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Screening and Environmental Factors Effecting on Growth of Heparinase-Producing Bacteria

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Abstract

Heparinases are heparin-degrading enzymes that eliminatively cleave certain sequences of heparin/ heparan sulfate specifically. Heparinases offer therapeutic and diagnostic applications such as heparin detection and affect reversal. In addition, they have been employed in preparation of Low-Molecular-Weight heparin (LMWH) possessing high efficiency than unfractionated heparin (UFH). Novel heparinase-producing microorganisms can lead to an elevated efficiency and effective production including innovative application. In this research, four (RYA En1, RYB En1, RYA HT1 and RYA HT1) and two (SKA En1 and SKA HT1) heparinase-producing bacteria were isolated from brackish sediment samples located at Krached canal in Rayong province and from marine sediment samples located at Don Hoy Lod in Samut Songkhram province, respectively. All heparinase-producing isolates are gram-negative, non-spore forming rod shape bacteria, positive for catalase test, negative for indole production and gelatin hydrolysis tests, and gave positive result for preliminary simple heparinase-producing detection via metachromatic reaction using Toluidine method. The isolated bacteria were capable of degrading more than 90% (w/w) of heparin containing in the culture medium at 24 hours of cultivation with 3.75 U L^{-1} h^{-1} of heparinase. Furthermore, these isolates were examined for intracellular constitutive heparinase that released from the periplasmic space by Azure A assay and it was found that the highest heparinase specific activity of RYA En1 at 82 U mg^{-1} was achieved. According to the cultivation conditions, optimal temperatures for growth of these isolates were 25-30 °C except RYA HT1 and SKA En1 that were able to grow at 40 °C. Corresponding to the sediment sources, RYA HT1, RYA En1 and RYB En1 were able to grow in media containing 0-5% (w/v) NaCl whereas SKA HT1, SKA EN1 and RYA HT1 were capable to grow at 10% (w/v) NaCl. The optimal pH for growth of most isolates ranged from 5-10.

Keywords: heparinase, heparin lyase, glycosaminoglycans

1. Introduction

Heparin, also known as unfractionated heparin (UFHs), has been clinically employed in the prevention and treatment of thromboembolic since 1935 (1). The structure of major heparin is highly sulfated linear polysaccharide comprised of 1→4 linked repeating sequences of the trisulfated disaccharide [(α-L-IdoA(2S))-(1→4)-(β-D-GlcNS(6S))] (2). β-D-glucuronic acid, N-acetyl-glucosamine and disaccharides repeating units with different degree of sulfation founded in heparin are minor constituents and existing at varying amount depending on the heparin origin. Low molecular weight heparin (LMWH) which is efficient anticoagulant compared to unfractionated heparin (UFH) is obtained by controlled chemical or enzymatic depolymerization of heparin. Heparin is widely used as blood anticoagulant normalizing prothrombin and thromboplastin times (3). It also possesses antilipaemic, antiheamostatic and antithrombotic activities (4). Heparin binding to antithrombin III (AT-III) subsequently increases the rate of AT-III inactivating proteolytic enzymes of the coagulation cascade. Recent studies revealed a distinctive pentasaccharide sequence which is present in approximately one-third of heparin polymer chains, contributes the anticoagulant activity (5, 6). Besides thromboembolic prevention and treatment applications, heparin derivatives demonstrated numerous potential novel therapeutic applications such as anti-inflammatory, anti-viral, antiatherosclerotic, antiproliferation, antitumor and antiangiogenesis (7). The biological activities contributed by unique oligosaccharide sequences of heparin have gained an interest for identification and modification of heparin or heparin analogous structures. In addition, detection heparin contaminants by analysis of heparin structure for confirmation of the purity has been required as a consequence of the adverse clinical event causing angioedema, hypotension and multiple deaths (8).

Heparin lyases or heparinases cleave the glycosidic linkage between hexosamines and uronic acids of heparin and heparin sulfate (HS) via β-elimination. According to the specificity of heparin lyases action on heparin or HS, heparinases can be classified into three groups (heparinase I; Hep I, heparinase II; Hep II and heparinase III; Hep III) as shown in Figure 1. Heparinase catalyzing depolymerization of heparin results in double bond of the uronic acid at the nonreducing terminus. Among those heparinases, Hep I (EC 4.2.2.7) is acting primarily at the →4)-α-D-GlcNS (6S or OH)(1→4)-α-L-IdoA2S(1→ linkages present in heparin. It is widely used for neutralizing heparin concentration in human blood and analyzing heparin including heparin-like glycosaminoglycans. Hep I was also employed for an improved preparation of LMWHs owing mild reaction condition which is advancing over conventional chemical degradation. Hep II (or heparitinase II) is acting at the →4)-α-D-GlcNS (6S or OH)(1→4)-α-L-IdoA(2S or OH) or -β-D-GlcA(1→ linkages present in both heparin and heparin sulfate (HS). Hep III (or heparitinase, EC 4.2.2.8) is acting on the →4)-α-D-GlcNS (Ac)(1→4)-β-D-GlcA(or IdoA(1→ linkages which is specifically found in heparan sulfate (9).

