Biocatalysis and Agricultural Biotechnology 6 (2016) 219–226

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Characterization of extracellular agarase production by *Acinetobacter junii* PS12B, isolated from marine sediments

T. Leema Roseline, N.M Sachindra *

Department of Meat and Marine Sciences, CSIR-Central Food Technological Research Institute, Mysore 570020, India

ARTICLE INFO

Article history:
Received 8 February 2016
Received in revised form
21 April 2016
Accepted 23 April 2016
Available online 25 April 2016

Keywords:
Agar
Agarase
Acinetobacter junii
Response surface methodology

ABSTRACT

A marine, gram negative, rod shaped bacterium that degrades agar was isolated from the east coast of India and was identified to be *Acinetobacter junii* PS12B based on 16S rRNA gene sequencing. The effect of different culture conditions, namely pH of the medium, time and temperature of incubation and the agar concentration in the medium, on the agarase production by the strain, was evaluated. The agarase production was significantly ($p < 0.05$) affected by the culture conditions. The optimum conditions as determined by response surface methodology were found to be a temperature of 35 °C, pH of 7.0 and time of fermentation of 33 h and agar concentration of 0.5%. Under the optimum conditions, the isolate produced 0.17 units of agarase per ml of the medium. Ammonium nitrate and sodium nitrate were the best nitrogen source in the medium for agarase production by the isolate. Supplementing the agar containing minimal media with simple sugars like glucose and galactose was found to enhance agarase production significantly by two fold. The isolate was also able to degrade carrageenan, which indicates its role in utilization of marine polysaccharides for the production of bioactive oligosaccharides.

1. Introduction

Most of the marine microbes have evolved degradation enzymes to use agar or agarose as an energy and carbon source (Pluvinage et al., 2013) Marine bacteria secrete a specific glycoside hydrolases (GH) enzyme agarase to utilize algal cell wall polysaccharides as a carbon and energy source. Agarases are classified based on the mode of action, into α-agarase (EC 3.2.1.158), which cleaves α-1, 3 linkages to produce agaro-oligosaccharides and β-agarase (EC 3.2.1.81) which cleaves β-1, 4 linkages to produce neoagaro-oligosaccharides. β-Porphyranases hydrolyze the β-(1, 4) glycosidic bonds of the porphyran moieties (G-L6S) in agar and produces oligosaccharides with reducing ends at G residue (Hehemann et al., 2010). Based on the amino acid sequence similarity, β-agarases are found in four distinct glycoside hydrolases (GH) families in the CAZy database includes GH16, GH50, GH86, and GH118, whereas α-agarase belong to GH96. To date, a vast number of agarolytic micro-organisms from taxonomically diverse genera, as well as agarases and their encoding genes have been well reported and summarized (Chi et al., 2012; Fu et al., 2010; Michel et al., 2006). The approaches to increase the productivity of agarases by agarolytic organisms would be to isolate hyper-producers or mutants for agarases or by cloning the genes encoding for agarases and expression of these genes by molecular genetic techniques.

Agarase producing bacteria has been isolated from different environments. Most of the agarase producing isolates were found to be of marine origin. However, Feng et al. (2012) isolated an agarolytic *Rhodococcus* sp. from printing and dyeing wastewater. Agarolytic bacteria have also been isolated from terrestrial soil (Suzuki et al., 2003; Hosoda et al., 2006; Lakshmikanth et al., 2006b). In recent years, several novel agarase producing strains were isolated from marine environments. Agarolytic *Aliagarivorans marinus and Aliagarivorans taiwanensis* (Jean et al., 2009), *Simiduia agarivorans* (Shieh et al., 2008) were isolated from seawater. *Flammeovirga* sp. MY04 (Han et al., 2012), *Agarivorans* sp. (Hu et al., 2009) isolated from marine sediments were found to produce β–agarase. Seaweeds were found to be one of the primary sources of agarolytic bacteria in the marine environment (Lee et al., 2013; Oh et al., 2010). Agarolytic bacteria have also been isolated from the gut of mollusks (Fu et al., 2008; Jung et al., 2012).

Applications of agarases are well-known in the areas of food, pharmaceuticals, cosmeceuticals, and biotechnology. The neoagarooligosaccharides have been considered to have high economic value, because of their physiological and biological activity without toxicity, as GRAS. These oligosaccharides especially neoagarotetraose and neoagarohexaose exhibit antioxidative activity, scavenging hydroxyl free radicals and superoxide anion radicals and inhibiting lipid peroxidation (Wang et al., 2004; Wu et al., 2005). Additionally, neoagarooligosaccharides acts as a low-calorie

* Corresponding author.
E-mail address: nmsachindra@cftri.res.in (N. Sachindra).

http://dx.doi.org/10.1016/j.bcab.2016.04.007
1878-8181/

additive to improve food quality, inhibit the growth of bacteria, and slow down the degradation of starch (Giordano et al., 2006). They also have a moisturizing effect on skin and a whitening impact on melanoma cells (Kobayashi et al., 1997; Ohta et al., 2004). Because of these functional implications, agar-derived oligosaccharides have broad applications in the health-food, pharmaceutical, and cosmetic industries potentially.

Single-factor optimization can be eliminated by using response surface methodology (RSM) which is used to explain the combined effects of all the factors in a biotechnological process (Anil kumar and Suresh, 2014). Therefore, the use of RSM in biotechnological processes is gaining much importance for the optimization of enzyme production (Beg et al., 2003; Kumar and Satyanarayana, 2004; Rao et al., 2006). Some statistical approaches have been employed for agarase production by *Agarivorans albus* YKW-34 (Fu et al., 2009), *Pseudoalteromonas* sp. JYBCL1 (Jung et al., 2012), and *Streptomyces lividans* (Park et al., 2014).

In the present work, a marine bacterial isolate identified as *Acinetobacter junii* PS12B was evaluated for agarase production under different culture conditions. The strain was characterized by investigating the growth and production of agarase in the presence of various parameters to exploit the organism for production of agarase. Further, the combined effect of different culture conditions was evaluated using RSM for optimization of enzyme production.

2. Materials and methods

2.1. Sampling, isolation, screening of agarolytic marine bacteria

Marine samples such as seaweeds, seawater, and sediment collected from the east coast of India (Rameshwaram, Mandapam and Tuticorin) were suspended in the sterilized minimal mineral salts medium (MMS) (Lakshmikanth et al., 2006b) containing (g/L) K_2HPO_4 (1.2), KH_2PO_4 (0.3), $MgSO_4$ (0.1), $FeCl_3$ (0.1), NH_4NO_3 (1.0), $CaCl_2$ (0.1) and agar (1.0) (pH 7.0). After incubation at 37 °C for 72 h, a loop full of the media was streaked on MMS media plates containing 1.5% agar. Following incubation, the colonies exhibiting an obvious clear zone around the colony or pit formation, indicative of agar degradation were selected as agarolytic bacteria and purified by repeated streaking. Further confirmation of agarolytic activity was carried out by spot inoculation of purified culture on MS agar plates and overlaying with Lugol's iodine after incubation for 24 h at 37 °C. The clear zone around the colony indicates the agarolytic activity.

