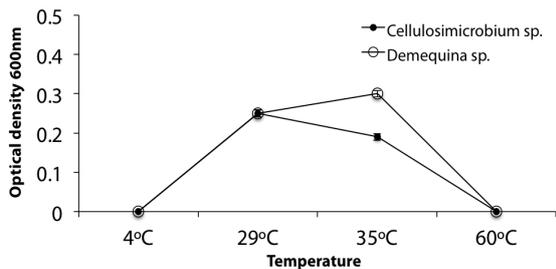


Figure 3. Effect of temperature on cell yield of *Cellulosimicrobium* sp. and *Demequina* sp. (λ 600nm). The data are the means of two replicates \pm standard deviation.

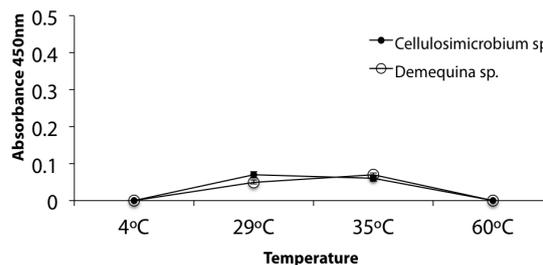


Figure 4. Effect of temperature on amylase production of *Cellulosimicrobium* sp. and *Demequina* sp. (λ 540nm). The data are the means of two replicates \pm standard deviation.

since it was not affected by Ampicillin and Penicillin G and was susceptible to only higher concentrations of Streptomycin. Probably this was due to isolation of our novel strain from different environment (marine). Additionally, different antibiotic susceptibility could be due to antibiotic resistance of some bacterial strains (Brown *et al.*, 2006).

Bacterial yield and amylase production

Effect of temperature

The results showed optimal temperature for cell growth and enzyme production between 29°C and 35°C for *Cellulosimicrobium* sp. and *Demequina* sp., respectively (Fig. 3 & 4). The two strains were inactivated at 4°C and 60°C which indicates the mesophilic characteristics of the two bacterial strains. This property can be related to the natural habitat of the two bacterial strains in the mesophilic sea environment. This is in accordance with what was reported by Asgher *et al.* (2007) and Kathiresan and Manivannan (2006) that the influence of temperature on amylase production is related to the growth of the microorganism.

Effect of salt concentration

The highest bacterial growth and enzyme production for *Cellulosimicrobium* sp. and *Demequina* sp. was obtained

in the presence of 3% NaCl (w/v) (Fig. 5 & 6), which is the normal salinity of sea water. These results might be related to the nature of the bacterial origin since they were isolated from marine environments. There was no amylase production in the absence of NaCl which clearly indicates the halophilic nature of the enzymes of both strains. *Cellulosimicrobium* sp. retained about 9% of its growth ability at 10% salt but both strains lost their enzyme productivity at this salinity. Prakash (2009b) reported that halophilic enzymes require high salt concentrations for their activity due to their adaptation to high salt concentrations while they undergo denaturation at NaCl concentrations below 1M. He also suggested that halophilic bacteria can produce enzymes that can tolerate high salt concentrations 3-15% NaCl.

Effect of carbon and nitrogen sources on bacterial yield and amylase production

The maximum optical density and maltose production by *Cellulosimicrobium* sp. in the presence of lactose was observed (Figure 7). This indicated higher cell growth and amylase production, with this carbon source. This amount of cell yield and amylase production was much higher than what was obtained in the presence of starch, which is known as the general substrate for amylase production. Hiller *et al.* (1997) demonstrated a similar enhanced cell growth and α -amylase production by Ba-

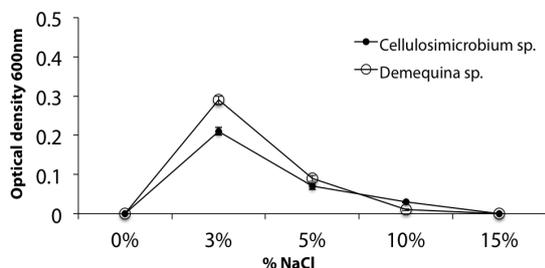


Figure 5. Effect of salt concentration on cell yield of *Cellulosimicrobium* sp. and *Demequina* sp. (λ 600nm). The data are the means of two replicates \pm standard deviation.