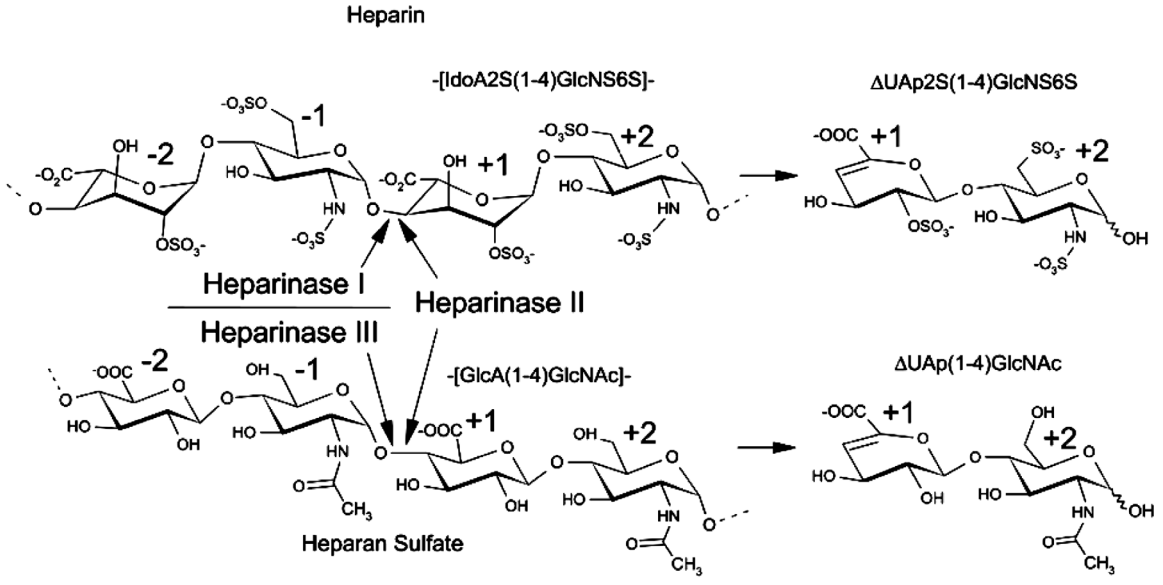

Figure 1. Schematic degradation of heparin and heparin sulfate by heparinases I–III (14).

A number of bacterial heparinases have been discovered, purified and characterized from several species such as *Pedobacter heparinus* (commercial heparinases species), *Sphingobacterium*, *Bacillus circulans* and *Bacteroides* (7, 9-13). In addition, influencing factors effecting on heparinase production had been paid interests (15-17). The objective of this research was to obtain heparinase-producing bacteria isolated from brackish and marine sediment samples. Furthermore, influence of environmental factors such as pH, temperature and sodium chloride concentration of cultivation conditions on cell growth were investigated.

2. Materials and methods

2.1 Isolation of heparin degrading bacteria

Brackish and marine soil sediment samples were collected from Krached canal in Rayong province and Don Hoy Lod located Samut Songkhram province in Thailand, respectively. Three samples were obtained from different areas of the sampling location. The samples were kept at 4°C during transportation to laboratory. Three soil sediment samples from Rayong province were referred as RYA, RYB and RYC whereas the samples from Samut Songkhram province were coded as SKA, SKB and SKC. One gram of soil sediment sample was added to centrifuge tube containing 9 ml of sterile distilled water. After well mixing, the mixture was centrifuged at 4000xg for 10 minutes. Thereafter, 10% (v/v) of soil suspension was transferred to 250 ml Erlenmeyer flasks containing enrichment (En) and Heparin (HT) media (18). En media consisted of 20 g Tryptone and 5 g NaCl in 1 L of 0.1 M phosphate buffer pH 7.0. For HT media contained 1 g $(NH_4)_2SO_4$ and 0.28 mg heparin sodium salt from porcine intestinal mucosa (Grade I-A, 180 USP/mg) in phosphate buffer. The flasks were incubated at 30°C with rotary shaking at 180 rpm for 3-7 days (optical density at 600 nm was above 1.0). The culture was tenfold serially diluted and appropriate dilution was plated onto En or HT agar plates. The En and HT agar plates were then incubated at 30°C for 2-5 days. Colonies with different morphology were picked up and purified by restreaking onto the same media until pure isolates were obtained. For colonies presented on En

agar, they were also examined ability to utilize heparin as carbon source by streaking on to HT agar. Colonies that were gram-negative, non-spore forming, catalase positive and gelatin liquefaction negative were collected and stored at 4°C for further examination.

2.2 Analytical methods

Heparinase activity was preliminarily examined through disappearance of heparin following by a decrease in metachromasia with toluidine blue. The assay was carried out in 96 microwell plate with flatted bottom. The bacterial isolates were inoculated in tryptone broth and incubated at 30°C and shaking at 180 rpm for 24-48 hours. Twenty microliters of the bacterial isolate culture was aseptically transferred into a well containing 90 µl of sterile tryptone broth and 10 µl of 10% (w/v) heparin. The microwell plate was incubated at 30°C for 24 hours. Subsequently, addition of 60 µl of 0.01% (w/v) aqueous solution of toluidine blue (total dye content 80%) in the well was conducted. Microwell plate was standed for 10 minutes to allow metachromasia formation. Electrostatically complex between toluidine blue dye molecules and polyanions of heparin including dimerization of the adjacent bound dye molecules occurred. The metachromasia took place as a result of both mentioned interactions (19). The complex resulted in red color against blue color background of accessed toluidine blue. This consequently contributed purple or purple-red color appearance depending upon the amount of heparin. The intensity of the purple color increased with an increase of amount of heparin. Heparin concentrations ranged from 0 to 10% (w/v) were subjected to the same assay in order to estimate the amount of heparin that degraded by the bacterial isolates.