2.2. Agarase activity measurement

The isolated and purified cultures were inoculated to the enzyme production medium (Lakshmikanth et al., 2006b) containing (g/L) K_2HPO_4 (0.38), $MgSO_4$ (0.20), $FeCl_3$ (0.05), NH_4NO_3 (1.0) supplemented with 0.3% agar as a sole source of carbon. The pH was adjusted to 7.0 before sterilization at 121 °C for 15 min. The culture was incubated at 37 °C on the orbital shaker at 180 rpm. After 24 h of incubation, the production media was centrifuged at 8000 rpm for 15 min at 4 °C and the supernatant collected was taken as a crude extracellular enzyme.

Agarase activity was measured by the release of reducing sugars according to the 3, 5-dinitro salicylic acid (DNS) method (Miller, 1959). Briefly, 1 ml of the crude enzyme solution was mixed with 1 ml of substrate (0.25% agar in 20 mM Tris-Cl buffer, pH 7.5), vortexed and incubated for 60 min at 37 °C. After incubation, 1 ml of DNS solution was added to the mixture and heated in boiling water bath for 10 mins, cooled, and absorbance was measured at 540 nm (Hitachi U 2900). One unit (U) of activity was defined as the amount of enzyme that released 1 μmol of galactose equivalents from the substrate per minute under the specified assay condition. The specific activity was expressed in one μmol galactose equivalents/min/mg protein. Protein concentration was determined by Lowry's method using bovine serum (BSA) as the standard (Lowry et al., 1951). Galactose was used as a reference for preparing the standard curve.

2.3. Random amplification of polymorphic DNA (RAPD) analysis of agarolytic isolates

In order to determine the similarity between the strains, Random Amplification of Polymorphic DNA (RAPD) was performed using a universal M13 primer (5′-GAGGGTGGCGGTTCT-3′) for microbial typing (Schillinger et al., 2003). The amplified product was run on 1.8% agarose gel. The RAPD banding pattern was further analyzed using GeneSys® software (SYNGENE, UK) and the similarity of the band profiles and the grouping of the RAPD-PCR patterns were calculated based on the Pearson's coefficient and agglomerative clustering with unweighted pairs group matching algorithm (UPGMA), and the dendrogram was constructed using GeneSys® software.

2.4. Biochemical and molecular characterization of the selected agarolytic isolate

The strain selected based on screening for the agarolytic activity was characterized by performing various biochemical tests such as gram staining, motility test, oxidase activity, catalase production, Methyl red-Voges Proskauer (MR-VP), oxidation and fermentation of sugars. Utilization of glucose, mannitol, inositol, sucrose and 31 other sugars was assessed using Hi-carbo kit (Hi-Media Laboratories, India). Hydrolysis of esculin, gelatin and starch were also performed using standard techniques. Growth in mineral salts media at different temperatures of 10 °C, 25 °C, 37 °C and 50 °C, at different pH of 4.0, 7.0 and 10.0 were performed to characterize the growth of organisms. Hemolytic activity of the cultures was tested using blood agar. Morphological analysis of the culture isolate was carried out by scanning electron microscopy (SEM) according to the method of McDougall et al. (1994). Briefly, selected strain grown in Tryptone soya broth were centrifuged, washed thrice with phosphate buffer saline (pH 7.0) to remove salts, fixed with glutaraldehyde (2%) and subjected to gradual alcoholic dehydration. The processed samples were then analyzed by SEM (Leo-435 VP, Leo Electron Microscope, Zeiss Ltd., and Cambridge, UK).

For molecular characterization of the agarolytic isolates, genomic DNA was extracted from the bacterial strain using the standard DNA isolation protocol (Sambrook and Russell, 2001). For 16S rRNA gene sequencing and phylogeny analysis, 16S rRNA gene was amplified using the universal bacterial forward and reverse primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (51-GGTTACCTTGTTACGACTT-31) respectively. Each PCR mixture of 25 μl contained template DNA (20 ng/μl), 0.2 μM of each primer, 0.25 mM of each deoxynucleoside triphosphate (dNTP), and 2.5 U of *Taq* DNA polymerase in a final concentration of 10 mM Tris–HCl, 50 mM KCl, and 1.5 mM $MgCl_2$. PCR was performed under the following cycle conditions: an initial denaturation step at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 2 min, and extension at 72 °C for 2 mins, with a final extension step at 72 °C for 10 min (Primus 25 Thermal cycler). The PCR product of was purified and sequenced at Amnion Biosciences, Bangalore, India. The 16S rRNA gene sequences obtained was subjected to nBLAST for similarity identification. Multiple alignment and sequence similarity with that of available sequences of reference strains from GenBank database were analyzed using

CLUSTAL X. The phylogenetic tree was constructed using the MEGA 6 program (Biodesign Institute, Tempe, AZ, USA) by the method of neighbour-joining (Kumar et al., 2008).

2.5. Effects of culture conditions on agarase production by selected isolate

The effect of incubation time (24, 48, 72, 96 and 120 h), temperature (25, 30, 37, and 50 °C), pH (4 to 10) and the initial concentration of agar in the fermentation medium (0.1–0.3% w/v) was evaluated by culturing the selected organism under specific conditions, by varying one parameter and keeping the other conditions constant (24 h, 37 °C, pH 7.0 and 0.3% agar). The growth of the organism was determined by centrifuging 2 ml of culture suspension, suspending the cell pellets in 2 ml physiological saline and measuring the absorbance of the suspension at 600 nm. To check multiple polysaccharide degrading activity of the selected strain, the isolate was inoculated into MS medium containing K-carrageenan, ι-carrageenan, λ-carrageenan, chitosan, alginate, and agar. After 24 h of incubation, the enzyme activity of the culture supernatant was assayed using the same polysaccharide as the substrate.

2.6. Evaluation of combined effect of culture conditions on agarase production

The combined effect of culture conditions was evaluated using three-level factorial design. The independent factors considered for optimization included temperature (X1; °C), time (X2; h), pH (X3) and agar concentration (X4; % w/v), while the enzyme activity was the dependent factor. The detail of factors and the experimental run design is presented in Table 1. The data was analyzed for determining the response profile of different factors, and the response surface plots were drawn using the software STATISTICA (StatSoft, 1999).

Table 1
Experimental design for studying the combined effect of different variables on agarase production.

Run number	Temperature X1 (°C)	Incubation time X2 (h)	pH X3	Agar concentration (% w/v) X4
1	20	6	4	0.1
2	20	6	7	0.5
3	20	6	10	0.3
4	20	24	4	0.5
5	20	24	7	0.3
6	20	24	10	0.1
7	20	42	4	0.3
8	20	42	7	0.1
9	20	42	10	0.5
10	35	6	4	0.5
11	35	6	7	0.3
12	35	6	10	0.1
13	35	24	4	0.3
14	35	24	7	0.1
15	35	24	10	0.5
16	35	42	4	0.1
17	35	42	7	0.5
18	35	42	10	0.3
19	50	6	4	0.3
20	50	6	7	0.1
21	50	6	10	0.5
22	50	24	4	0.1
23	50	24	7	0.5
24	50	24	10	0.3
25	50	42	4	0.5
26	50	42	7	0.3
27	50	42	10	0.1

2.7. Effect of carbon and nitrogen sources on production of agarase

Different inorganic (ammonium chloride, ammonium nitrate, sodium nitrate) and organic (yeast extract, peptone, casein hydrolysate) nitrogen sources were tested at a concentration of 0.1% in the minimal salt medium. Different carbon sources were tested by supplementing the MS medium containing ammonium nitrate (0.1%) and agar (0.3%) with monosaccharide's (glucose, fructose and galactose), disaccharides (ribose, raffinose, sucrose) and cellulose at 0.1% level. Optimized culture conditions were employed during the study.