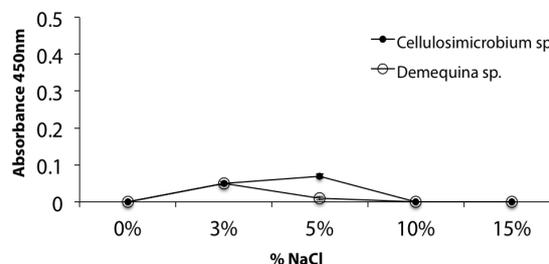


Figure 6. Effect of salt concentration on amylase production of *Cellulosimicrobium* sp. and *Demequina* sp. (λ 540nm). The data are the means of two replicates \pm standard deviation.

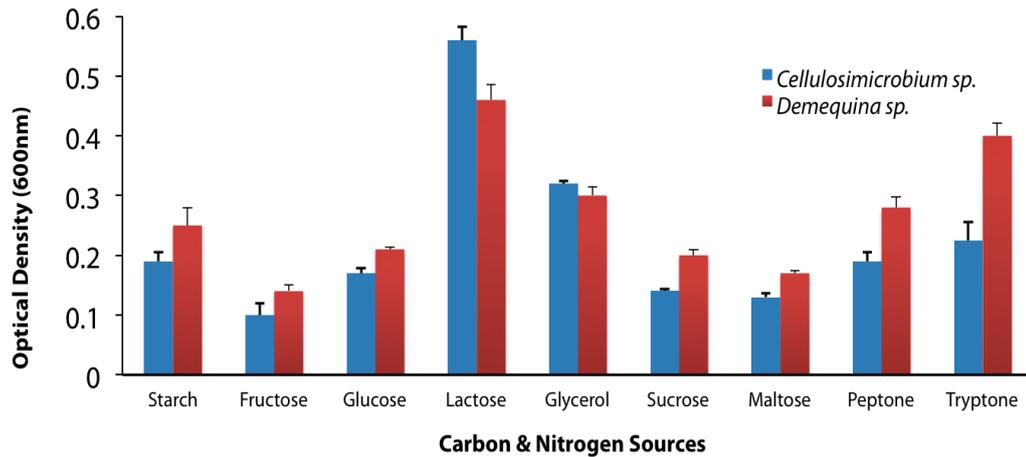


Figure 7. Effect of carbon and nitrogen sources on cell yield of *Cellulosimicrobium sp.* and *Demequina sp.* ($\lambda = 600\text{nm}$). The data are the means of two replicates +1 standard deviation.

cillus amyloliquefaciens from lactose. The lowest bacterial growth and enzyme secretion was observed in the presence of fructose. Glucose was found to yield very minimum amounts of amylases from both of the bacterial strains regardless of its effect on cell growth. Repression of amylase synthesis by glucose was also reported in thermophilic and alkaliphilic *Bacillus sp.* TS-23 (Lin *et al.*, 1998), *Sulfolobus solfataricus* (Haseltine *et al.*, 1996) and *Bacillus sp.* isolate L1711 (Bernhardsdotter *et al.*, 2005). Haseltine *et al.* (1996) justified that glucose prevents gene expression of α -amylase and represses its secretion. Although maximum cell growth of *Demequina sp.* was obtained in the presence of lactose, the highest enzyme production was induced by fructose and glycerol (Figure 8). Glycerol was reported to increase enzyme production in *Bacillus sp.* PS-7 (Tanyildizi, 2005) and *Bacillus sp.* I-3 (Goyal, 2005).

Higher optical density was obtained in the presence

of tryptone which indicates that it enhanced the cell growth of both strains more than peptone (Figure 9 & 10). However, more maltose was produced by the addition of peptone which reflected its higher stimulation of amylase production. The decrease in the amounts of maltose as the end product of starch hydrolysis in the presence of tryptone may be due to the intolerance of the bacterial strains to 0.5% tryptone. Similar results were reported previously (Chakraborty *et al.* 2008a). These authors found a decrease in amylase production by *Saccaropolyspora* species A9 at tryptone concentrations beyond 0.4%.