Later, the isolates that gave positive results for heparin degradation monitored by preliminary toluidine complex method were cultivated in 1L FM1 medium (media for *Flavobacterium*) containing the following ingredients; 0.5% (w/v) proteose peptone, 0.2 % (w/v) beef extract, 0.1 % (w/v) yeast extract and 0.3 % (w/v) NaCl at pH 7.0. The 10% (v/v) of inoculum was then transferred to the medium and incubated at 30 °C with 180 rpm rotary shaking for 24 hours. Cultures were harvested and centrifuged at 4°C for 15 minutes at 4000xg. Supernatants were discarded and 5 ml sterile MilliQ water was added to the cell pellets. Cells suspensions were subsequently subjected to sonication with 125 W adjusted to 40% duty and pulse mode for 12 minutes. The cells suspension was always retained at 4°C during sonication. Crude cells extract was centrifuged at 4000xg and 4°C for 15 minutes. Supernatants were obtained and examined constitutive heparinase via metachromatic activity with Azure A (20-21). Crude cells extract aliquot was mixed with the same volume of heparin solution (25 mg ml^{-1} in 0.25 M sodium acetate – 0.0025 M calcium acetate at pH 7) and incubated at 30°C. After 1 hour of incubation for the heparin degradation by heparinase comprised in crude cells extract, 10 µl of the mixture was transferred to the clean test tube and 10 ml of Azure dye solution (conc. 0.02 g L^{-1}) was added. The conversion from blue to red color indicated presence of heparin henceforth contributed metachromasia. The change of absorbance at 620 nm was measured and compared with standard curves of heparin concentration (0-15 mg ml^{-1}). One unit of heparinase activity is referred to the amount of enzyme required to degrade 1 mg of heparin per hour (at stated pH and temperature). The proteins concentration of the crude cells extracts were determined by Bradford assay.

2.3 Phenotypic features examinations

Some characteristics of heparin degrading isolates were examined. Acid and gas production from 1% (w/v) glucose, sucrose, lactose, L-arabinose, rhamnose, mannose, maltose, xylose, xylitol, sorbitol, mannitol and adonitol by the isolates were determined

using carbohydrate fermentation broth. Motility was determined using semisolid agar tall. Triple sugars iron (TSI) was employed to deter glucose, lactose and sucrose fermentation. Oxidation-fermentation (OF), urease, oxidase, IMVIC (indole, methyl red, Voges-Proskauer and citrate) tests were also carried out.

All heparinase-producing isolates were investigated sensitivity to antibiotics; 30 µg Chloramphenicol (C), 30 µg Tetracycline (TE), 300 µg Streptomycin (S), 300 U Polymyxin B (PB), 30 µg Kanamycin (K), 5 µg Ciprofloxacin (CIP), 30 µg Vancomycin (VA) and 30 µg Cephalothin (KF) disks. The isolates were grown in FM1 media for 2 days and bacterial cells then were separated by centrifugation. Cells were washed twice and suspended with sterile normal saline. The turbidity of cell suspension was adjusted to Mcfarland number 4 prior spreading onto En and HT agar (OD_{600} = 0.669, initial bacterial cells were approximately 1.2 x 109 CFU ml^{-1}). Antibiotic disks were placed onto surface of both En and HT agar. Incubation was carried out at 30°C for 2 days. Sensitivity to antibiotics was determined by inhibition zone diameter.

2.4 Effects of pH, temperature and NaCl concentration on growth of heparinase-producing isolates

Inoculum of isolated heparinase-producing bacteria was cultivated using FM1 media and incubated at 30°C with rotary shaking at 180 rpm for 24 hours. To determine influence of temperature on bacterial growth and heparinase production, flask containing FM1 media and 10% (v/v) of inoculum were incubated at different temperatures; 4, 10, 30, 40 and 50°C and shaking at 180 rpm for 24 hours. Investigation of pH influencing on the isolate growth was performed by adding 10% (v/v) of the inoculum to FM1 media that adjusted initial pH from 2-12 using increments of 1.0 pH units. The cultures were incubated at 30°C at 180 rpm for 24 hours. Growth was simply observed by optical density at 600 nm. Influence of NaCl concentrations on growth of heparinase-bacterial isolates was determined with similar fashion except that FM1 media supplemented with varied NaCl concentrations (0, 1, 2, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0% (w/v) NaCl).

3. Results and Discussion

3.1 Isolation of heparin-degrading bacteria

Brackish soil sediment samples from Rayong (RYA, RYB and RYC) had slightly lower pH measured on-site (pH 7.10-8.16) compared to marine soil sediment samples (SKA, SKB and SKC) that had pH 7.60-8.41. The temperature of soil sediment samples from Rayong and SamutSongkhram were 32 and 31 °C, respectively. A total of 36 bacterial isolates with different colony morphology were obtained from 6 soil sediment samples using En and HT media for cultivation. Among those isolates, only 6 isolates was able to degrade heparin preliminarily examined by toluidine complex assay. The appearances of isolated heparinase-producing bacteria grown on En or HT agar plates were shown in Figure 2. The heparin-degrading bacterial strains were assigned as RYA En1, RYB En1, SKA En1, RYA HT1, RYB HT1 and SKA HT1.