2.8. Statistical analysis

Statistical analysis was performed for each experiment by analysis of variance (ANOVA) techniques using the statistical software STATISTICA (StatSoft, 1999). All experiments were conducted in triplicate, and the mean values are reported.

3. Results

3.1. Screening, isolation and selection of agarolytic bacteria

From the marine samples, 65 bacterial strains were isolated, and six of the isolates had shown good agarolytic activity as demonstrated by zones of clearing around the colony after flooding with iodine, and were selected for further studies (Fig. 1(a)). The geographic details of selected isolates is given as supplementary data. RAPD fingerprints of the potential agarolytic isolates are shown in Fig. 1(b) with distinct banding patterns. The number of bands varied between 3 and 9, with fragment size ranging between 250 and 2370 bp. According to dendrogram and cluster analysis, isolates were grouped into two major, and each group was subdivided into two sub-groups. The selected cultures (PS12B, PS18, AS, SAR, CS15M, MBW, SW17 groups) with banding patterns as observed by RAPD profiles proves that the agarolytic cultures are diverse (Fig. 1(b)). Six selected isolates were screened for agarase activity by submerged fermentation studies. The agarase production significantly ($p < 0.05$) differed between isolates, with isolate PS12B showing highest specific activity of 1.72 ± 0.23 followed by the isolate SAR 1.52 ± 0.13, while the isolate SW17 (0.45 ± 0.07) showed the lowest activity (Fig. 1(c)). Hence, isolate PS12B was selected for further study.

3.2. Biochemical, physiological profile and phylogenic analysis of the potential agarolytic strain PS12B

As the isolate PS12B showed highest agarase activity, the isolate was further characterized. Colonies produced on minimal mineral salts medium at 37 °C for 2–4 days were circular, off-white, opaque and rod-shaped. The isolate was a gram-negative, catalase-positive and oxidase-negative. The isolate was able to grow at temperatures between 25 and 40 °C, at a pH range of 5–10. The isolate could not hydrolyze esculin, gelatin, and cellulose and was non-hemolytic. Additionally, PS12B strain was able to utilize the sugars, lactose, xylose. maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose, inulin, sodium gluconate, salicin, glucosamine, dulcitol, inositol, sorbitol, mannitol, adonitol and unable to utilize the sugars α-methyl-D-glucoside, rhamnose, cellobiose, melezitose, α-methyl-D-mannoside, xylitol, ONPG, esculin, D-arabinose, citrate, malonate, sorbose. Detailed physiological and biochemical characterization are provided as supplementary data. Phylogenetic analysis (Fig. 2) based on 16S rRNA gene sequences revealed that agarolytic strain PS12B closely matches with *Acinetobacter junii.*, with 99%

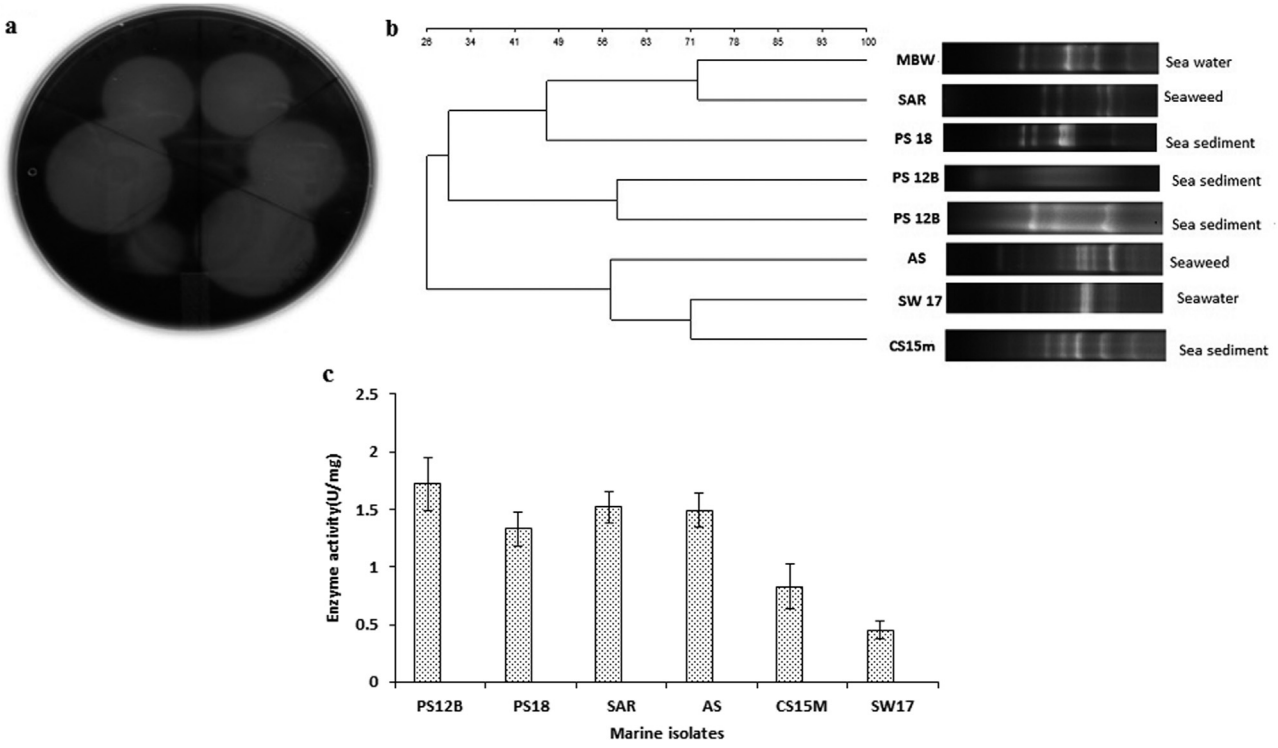

Fig. 1. Screening of agarolytic isolates. a) Detection of agarolytic activity on agar plate, b) Dendogram drawn using RAPD profile of bacterial isolates, c) Agarolytic activity of organism isolated from marine environment (mean ± SD, n=3).

