

Saccharification of *Ulva lactuca* via *Pseudoalteromonas piscicida* for biofuel production

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Abstract: *Pseudoalteromonas piscicida* WM21 was isolated from seawater at Hurghada, Red Sea, Egypt. It was promising to hydrolyze the polysaccharides of *Ulva lactuca*. *Ulva lactuca* contained 44% carbohydrates, 5% lipids, 16% proteins, 12% Fibers and 23% ash. Optimization of reducing sugars production by *P. piscicida* WM21 was investigated using Plackett-Burman design. The main effect data as well as the *t*-test results suggested that the beef extract and inoculum size are the most effective variables that controlled the reducing sugar produced by *P. piscicida*. Considerable positive effects of the high levels of substrate concentration and low levels of incubation period were also suggested. On the other hand, variations within the examined levels of pH levels, NaCl and peptone recorded slight effects. While the main effect data as well as the *t*-test results suggested that the substrate concentration and incubation period were the most effective variables that controlled amylase activity produced by *P. piscicida*. To evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out. The predicted near optimum and far from optimum levels of the independent variables were examined and compared to the basal condition settings. The applied near optimum condition, resulted in approximately 56 mg/g increase in reducing sugar with 6 mm amylase activity by *P. piscicida* when compared to the basal medium formulation, while the conditions predicted to be far from optimal recorded approximately 45 mg/g decreases in reducing sugar with 3 mm amylase activity. These results supported the predictions of the applied Plackett-Burman experiment for enhancement of reducing sugar production by marine microorganisms.

Keywords: Reducing sugar, *Ulva lactuca*, *Pseudoalteromonas piscicida*, Saccharification process, Biofuel

1. Introduction

Conversion of biomass from marine algae into ethanol could be economically feasible since some algae hydrolysate can contain more total carbohydrate and hexose sugars than some terrestrial, lignocellulosic biomass feedstock (Philippidis *et al.*, 1993; Chynoweth, 2002; Sluiter, 2006; John *et al.*, 2011). The green macroalgae (chlorophyceae), like *Ulva lactuca* has been considered as a potential aquatic energy crop as early as in the Aquatic Species Program in the USA back in 1978–1996, due to its high potential growth rates and high content of carbohydrates (Ryther *et al.*, 1984). The process of breaking a complex carbohydrate into its monosaccharide components is called saccharification

process. Marine bacteria act a vital role in production of industrial enzymes. Marine bacterial enzymes have several advantages for industrial utilization (Ventosa and Nieto, 1995; Hong *et al.*, 2013; Koppram *et al.*, 2013). Statistical experimental designs are powerful tools for searching the key factors rapidly from a multivariable system and minimizing the error in determining the effect of parameters and the results are achieved in an economical manner (El-Helow and El-Ahawany, 1999; Xiong *et al.*, 2008). The application of statistically based experimental designs is a more efficient approach to deal with a large number of variables (Ooijkaas *et al.*, 1998). Using the Plackett-Burman experimental

designs has resulted in increased optimization of fermentation titers, and an ability to predict the presence of mixtures and to select substitutes for complex medium ingredients (Monaghan and Koupal, 1989). The Plackett-Burman experimental design, a fractional factorial design (Plackett and Burman; 1946, Yu *et al.*, 1997) was applied in this research to reflect the relative importance of various environmental factors on the saccharification process in liquid cultures. The current study was suggested for enhancing the ability of isolated marine bacteria to hydrolyze the polysaccharides of *Ulva lactuca* by Plackett-Burman experimental designs.

2. Material and Methods

2.1. Sample Collection

Sea water and sediment samples were collected from marine environment at Hurghada coastline, Red Sea, Egypt, summer 2011. While *Ulva lactuca* were harvested from Red Sea, Egypt coastline, to be used as substrates of reducing sugars production.

2.2. Screening of Hydrolysis Enzymes Producing Marine Bacteria

Isolation of marine bacteria from the seawater and sediment samples was performed by serial dilution and spread plate method. A volume of 1 ml of each dilution was transferred aseptically to starch nutrient agar plates containing: starch; 10 g/L and nutrient agar; 23 g/L. The plates were incubated at $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 24 h. Tooth picking technique was used to test the ability of isolated bacteria to produce amylase (Margesin *et al.*, 2003). The plates were stabbed consequently using a sterile clean tooth pick in each time with a single colony of each of the tested bacteria. After 3 days the stabbed plates were flooded with iodine solution for detecting the amylase enzyme (Horikoshi, 1999).