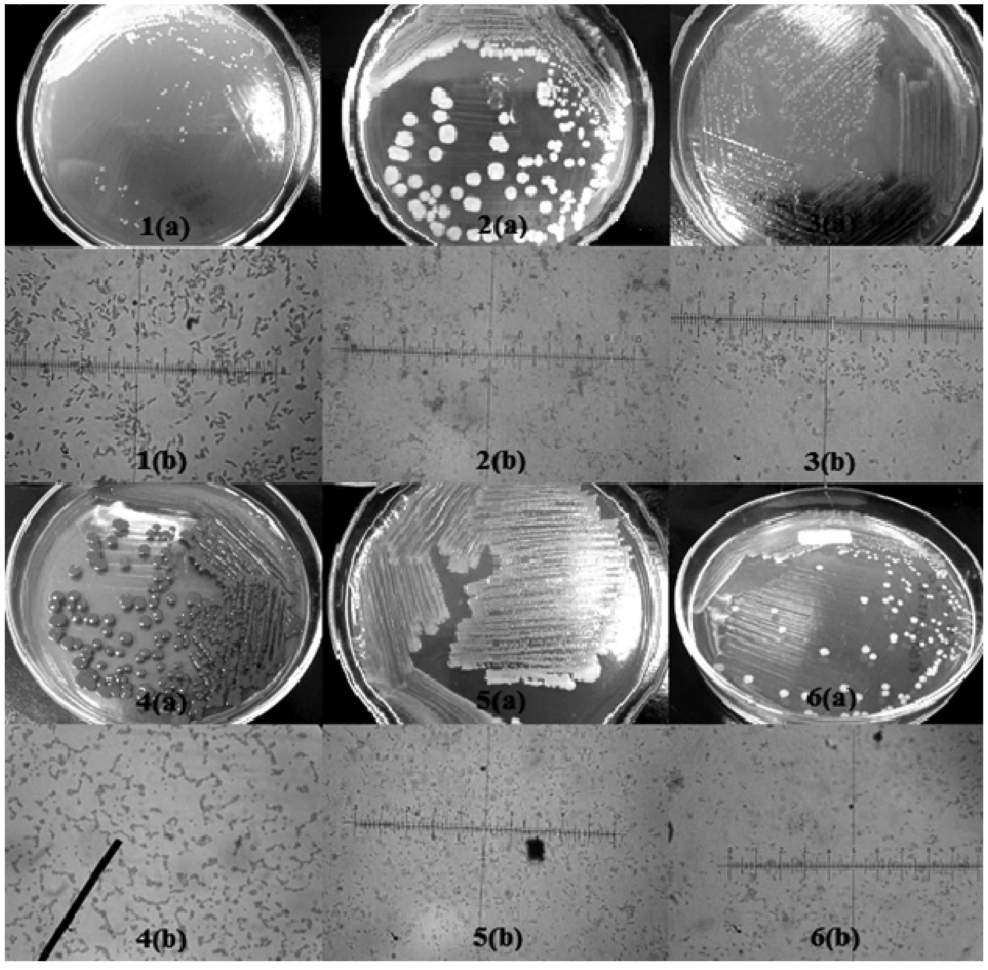

Figure 2. Colony appearance on En or HT agar (a) and Gram-staining (b) of heparinase-producing bacterial isolates visualized under a microscope with 1000X resolution. 1. RYA En1, 2. RYB En1, 3. SKA En1, 4. RYA HT1, 5. RYB HT1 and 6. SKA HT1

According to the toluidine complex assay, efficiency in heparin degradation (indirectly determination of heparinase producing) increased with a decrease of metachromasia formation which could be observed from conversion of blue color to purple or purple-red color. The assay was employed to examine induced extracellular heparinase produced of the isolates. Nevertheless, heparinase activities approximated by this assay were also depending on growth, heparinase production and enzyme secreted rates of isolates. It was found that all isolates except RYA HT1 degraded more than 90% (w/w) of initial amount of heparin containing in the medium. The heparinase activity was approximately above 3.75 U ml^{-1} (incubation temperature was at 30°C). The red pigment of RYA HT1 interfered metachromasia observation therefore it was inconclusive for its heparinase activity (Figure 3).

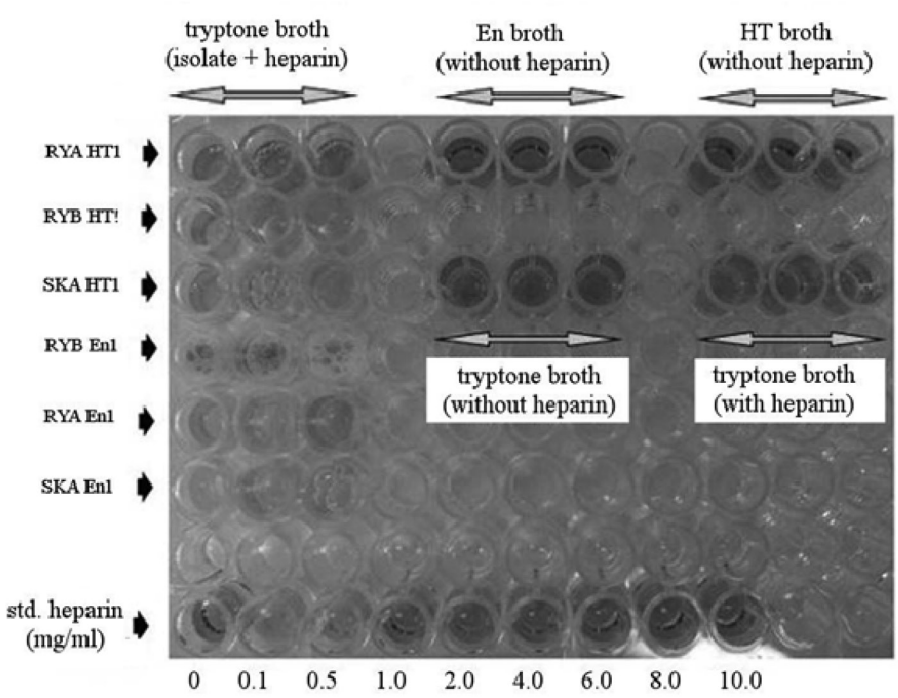

Figure 3. Toluidine blue assay of heparinase-producing bacterial isolates; RYA HT1, RYB HT1, SKA HT1, RYA En1, RYB En1 and SKA En1. Standard heparin concentration ranged 0-10 mg ml^{-1}.