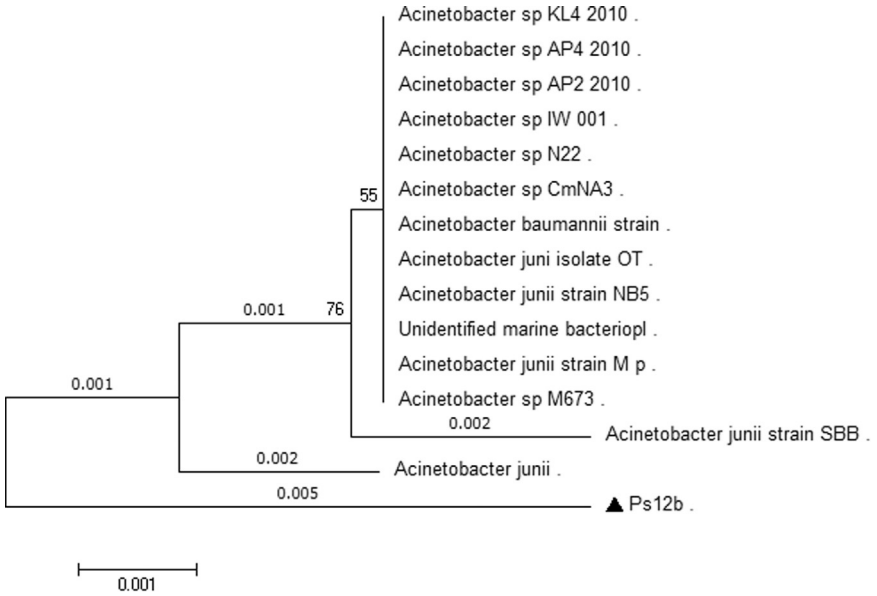

Fig. 2. Phylogenetic tree of *Acinetobacter* sp. PS12B associated with other members of the genus *Acinetobacter* using 16S rRNA sequence.

identity.

3.3. Effects of culture conditions on agarase production

The optimal conditions for agarase production with respect to initial pH of the medium, temperature, concentration of agar and incubation time by the selected isolate *Acinetobacter junii.* PS12B were investigated using one-factor-at-a-time experiments. The pH of the growth medium had a significant ($p < 0.05$) effect on enzyme production, and significantly ($p < 0.05$) higher enzyme production (0.16±0.007 U/ml) was observed at pH 7.0 compared to other pH (Fig. 3(a)). Growth of the organism was also highest pH 7.0, indicating that higher growth at pH 7.0 resulted in higher enzyme production.

It is well known that temperature is one of the most critical parameters that have to be controlled in the bioprocess. The effect of temperature on the production of agarase by *Acinetobacter junii* PS12B was clearly evident as the ideal temperature for increased agarase production was between 30 and 37 °C (Fig. 3(b)). The enzyme production was highest at 30 °C (0.17±0.001 U/ml) followed by at 37 °C (0.14 ± 0.01 U/ml). The enzyme production was significantly ($p < 0.05$) reduced when the growth temperature was 25° or 50 °C. However, the growth was marginally higher at 37 °C compared to 30 °C, but was lowest at 25° and 50 °C. The

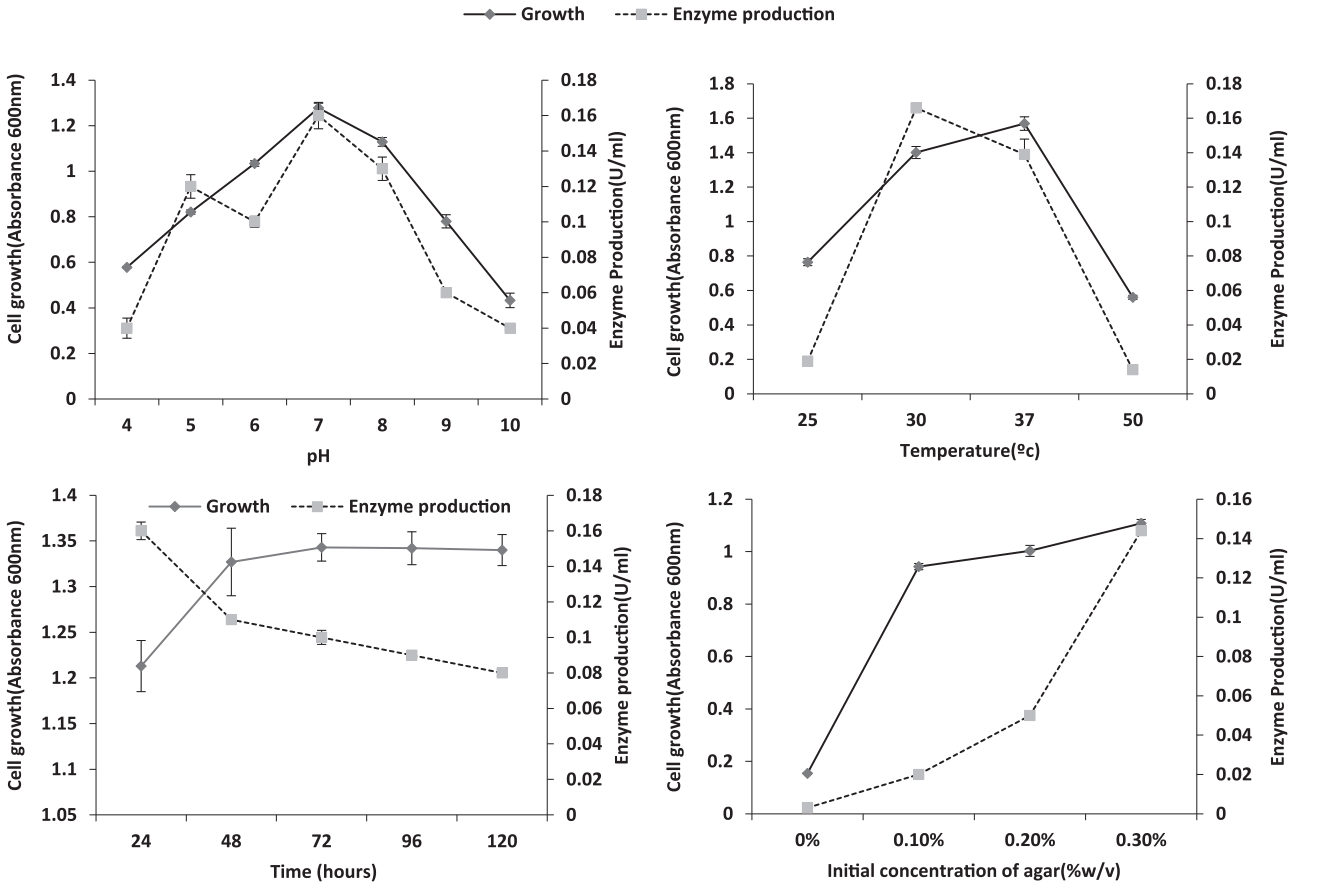

Fig. 3. Effect of a) pH, b) temperature, c) the time course and d) concentration of agar on the growth and production of agarase enzyme (mean ± SD, n=3).

incubation period was found to affect the enzyme production, as increased fermentation period above 24 hrs resulted in significant ($p < 0.05$) reduction enzyme production even though the growth increased after 24 h (Fig. 3(c)). The agarase activity was highest at 0.17 ± 0.005 U/ml after 24 h of the fermentation that reduced when incubated further. The decrease in enzyme production at longer incubation periods even with increased growth may probably be due to the degradation of the enzyme by proteases, as the isolate was also found to be proteolytic (Data not shown).