Conclusions

Two amylases were isolated from the two bacterial strains *Cellulosimicrobium sp.* and *Demequina sp.*, which were correspondingly obtained from the green

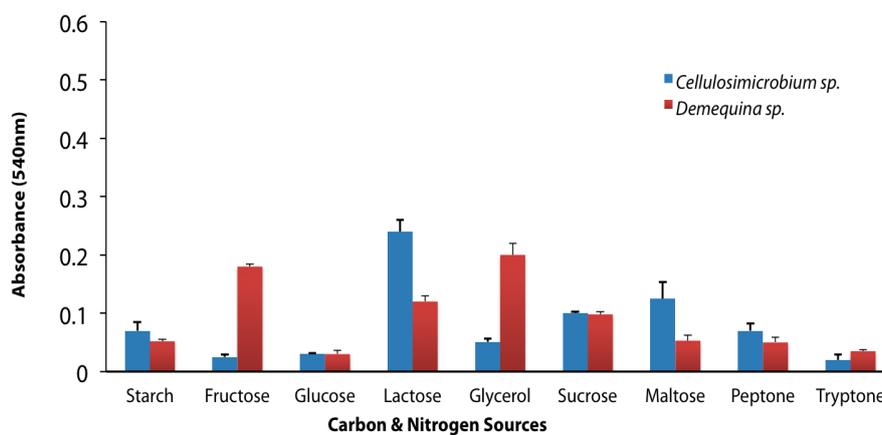


Figure 8. Effect of carbon and nitrogen sources on amylase production of *Cellulosimicrobium sp.* and *Demequina sp.* ($\lambda = 540\text{nm}$). The data are the means of two replicates +1 standard deviation.

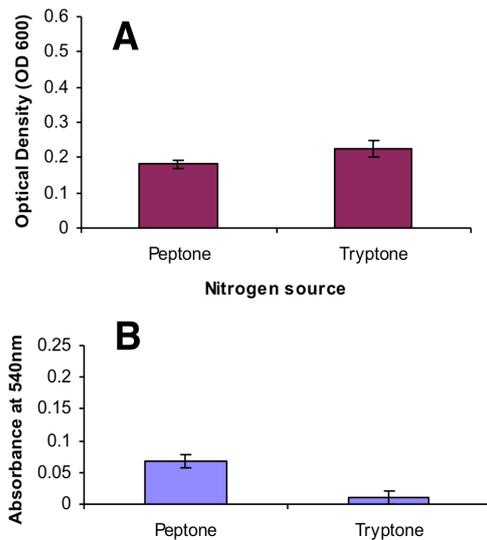


Figure 9. (A) Effect of nitrogen source on cell yield of *Cellulosimicrobium* sp. measured at $\lambda = 600$ nm. (B) Effect of nitrogen source on amylase production by *Cellulosimicrobium* sp. measured at $\lambda = 540$ nm. The data are the means of two replicates \pm standard deviation.

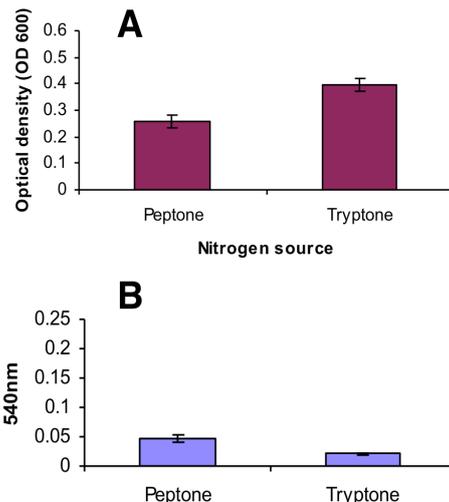


Figure 10. (A) Effect of nitrogen source on cell yield of *Demequina* sp. measured at $\lambda = 600$ nm. (B) Effect of nitrogen source on amylase production by *Demequina* sp. measured at $\lambda = 540$ nm. The data are the means of two replicates \pm standard deviation.

alga *Ulva rigida* and the sponge *Mycale* sp. Maximum cell yield from the two bacterial strains was obtained at temperatures between 29°C and 35°C and 3% salinity, which indicate that the two strains have mesophilic and halophilic properties. Addition of lactose and tryptone significantly increased the growth of both of the strains. Both strains produced highest amount of amylase at temperatures between 29°C and 35°C, 3% salinity and in the presence of peptone. Addition of lactose increased amylase production by *Cellulosimicrobium* sp., whereas supplementation of the media with fructose and glycerol resulted in maximal amylase production by *Demequina* sp. The present study demonstrated that all eight investigated marine organisms are promising sources of amylases producing bacteria. This finding provides the possibility to isolate more enzymes-producing bacteria and search for novel bioactive compounds from the marine environment in Oman.

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