Constitutive heparinase from crude cell extracts was determined by Azure A assay. Dimerization of Azure A dye molecules bound to adjacent negative charges of heparin allowed indirect determination of heparinase. Optical density at 620 nm (OD_{620}) which is characteristic of Azure dimer bond decreased when heparin was degraded resulting in less adjacent dye bound to the negative charge of heparin and henceforth less Azure dye dimers were formed. Therefore the decrease of OD_{620} was reversal to heparinase activity. Crude cells extract of RYA En1 had highest heparinase specific activity of 82 U mg^{-1} proteins and heparinase productivity of 792 U L^{-1} (Figure 4). Heparinase specific activities obtained from RYB HT1 (60 U mg^{-1} proteins) and SKA HT1 (47 U mg^{-1} proteins) were slightly lower than heparinase activity derived from RYA En1. Nevertheless, the other isolates yielded approximately 20 U mg^{-1} proteins heparinase activities. In addition, all isolates were capable to degrade 60-76 % of initial heparin containing in the media.

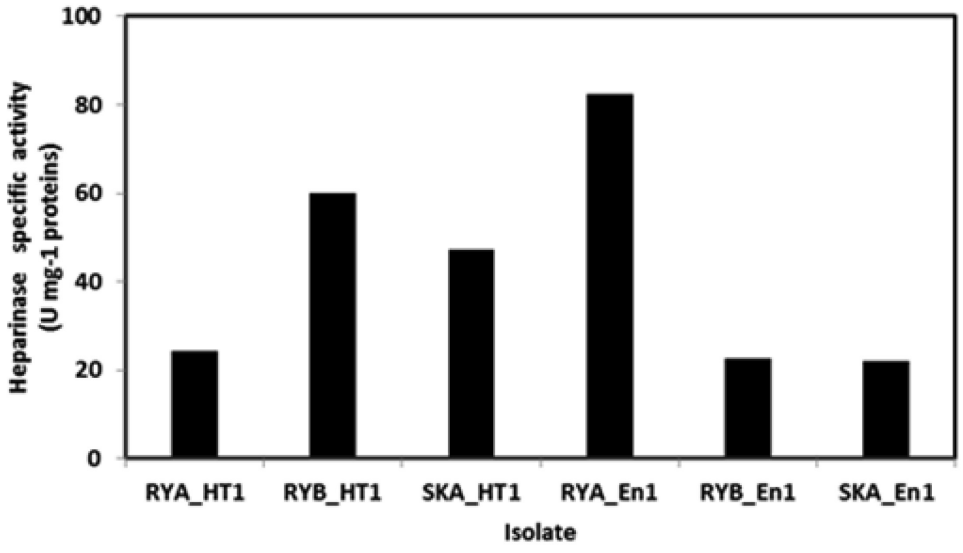

Figure 4. Specific activities of intracellular constitutive heparinase (U mg^{-1} proteins) from crude cells extract of bacterial isolates grown in FM1 incubated at 30°C for 24 hours. Heparinase was indirectly determined by Azure A assay.

Intracellular heparinase obtained from RYA En1 was produced approximately similar amount to *Aspergillus oryzae* (754 U L^{-1}) and only half compared to amount of haparinase derived from *Aspergillus flavus* (1784 U L^{-1}) cultivated in optimized basal medium (17, 20). *Flavobacterium heprinum* yielded specific heparinase activity only 8 U mg^{-1} proteins that was accounted only 10% of specific heparinase activity obtained from RYA En1 without heparin as an inducer (16). Specific heparinase activity of 18 mU mg^{-1} proteins was observed in cell-free extract of *Bacillus circulans* and only 0.8 U mg^{-1} proteins obtained after purification applied (13). This suggested that RYA En1, RYB HT1 and SKA HT1 could be good candidates for optimized and enhanced heparinase production.

3.2 Partial phenotypic and chemotaxonomic analyses of isolated heparinase-producing bacteria

From purification of isolates using En or HT media, RYA HT1, RYB En1 and SKA HT1 had colony diameter ranged from 3.0-5.0 mm whereas RYA En1, RYB HT1 and SKA En1 had smaller colonies with diameter of 0.5-2.0 mm (Table 1). Only RYA HT1 produced red pigment and therefore colonies appeared in red color as shown in Figure 1.; 4(b). All isolated heparinase-producing bacteria were Gram-negative, non-endospore forming and non-motile rods. They were capable to grow on nutrient agar (NA), En and FM1 media. Only RYA HT1, RYB HT1 and SKA HT1 were able to grow on HT media though they demonstrated heparin degrading capability by toluidine complex assay and Azure A assay. Requiring of heparin as inducer could be a reason to explain this scenario.

Table 1. Microscopic and colony charateristics of the isolates compared to some type strains of genus Pedobacter