The initial concentration of agar plays a crucial role in the production of agarase as the strain utilizes only agar as the sole energy source, and agarase enzyme was induced by agar in the fermentation medium. It is evident that the increase in agar concentration significantly ($p < 0.05$) increased the agarase activity reaching higher activity of 0.13±0.002 U/ml at 0.3% agar concentration tested and increased production coincided with increased growth (Fig. 3(d)). However, as the culture medium becomes more viscous when the concentration of agar increased, it would be difficult to recover the crude enzyme.

The activity of the isolate *Acinetobacter junii* PS12B to break down other marine polysaccharides was evaluated by growing the organism in minimal medium containing different polysaccharides and enzyme activity was determined as mentioned Section 2. The isolate showed significantly ($p < 0.05$) higher specific activity (1.90±0.10 U/mg) with respect to agarase (Fig. 4). However, the isolate also exhibited carrageenase activity as it could break down all the three types (kappa, iota, and lambda) of carrageenan. The activity was lowest with respect to alginate lyase and chitosanase activity. The organism also could degrade the agar containing red seaweed as it showed enzyme activity of 0.37±0.12 U/mg when red seaweed was used as carbon source in the growth medium and as the substrate in the enzyme assay.

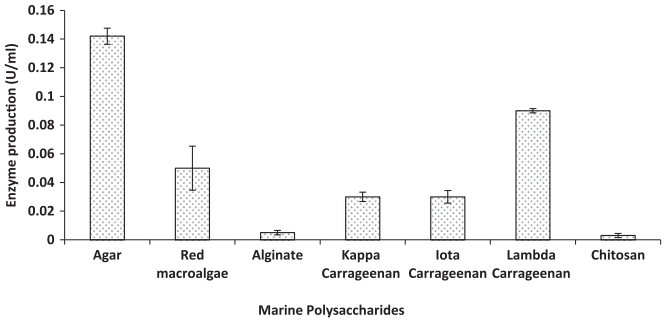

Fig. 4. Multiple polysaccharide degrading activity of *Acinetobacter junii.* PS12B (mean ± SD, n=3).

3.4. Combined effect of growth conditions on agarase production

One factor at a time experiment indicated that maximum agarase production was affected by pH of the medium, time and temperature of incubation and agar concentration of the medium. Hence, this study was carried out to evaluate the combined effect of these variables on agarase production by *Acinetobacter junii* PS12B using response surface methodology to arrive at the optimized condition for maximum agarase production. Among the variables screened, pH of the medium was identified as the most significant ($p \leq 0.05$) variables affecting the production of agarase. The interaction of four variables was determined by constructing three-dimensional surface plots (Fig. 5(a)–(d) which compare the effect of two variables on agarase production, keeping the other two variables at the center of the level. In Fig. 5(a), agarase production was plotted against concentration of agar and pH, which indicated that enzyme production increased with increase in pH

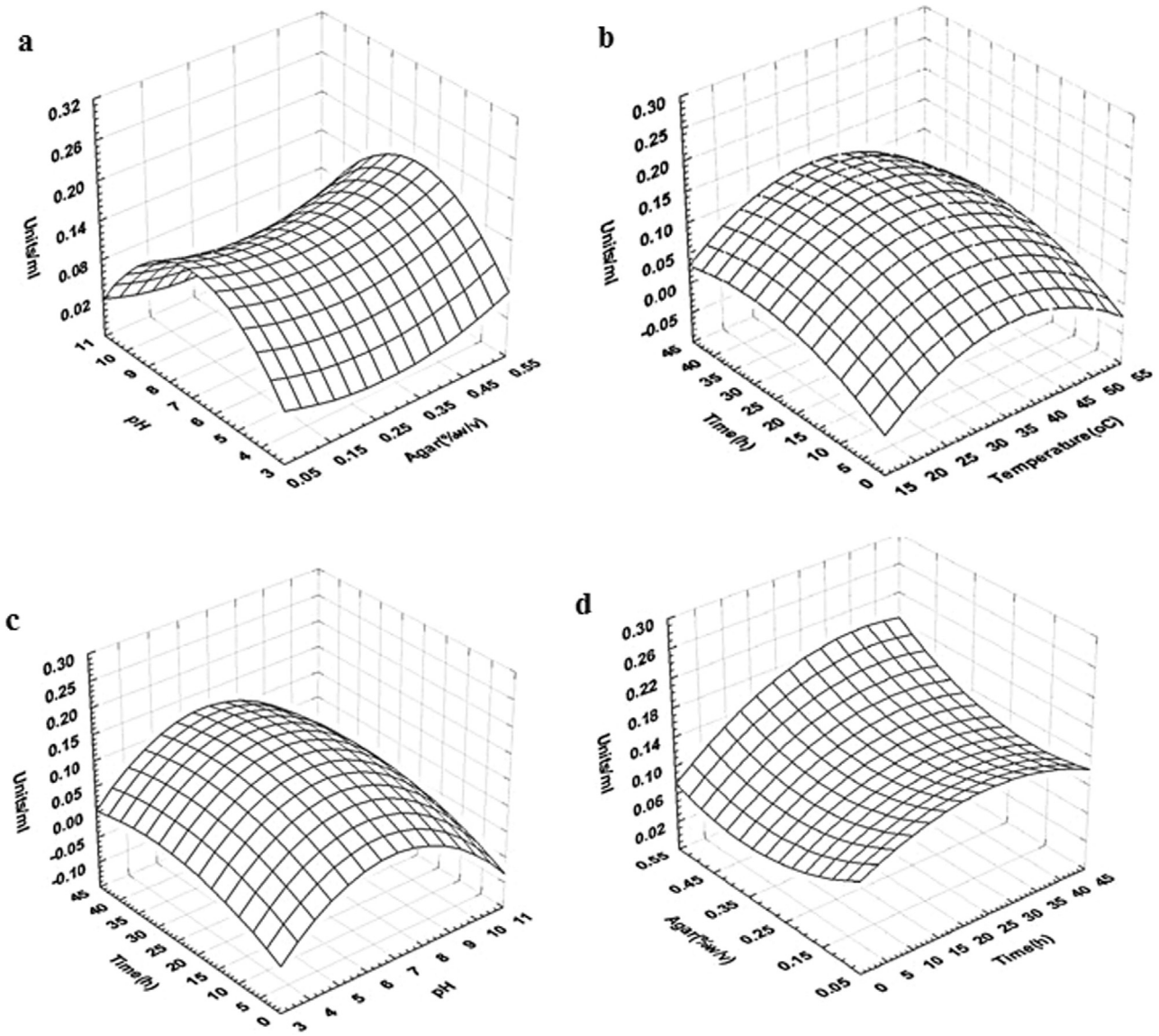

Fig. 5. Response surfaces plots of effect of a) agar concentration and pH, b) temperature and time, c) pH and time and d) time and agar concentration on agarase production (other variables kept at the center level).

upto 7.0 and then reduced, while increase in agar concentration resulted in a marginal increase in enzyme production. The rise in temperature also increases enzyme production, but at high-temperature agarase production reduces. Time also showed a minimal effect on agarase production. The optimum levels of the four independent variables determined from the response profile (provided as supplementary data) for maximum agarase production of 0.17 units, was found to be 0.5% (w/v) of agar, pH of 7.0 and incubation at 35 °C for 33 h. Optimized conditions were found to very close to the conditions obtained during one factor at a time experiment.