Characteristic / Isolated bacteria	Sub-characteristic	*P. heparinus	*P. afticanus	*P. piscium	*P. saltans	RYA HT1	RYB HT1	SKA HT1	RYA En1	RYB En1	SKA En1
Microscopic characters	Habitat	soil	soil, sludge	fish	soil	Brackish soil sediment	Brackish soil sediment	Marine soil sediment	Brackish soil sediment	Brackish soil sediment	Marine soil sediment
	Gram-staining	negative (-)	negative (-)	negative (-)	negative (-)	negative (-)	negative (-)	Negative (-)	negative (-)	negative (-)	negative (-)
	Shape	rod (short)	rod (short)	rod (short)	rod (short)	rod (short)	rod (short)	rod (short)	rod (long)	rod (short)	rod (short)
	Cell size w x l (μm)	0.5 x 0.7-6 μm	0.5 x 0.7-6 μm	0.5 x 0.7-6 μm	0.5 x 0.7-6 μm	0.5 x 1.0	0.5 x 1.0	0.5-1.0	1.0 x 3.0	0.5 x 1.0	0.5x 1.0
	Endospore	negative (-)	negative (-)	Negative (-)	Negative (-)	negative (-)	negative (-)	negative (-)	negative (-)	negative (-)	negative (-)
Physical characters	Motility	variable	gliding	Non-motile	gliding	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile
	Respiration	obligate aerobic	obligate aerobic	obligate aerobic	obligate aerobic	facultative aerobic	facultative aerobic	facultative aerobic	facultative aerobic	facultative aerobic	facultative aerobic
	Colony diameter (mm.)	1-4	2-4	2-4	2-4	1.0-2.0	1.0-2.0	3.0-5.0	3.0-5.0	3.0-5.0	0.5-1.0
	colony form	round	round	round	round	round	round	round	round	round	round
	Colony elevation	convex	convex	convex	convex	convex	convex	convex	convex	convex	convex
	Colony margin	entire	entire	entire	entire	entire	entire	entire	entire	entire	entire
	Colony color	Translucent yellow	Translucent yellow	Yellow or creamish white	Light yellow	red	creamy	creamy	creamy	yellow (light)	white
growth condition characters	Grow on medium	n/a	n/a	n/a	n/a	En, HT	En, HT	En, HT	En	En	En
	Growth temp. range (°C)	5-30	n/a	5-30	n/a	10-40	10-30	30-40	10-40	10-40	30-45
	pH range	4.5-10	4.5-10	4.5-10	4.5-10	4-11	5-11	5-11	5-11	5-11	5-11
	Incubation time (solid media, days)	n/a	n/a	n/a	n/a	2 days	5 days	5 days	5 days	5 days	5 days
	Incubation time (liquid media;days)	n/a	n/a	n/a	n/a	5 days	5 days	5 days	5 days	5 days	5 days

According to Oxidation-Fermentation (OF) test, only RYB HT1 and SKA HT1 were strict aerobes while the rest of isolates were facultative anaerobes (Table 2). Similar characteristics such as negative for activity of gelatinase, acid production from rhamnose, indole production and Voges-Proskauer test including positive for activity of catalase were found for all of the isolates. Only RYA En1 was positive for acid production from lactose and citrate lyase activity carbohydrate utilization tests and had negative result for oxidase activity. Noticeably, bacteria isolated from the same soil sediment sample had similar capabilities in carbohydrate utilization. In addition, positive results for catalase and oxidase activities, negative results for indole production and acid production from sorbitol and adonitol including gelatinase and citrate lyase activities were described as characteristics often found in *Pedobacter* and *Sphingobacterium* type strains. Some characteristics such as being positive for heparinase and acid production from sucrose were usually found in the genus *Pedobacter* and not presenting in the genus *Sphingobacterium*. RYB HT1, SKA HT1, RYB En1 and SKA En1 had characteristics corresponding to several type strains of the genus *Pedobacter*.

Table 2. Partial phenotypic and chemotaxonomic of the isolates compared to some type strains of genus *Pedobacter* and *Sphingdobacterium*

Characteristic test	*Pedobacter**				*Sphingobacterium**			Isolates obtained from this research					
	P1	P2	P3	P4	S1	S2	S3	RYA HT1	RYB HT1	SKA HT1	RYA En1	RYB En1	SKA En1
Culture medium													
NA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	+	+	+	+	+	+
En								+	+	+	+	+	+
HT								+	+	+	-	-	-
FM1								+	+	+	+	+	+
Motility	gliding	n/a	gliding	-	n/a	n/a	n/a	-	-	-	-	-	-
Heparinase	+	v	+	-	-	-	-	+	+	+	+	+	+
Gelatinase	-	-	-	-	-	v	-	-	-	-	-	-	-
Urease	v	-	-	-	+	+	+	-	-	+	-	-	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	-	+	+
H_2S production	-	-	-	n/a	n/a	n/a	n/a	-	-	-	-	+	+
Acid production from:													
Glucose	v	v	v	+	+	+	+	+	-	+	+	-	+
Sucrose	v	-	-	+	+	+	+	+	-	-	+	-	-
Lactose	v	n/a	-	+	+	+	+	-	-	-	+	-	-
L-Arabinose	n/a	v	v	v	-	+	+	-	-	-	+	+	-
Rhamnose	-	-	-	+	-	v	+	-	-	-	-	-	-
Mannose	-	n/a	n/a	n/a	n/a	n/a	n/a	+	-	+	+	-	-
Maltose	+	v	+	+	+	+	+	+	-	-	+	+	-
Xylose	+	v	+	+	+	+	+	-	-	+	-	-	+
Xylitol	+	v	+	+	+	+	+	+	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	+	-	-	+	-	-
Mannitol	-	+	-	v	+	-	-	+	-	-	+	-	-
Adonitol	-	-	-	-	-	-	-	+	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-
MR-VP	n/a	n/a	n/a	n/a	n/a	n/a	n/a	-,-	-,-	-,-	+,-	-,-	+,-
Citrate lyase	-	-	-	-	-	-	-	-	-	+	-	-	-

Note: P1; P. *heparius*, P2; P. *africanus*, P3; P. *saltans*, P4; P. *piscium*, S1; S. *spiritivorum*, S2; S. *multivorum*, S3; S. *thalpohilum*, +; positive result, -; negative result, n/a; no information, v; variable result *; data from other resources (22-23).

The heparinase-produing isolates were investigated their sensitivities to a number of antibiotics when they were grown on En or HT and FM1. All of the isolates were sensitive to Polymyxin B (PB), Chloramphenicol (C), Tetracycline (TE), Streptomycin (S), Kanamycin (K) and Gentamicin (GN) and Ciprofloxacin (CIP) when grown on FM1 agar except SKA En1 that resisted to PB and TE. According to mode of action of antibiotics, PB disrupts the outer membrane of Gram-negative bacteria. C inhibits protein synthesis by binding to the 50S ribosomal subunit whereas S, K and GN irreversibly bind to the 30S ribosomal subunit. CIP inhibits DNA synthesis and function by binding to the A subunit of DNA gyrase and preventing supercoiling of DNA. Growing the isolates on HT agar seemed to promote resistance to C, TE, S, K and CIP of RYA HT1 and SKA HT1. This was also true for En agar that RYA En1, RYB En1 and SKA En1 enhanced resistances for C, TE, S, K, GN and CIP (Table 3). Only SKA HT1, RYA En1, RYB En1 and SKA En1 grown on FM1 agar were marginally sensitive to Vancomycin (VA) and Cephalothin (KF) which inhibit the synthesis of peptidoglycan. This was corresponding to cell wall characteristic of all isolates that were Gram-negative bacteria.

Table 3. Sensitivities to some antibiotics of the heparinase-producing isolates grown on HT, En and FM1 agar.

Isolate	media	VA	KF	PB	C	TE	S	K	GN	CIP
RYA HT1	HT	-	-	+ (2.40)	+ (1.35)	-	-	-	-	-
	FM1	-	-	+ (1.40)	+ (2.30)	+ (1.30)	+ (0.95)	+ (2.05)	+ (1.80)	+ (2.75)
RYB HT1	HT	-	-	+ (1.30)	+ (2.00)	+ (3.10)	+ (1.00)	+ (1.75)	+ (1.80)	+ (2.35)
	FM1	-	-	+ (1.55)	+ (1.40)	+ (2.80)	+ (2.35)	+ (2.30)	+ (2.50)	+ (2.30)
SKA HT1	HT	-	-	+ (2.20)	-	-	-	+ (1.85)	+ (2.00)	-
	FM1	-	+ (0.80)	+ (0.90)	+ (2.90)	+ (1.75)	+ (1.25)	+ (2.25)	+ (1.70)	+ (2.30)
RYA En1	En	-	-	+ (1.55)	+ (3.20)	+ (2.70)	-	+ (1.70)	+ (1.80)	+ (3.80)
	FM1	+ (1.40)	-	+ (1.35)	+ (3.60)	+ (2.85)	+ (1.80)	+ (2.50)	+ (2.20)	+ (3.90)
RYB En1	En	-	-	+ (1.45)	+ (3.05)	+ (2.00)	-	+(1.25)	+ (1.50)	+ (2.45)
	FM1	+ (1.00)	-	+ (1.20)	+ (3.15)	+ (2.25)	+ (1.95)	+ (2.30)	+ (2.00)	+ (3.00)
SKA En1	En	-	-	-	+ (2.15)	-	-	+ (1.20)	+ (1.15)	+ (3.60)
	FM1	-	+ (1.70)	-	+ (3.10)	-	+ (1.95)	+ (2.35)	+ (2.05)	+ (3.70)

Note; VA; Vancomycin, KF; Cephalotin, PB; Polymyxin B, C; Chloramphenicol, TE; Tetracycline, S; Streptomycin, K; Kanamycin GN; Gentamycin CIP; Ciprofloxacin

-; resistance to antibiotic + (number); growth inhibition by antibiotic (diameter of inhibition zone; cm)

2.5 Influences of pH, temperature and NaCl concentration on isolated heparinase-producing bacteria

Optical density at 600 nm (OD_{600}) was measured to determine growth of the isolates in FM1 media influenced by pH, incubation temperatures and NaCl concentrations. Growth of the isolates occurred at pH values from 5 to 11 except RYB HT1 that maximum pH for growth was 10. Optimum pH for growth of RYA HT1 and RYB HT1 was 5-8 unlike RYA En1 and SKA En1 that had optimal pH 6-8. SKA En1 had constricted optimal pH at 5-6 while wider range of optimal pH of 5-10 as shown in Figure 5.

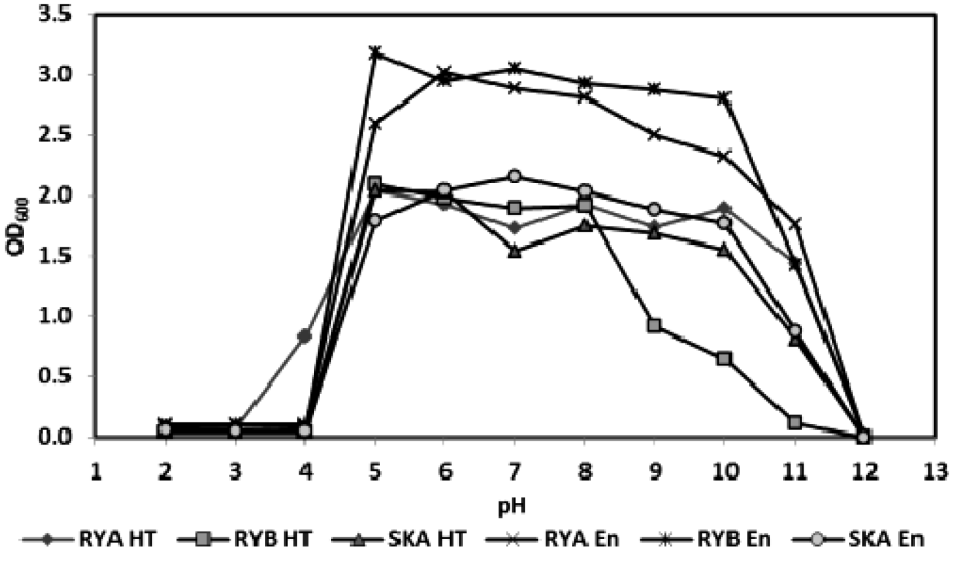

Figure 5. Influence of pH on growth of the isolates when grew in FM1 media adjusted initial pH 2-12 with increments of 1.0 pH unit. The culture was incubated for 24 hours at 30°C with rotary shaking at 180 rpm.