3.5. *Influence of nitrogen and carbon sources on agarase production*

As optimization experiment did not yield higher enzyme production, in order to increase the enzyme production, the effect of addition of different carbon sources and nitrogen sources to the minimal medium under optimal conditions on enzyme production was evaluated. The evaluation of effect of substituting ammonium nitrate with other nitrogen sources on agarase production indicated that the significantly higher agarase production was obtained when ammonium nitrate (0.14+0.006 U/ml) or sodium nitrate (0.13+0.006 U/ml) was used as nitrogen source in the medium (Fig. 6(a)). Substitution of ammonium nitrate with other nitrogen sources (yeast extract peptone or casein) decreased the agarase production significantly ($p < 0.05$), indicating that *Acinetobacter junii* PS12 B is not able to efficiently utilize complex nitrogen source in the medium for agarase production.

Several reports have indicated that different carbon sources have different influences on extracellular enzyme production by various strains. Therefore, the effect of agar and agar with different sugars like glucose, galactose, sucrose, agarose, ribose, raffinose and cellulose on agarase production by *Acinetobacter junii* PS12B was examined. Combinations of agar with monosaccharide's, glucose, and galactose, significantly $p < 0.05$) increased agarase production by more than 2 fold (Fig. 6(b)). Cellulose had an adverse effect on agarase production.

4. Discussion

Numerous species of bacterial genera have been reported to be producers of agarase. Most of the reports on agarolytic bacteria are

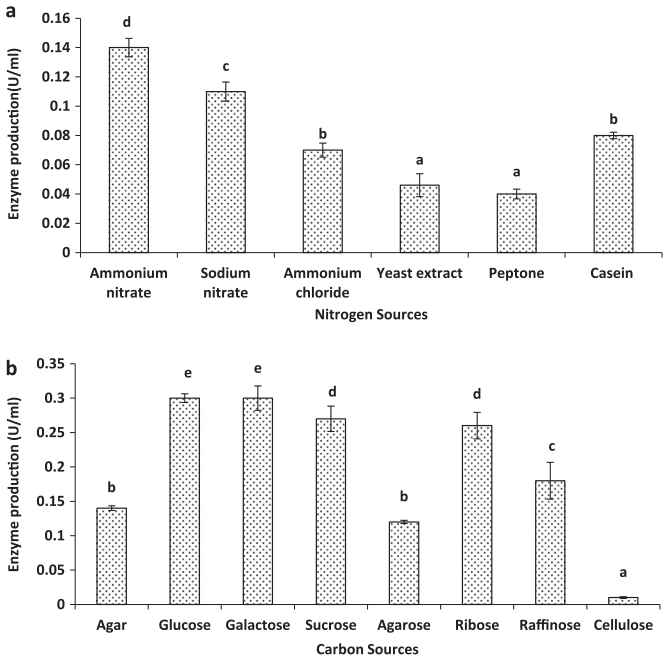

Fig. 6. Effects of different nitrogen sources (a) and carbon sources (b) on agarase production by *Acinetobacter junii* PS12b (mean ± SD, n=3).

from those isolated from the marine environment (Chi et al., 2012; Fu et al., 2010). Several marine bacteria are known to be agarolytic, examples being *Pseudoalteromonas* sp. (Jung et al., 2012), *Bacillus cereus* ASK202 (Kim et al., 1999), *Janthinobacterium* sp. SY12 (Shi et al., 2008), *Micrococcus* sp. GNUM-08124 (Choi et al., 2011), *Catenovulum* sp. X3 (Xie et al., 2013) *Agarivorans* sp. LQ48 (Long et al., 2010), *Bacillus megaterium* (Khambhaty et al., 2008).

Based on phylogenetic analysis, the strain PS12B found to be *Acinetobacter junii. Acinetobacter* species are widely distributed in nature and the hospital environment. Tsai et al. (2012) showed that *A. junii* is a bacterium of low virulence and low mortality rates. However, only certain *Acinetobacter* species are pathogenic to humans, causing disease mainly in neonates and immunocompromised individuals. The genus *Acinetobacter* comprises of more than 23 known species and 11 unknown species, which are commonly gram-negative, rod-shaped, non-pigmented, heterotropic, strictly aerobic, polar-flagellated bacteria. There are several species of *Acinetobacter* from marine environment are known to degrade oil, edible and mineral oils (Tanaka et al., 2010; Luo et al., 2013), and produce enzymes (Fu et al., 2014). *Acientobacter junii* PS12B isolated in the study was found to be a good candidate for agarase production. Comparing with *Acinetobacter* sp. reported earlier for agarase isolated from soil environment, the bacterium from the present study could thrive under different conditions as the organism was isolated from marine environment and could degrade marine polysaccharide such as carrageenan. Only one species of *Acinetobacter* has been reported as agarase producer from the terrestrial environment (Lakshmikanth et al., 2006a). This is the first report on *Acinetobacter* sp. from marine environment producing agarase enzyme.

Culture conditions are known to influence the enzyme production by microorganisms. According to Hu et al. (2009) the *Agarivorans* sp. HZ105 isolated from marine sediment could grow at a wide pH range of 6–11. The isolate in this study, *Acinetobacter junii* PS12B could grow at wide pH range from 5 to 9, indicating that the strain is alkali tolerant in nature and was found to produce higher amount of agarase at pH 7.0. Lakshmikanth et al. (2006b) reported that *Acinetobacter* sp. isolated from terrestrial soil also produces a higher amount of agarase at pH 7.0. In case *Pseudomonas aeruginosa*, maximal agarase production was observed at pH 8.0 even though growth could occur at a pH range of 5–11 (Lakshmikanth et al., 2006a). *Bacillus megaterium* isolated from marine environment exhibited higher agarase production at pH 6.6 (Khambhaty et al., 2008).

Pseudomonas aeruginosa (Lakshmikanth et al., 2006a) and *Rhodococcus* sp. (Feng et al., 2012) were found to have maximum agarase production at a growth temperature of 30 °C. The optimal temperature for production of agarase by marine microorganisms was reported to be in the range of 20–40 °C (Lakshmikanth et al., 2006a, 2006b; Fu et al., 2009; Khambhaty et al., 2008; Van der Meulen and Harder, 1975), except for thermophilic organisms such as *Halococcus* sp. 197A with an optimum temperature of 70 °C (Minegishi et al., 2013). *Acinetobacter* sp. isolated from terrestrial soil also produced agarase within a short period of time of 16–18 h (Lakshmikanth et al., 2006b). Similar results were obtained by strain *Agarivorans* sp. HZ 105 (Hu et al., 2009) where an increase in agar concentration from 0.1–0.25% induced higher production of agarase. In contrast Khambhaty et al. (2008) observed that low agar concentration of 0.03% is sufficient for induction of agarase production. According to Jonnadula and Ghadi (2011), *Microbulbifer* sp. CMC-5 could degrade multiple marine polysaccharides. However, *Acinetobacter* sp., AG LSL-1 isolated from terrestrial soil was not able to utilize other polysaccharides (Lakshmikanth et al., 2006b). Similarly, agarolytic marine isolate *Vibrio* sp. Strain JT0107 was also not able to degrade other polysaccharides (Sugano et al., 1993). Lakshmikanth et al. (2006b) reported that agarase secreted by the *Acinetobacter* strain was inducible by agar and are not repressed by other simple sugars when supplemented along with agar in the medium.

Currently, there is an immense interest in the scientific community around the world in exploiting novel micro-organisms. Marine microorganisms, with their distinctive nature, differ much in many aspects from their terrestrial counterparts and are well-known to produce diverse spectra of novel bioactive substances. In the present study a potential agarolytic strain identified as *Acinetobacter junii* PS12b. was isolated and evaluated for agarase production under different conditions. The enzyme production was influenced by the pH of the growth medium, time, and temperature of incubation and agar concentration in the medium. Under optimized conditions of pH (7.0), incubation time (33 h), temperature (35 °C) and agar concentration (0.5%) the isolate produced 0.17 units of agarase per ml of the growth medium. Ammonium nitrate or sodium nitrate was found to the best nitrogen source for enzyme production. Supplementation of minimal medium containing agar with simple sugars enhanced the enzyme production by 2 folds. The isolate was also exhibited activity towards degradation of other marine polysaccharides like carrageenan, indicating its potential for exploitation of marine polysaccharides for the production of the bioactive oligosaccharides.

Acknowledgements

The authors wish to thank the Director, CSIR-CFTRI for encouragement and for the facilities provided. First author thanks, UGC, Govt. of India (Grant No. 1269/NET-June 2011) for the support in the form of fellowship.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bcab.2016.04.007.

References

Anil Kumar, P.K., Suresh, P.V., 2014. Biodegradation of shrimp biowaste by marine *Exiguobacterium* sp. CFR26M and concomitant production of extracellular protease and antioxidant materials: production and process optimization by response surface methodology. Mar. Biotechnol. 16, 202–218.

Beg, Q.K., Vikram, S., Gupta, R., 2003. Statistical media optimization and alkaline protease production from Bacillus mojavensis in a bioreactor. Process Biochem. 39, 203–209.

Chi, W.J., Chang, Y.K., Hong, S.K., 2012. Agar degradation by microorganisms and agar-degrading enzymes. Appl. Microbiol. Biotechnol. 94, 917–930.

Choi, H.J., Hong, J.B., Park, J.J., Chi, W.J., Kim, M.C., Chang, Y.K., Hong, S.K., 2011. Production of agarase from a novel *Micrococcus* sp. GNUM-08124 strain isolated from the East Sea of Korea. Biotechnol. Bioprocess Eng. 16, 81–88.

Feng, Z., Peng, L., Chen, M., Li, M., 2012. *Rhodococcus* sp. Q5, a novel agarolytic bacterium isolated from printing and dyeing wastewater. Folia Microbiol. 57, 379–386.

Fu, X.,T., Lin, H., Kim, S.M., 2009. Optimization of medium composition and culture conditions for agarase production by *Agarivorans albus* YKW-34. Process Biochem. 44, 1158–1163.

Fu, X.T., Kim, S.M., 2010. Agarase: review of major sources, categories, purification method, enzyme characteristics and applications. Mar. Drugs 8, 200–218.

Fu, X.T., Lin, H., Kim, S.M., 2008. Purification and characterization of a novel beta-agarase, AgaA34, from *Agarivorans albus* YKW-34. Appl. Microbiol. Biotechnol. 78, 265–273.

Fu, X., Wang, W., Hao, J., Zhu, X., Sun, M., 2014. Purification and characterization of catalase from marine bacterium *Acinetobacter* sp. YS0810. Biomed. Res. Int., org/10.1155/2014/409626

Giordano, A., Andreotti, G., Tramice, A., Trincones, A., 2006. Marine glycosyl hydrolases in the hydrolysis and synthesis of oligosaccharides. Biotechnol. J. 1, 511–530.

Han, W., Gu, J., Yan, Q., Li, J., Wu, Z., Gu, Q., Li, Y.A., 2012. Polysaccharide-degrading marine bacterium *Flammeovirga* SP. MY04 and its extracellular agarase system. J. Ocean Univ. China 11, 375–382.

Hehemann, J.H., Michel, G., Barbeyron, T., Czjzek, M., 2010. Expression, purification and preliminary X-ray diffraction analysis of the catalytic module of a beta-agarase from the *Flavobacterium zobellia galactanivorans*. Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Commun. 66, 413–417.

Hosoda, A., Sakai, M., 2006. Isolation of *Asticcacaulis* sp. SA7, a novel agar-degrading Alpha proteobacterium. Biosci. Biotechnol. Biochem. 70, 722–725.

Hu, Z., Lin, B.K., Xu, Y., Zhong, M.Q., Liu, G.M., 2009. Production and purification of agarase from a marine agarolytic bacterium *Agarivorans* sp. HZ105. J. Appl. Microbiol. 106, 181–190.

Jean, W.D., Huang, S.P., Liu, T.Y., Chen, J.S., Yang, W., Shieh, W.Y., 2009. *Aliagarivorans marinus* gen. nov., sp. nov. and *Aliagarivorans taiwanensis* sp. nov., facultatively anaerobic marine bacteria capable of agar degradation. Int. J. Syst. Evol. Microbiol. 59, 1880–1887.

Jonnadula, R., Ghadi, C.S., 2011. Purification and characterization of β agarase from seaweed decomposing bacterium *Microbulbifer* sp. Strain CMC-5. Biotechnol. Bioprocess Eng. 16, 513–519.

Jung, C., Kwon, H., Park, C., Lee, J., 2012. Optimization of *Pseudoalteromonas* sp. JYBCL 1 culture conditions, medium composition and extracellular *β*-agarase activity. Biotechnol. Bioprocess Eng. 17, 937–945.

Khambhaty, Y., Modi, K., Jha, B., 2008. Purification, characterization and application of a novel extracellular agarase from a marine *Bacillus megaterium*. Biotechnol. Bioprocess Eng. 13, 584–591.

Kim, B.J., Kim, H.J., Ha, S.D., Hwang, S.H., Byun, D.S., Lee, T.H., Kong, J.Y., 1999. Purification and characterization of β-agarase from marine bacterium *Bacillus cereus* ASK202. Biotechnol. Lett. 21, 1011–1015.

Kobayashi, R., Takimasa, M., Suzuki, T., Kirimura, K., Usami, S., 1997. Neoagarobiose as a novel moisturizer with whitening effect. Biosci. Biotechnol. Biochem. 61, 162–163.

Kumar, S., Satyanarayana, T., 2004. Statistical optimization of a thermostable and natural glucoamylase production by thermophilic mold Thermomucor indicae-seudaticae in solid state fermentation. World J. Microbiol. Biotechnol. 20, 895–902.