Growth of the isolate occurred between 10 and 40 °C except SKA HT1 and SKA En1. According to examined incubation temperatures, optimal temperature for growth of the isolates was 30°C (Figure 6). Remarkably, growths of SKA En1 and RYA HT1 at 40°C were observed. Several type stains of Pedobacter have maximum temperature for growth at 37°C and temperature for growth ranged from 4-30 °C in R2A medium (Table 1). The isolates grew on FM1 supplemented with 0-15 % (w/v) NaCl. Optimal growth for RYA HT1 and RYB HT1 was observed with no NaCl. Optimal growth of RYA En1, RYB En1 and SKA En1 were obtained with 1% (w/v) NaCl supplemented in the media. 0-6% (w/v) NaCl supplemented in FM1 broth was found to promote growth of SKA HT1 (Figure 7).

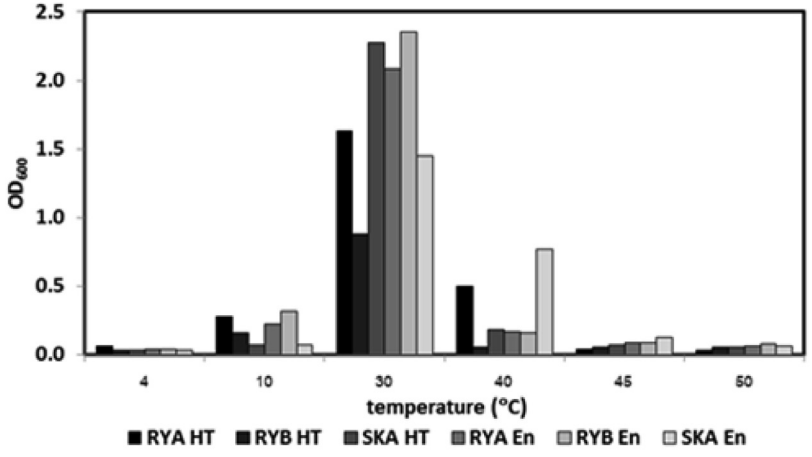

Figure 6. Influence of incubation temperature on growth of the isolates when grew in FM1 media. The culture was incubated for 24 hours at 4, 10, 30, 40 45 and 50°C with rotary shaking at 180 rpm.

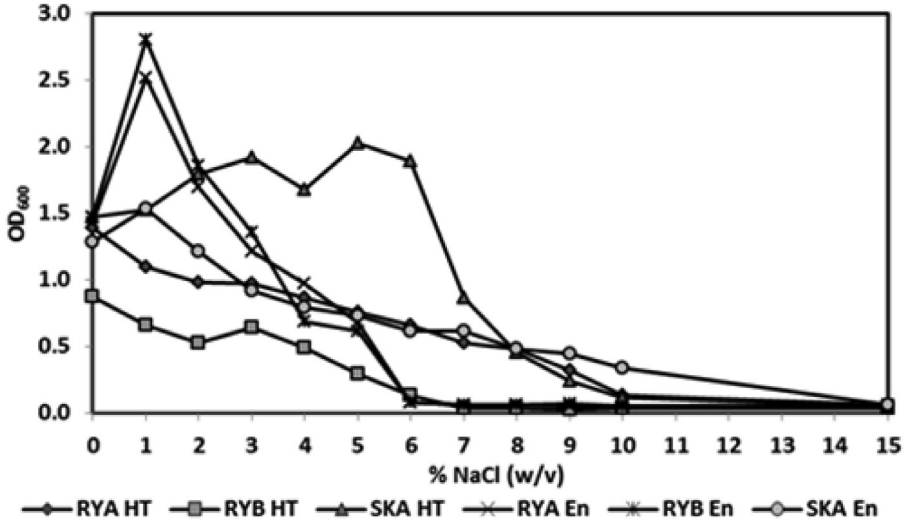

Figure 7. Influence of NaCl concentration on growth of the isolates when grew in FM1 media containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 15% (w/v) NaCl. The culture was incubated for 24 hours at 30°C with rotary shaking at 180 rpm.

4. Conclusion

Six heparinase-producing bacterial isolates obtained from brackish and marine soil sediments were capable to degrade heparin above 90% (w/v) within 24 hours of cultivation with 3.75 U L^{-1} h^{-1} of heparinase determined by toluidine complex assay. The highest specific intracellular constitutive heparinase activity of RYA En1 of 82 U mg^{-1} proteins and extracellular induced heparinase productivity of 792 U L^{-1} heparin were derived. Although RYB En1, SKA En1, RYB HT1 and SKA HT1 possessed several characters corresponding to type strains of the genus Pedobacter, 16S rRNA gene sequencing and fatty acid methyl ester analysis were suggested for further bacterial identification. Base on heparinase activities and growth responses for pH, temperature and NaCl concentration, RYA En1 and SKA En1 could be potential alternative strains for enhanced heparinase production.

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