Kumar, S., Nei, M., Dudley, J., Tamura, K., 2008. MEGA: biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief. Bioinform. 9, 299–306.

Lakshmikanth, M., Manohar, S., Souche, Y., 2006b. Extracellular β-agarase LSL-1 producing neoagarobiose from a newly isolated agar-liquefying soil bacterium, *Acinetobacter* sp., AG LSL-1. World J. Microbiol. Biotechnol. 22, 1087–1094.

Lakshmikanth, M., Manohar, S., Patnakar, J., Vaishampayan, P., Shouche, Y., Lalitha, J., 2006a. Optimization of culture conditions for the production of extracellular agarases from newly isolated *Pseudomonas aeruginosa* AG LSL-11. World J. Microbiol. Biotechnol. 22, 531–537.

Lee, Y., Oh, C., De Zoysa, M., Kim, H., Wickramaarachchi, W.D., Whang, I., Kang, D.H., Lee, J., 2013. Molecular cloning, overexpression, and enzymatic characterization of glycosyl hydrolase family 16 β-Agarase from marine bacterium *Saccharophagus* sp. AG21 in *Escherichia coli*. J. Microbiol. Biotechnol. 23, 913–922.

Long, M., Ziniu, Y., Xun, X., 2010. A novel β-agarase with high pH stability from marine *Agarivorans* sp. LQ48. Mar. Biotechnol. 12, 62–69.

Lowry, O.H., Rosebrough, A.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–273.

Luo, Q., Zhang, G.J., Shen, X.R., Fan, Q.Z., He, Y., Hou, D.Y., 2013. Isolation and characterization of marine diesel oil-degrading *Acinetobacter* sp. strain Y2. Ann. Microbiol. 63, 633–640.

McDougall, L.A., Holzapfel, W.H., Schillinger, U., Feely, D.E., Rupnow, J.H., 1994. Scanning electron microscopy of target cells and molecular weight determination for bacteriocin produced by *Lactococcus lactis* D53. Int. J. Food Microbiol. 24, 295–308.

Michel, G., Nyval, P., Barbeyron, T., Czjzek, M., Helbert, W., 2006. Bioconversion of red seaweed galactans: A focus on bacterial agarases and carrageenases. Appl. Microbiol. Biotechnol. 71, 23–33.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.

Minegishi, H., Shimane, Y., Echigo, A., Ohta, Y., Hatada, Y., Kamekura, M., Maruyama, T., Usami, R., 2013. Thermophilic and halophilic β-agarase from a halophilic archaeon *Halococcus* sp. 197A. Extremophiles 17, 931–939.

Oh, C., Nikapitiya, C., Lee, Y., Whang, I., Kang, D.H., Heo, S.J., 2010. Molecular cloning, characterization and enzymatic properties of a novel beta agarase from a marine isolate *Pseudoalteromonas* sp. AG52. Braz. J. Microbiol. 41, 876–889.

Ohta, Y., Hatada, Y., Nogi, Z., Li, M., Akita, Y., Hidaka, Y., Goda, S., Ito, S., Horikoshi, K., 2004. Enzymatic properties and nucleotide and amino acid sequences of a thermostable β-agarase from a novel species of deep-sea *Microbulbifer*. Appl. Microbiol. Biotechnol. 64, 504–514.

Park, J., Hong, S.K., Chang, Y.K., 2014. Production of DagA, a β-Agarase, by *Streptomyces lividans* in glucose medium or mixed-sugar medium simulating microalgae hydrolysate. J. Microbiol. Biotechnol. 24, 1622–1628.

Pluvinage, B., Hehemann, J.H., Boraston, A.B., 2013. Substrate recognition and hydrolysis by a family 50 exo-β-agarase, Aga50D, from the marine bacterium *Saccharophagus degradans*. J. Biol. Chem. 288, 28078–28088.

Rao, Y.K., Lu, S., Liu, B., Tzeng, Y., 2006. Enhanced production of an extracellular protease from *Beauveria bassiana* by optimization of cultivation. Biochem. Eng. J. 28, 57–66.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press,, New York.

Schillinger, U., Yousif, N.M.K., Sesar, L., Franz, C.M.A.P., 2003. Use of group-specific and RAPD-PCR analyses for 400 rapid differentiations of *Lactobacillus* strains from probiotic yogurts. Curr. Microbiol. 47, 453–456.

Shi, Y.L., Lu, X.Z., Yu, W.G., 2008. A new β-agarase from marine bacterium *Janthinobacterium* sp. SY12. World J. Microbiol. Biotechnol. 24, 2659–2664.

Shieh, W.Y., Liu, T.Y., Lin, S.Y., Jean, W.D., Chen, J.S., 2008. *Simiduia agarivorans* gen. nov., sp. nov., a marine agarolytic bacterium isolated from shallow coastal water from Keelung, Taiwan. Int. J. Syst. Evol. Microbiol. 58, 895–900.

StatSoft., 1999. STATISTICA for windows. StatSoft, Inc., Tulsa.

Sugano, Y., Terada, I., Arita, M., Yamazaki, Y., Noma, M., 1993. Purification and characterization of a new agarase from a marine bacterium, *Vibrio* sp. Strain JT0107. Appl. Environ. Microbiol. 59, 1549–1554.

Suzuki, H., Sawai, Y., Suzuki, T., Kawai, K., 2003. Purification and characterization of an extracellular β-agarase from *Bacillus* sp. MK03. J. Biosci. Bioeng. 95, 328–334.

Tanaka, D., Takashima, M., Mizuta, A., Tanaka, S., Sakatoku, A., Nishikawa, A., Osawa, T., Noguchi, M., Aizawa, S., Nakamura, S., 2010. *Acinetobacter* sp. Ud-4 efficiently degrades both edible and mineral oils: isolation and characterization. Curr. Microbiol. 60, 203–209.

Tsai, H.Y., Cheng, A., Liu, C.Y., Huang, Y.T., Lee, Y.C., Liao, C.H., Hsueh, P.R., 2012. Bacteremia caused by *Acinetobacter junii* at a medical center in Taiwan, 2000–2010. Eur. J. Clin. Microbiol. Infect. Dis. 31, 2737–2743.

Van der Meulen, H.J., Harder, W., 1975. Production and characterization of the agarase of *Cytoplaga flevensis*. Antonie Van Leeuwenhoek 41, 431–447.

Wang, J., Jiang, X., Mou, H., Guan, H., 2004. Anti-oxidation of agar oligosaccharides produced by agarase from a marine bacterium. J. Appl. Phycol. 16, 333–340.

Wu, S.C., Wen, T.N., Pan, C.L., 2005. Algal-oligosaccharide-lysates prepared by two bacterial agarases stepwise hydrolyzed and their anti-oxidative properties. Fish. Sci. 71, 1149–1159.

Xie, W., Lin, B., Zhou, Z., Lu, G., Lun, J., Xia, C., Li, S., Hu, Z., 2013. Characterization of a novel β-agarase from an agar-degrading bacterium *Catenovulum* sp. X3. Appl. Microbiol. Biotechnol. 97, 4907–4915.