2.3. Phenotypic Characterization of the Promising Marine Bacterial Isolate

Phenotypic characteristics such as Gram's staining, motility, cultural characteristics, catalase, oxidase and IMViC test of the marine bacterial isolate was studied by adopting standard procedures. Effect of sodium chloride, pH level, and temperature on growth was tested.

2.4. Electron Microscope Investigation

For scanning electron microscopy (SEM), bacterial cells, grown in S.N.B medium, were harvested by mild centrifugation, washed with phosphate buffer pH 8 and fixed with 2% glutaraldehyde followed by 1% osmium tetroxide treatment. After completion of fixation, sample was washed in buffer solution, and the washed cells were dehydrated in ascending order of ethanol concentrations. The sample was dried completely in a critical point dryer, and finally coated with gold in JEOL-JFG1100 E ion-sputter-coater. The

specimen was viewed in JEOL-JSM 5300 scanning electron microscope operated at 20 kV with a beam specimen angle of 45° .

2.5. Genotypic Characterization the Promising Marine Bacterial Isolate

The promising bacterial isolate was cultured in SN liquid medium for 2 days and genomic DNAs were extracted with genomic DNA extraction protocol of GeneJet genomic DNA purification Kit (Fermentas). PCR using Maxima Hot Start PCR Master Mix (Fermentas) and PCR clean up to the PCR product was performed using GeneJET™ PCR Purification Kit (fermentas). The sequencing to the PCR product on GATC Company was made by using ABI 3730xl DNA sequencer by using universal primaries (16S 27F and 16S 1492R) Table 1.

Table 1. The primers used in PCR amplification and sequencing.

Primers	Sequence (5' to 3')
16S 27F	AGAGTTTGATCCTGGCTCAG
16S 1492R	GGTACCTTGTTACGACTT

2.6. Chemical Composition of *Ulva Lactuca*

Humidity, organic matter and ash were determined by standard methods (Pádua *et al.*, 2004). Protein content was measured with Kjeldahl method using a factor of $N = 6.25$ (AOAC, 2000; method 976.05). Lipid content was determined with the Soxhlet method (AOAC, 2000; method 920.39). Subtraction of the sum of humidity, protein, lipids, fibers and ash values from 100 was the carbohydrate contents in percentage (Pádua *et al.*, 2004).

2.7. Pretreatment of *Ulva Lactuca*

The algal sample was dried at 70°C over night. All dried algae were milled using a laboratory hammer mill in order to obtain a chip size less than 0.2 mm. the dilute acid pretreatment of biomass was optimized at 121°C in an autoclave for with sulfuric acid concentrations 1N, w/v for one hour (Taherzadeh and Karimi, 2008).

2.8. The Saccharification Medium

The saccharification process was carried out using medium containing pretreated substrate. A volume of 100 ml of sterile starch nutrient broth medium was added to 3 g from pretreated substrate as sole carbon source and inoculated with 3 ml freshly prepared inoculum of bacteria. The flasks were loaded on a rotary shaker incubator at a speed of 120 ± 2 rpm at $30 \pm 2^{\circ}\text{C}$ for 24 h. After incubation, the production broths were centrifuged at 5000 rpm for 15 min. The supernatants were collected for determining the reducing sugars concentration and enzymatic activity.

2.9. Estimation of Reducing Sugars

The amount of reducing sugars was estimated by dinitrosalicylic acid (DNSA) method (Miller, 1959).

2.10. Optimization of Saccharification Process by Experimental Design

Seven independent variables were screened in nine combinations organized according to the Plackett-Burman design matrix. For each variable, a high (+) and low (-) level was tested. The factors tested were given in Table 2. All trials were performed in duplicates and the averages of observation results were treated as the responses. The main effect of each variable was determined using the following equation:

$$E_{xj} = (M_{i+} - M_{i-})/N$$

Where E_{xj} is the variable main effect, M_{i+} and M_{i-} are enzyme activity & reducing sugar (glucose conc.) in trials where the independent variable (xi) was present in high and low levels, respectively, and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the high level of this variable is nearer to optimum. Using Microsoft Excel, statistical *t*-values for equal unpaired samples were calculated for determination of variable significance (Plackett and Burman; 1946, Yu *et al.*, 1997).

Table 2. Factors examined as independent variables affecting the production of enzymes production by *P. piscicida* and its levels in the Plackett-Burman experiment.

Factor	Symbol	Level		
		-1	0	+1
Substrate conc. (g/L)	SC	10	30	50
Incubation period (h)	IP	24	72	120
NaCl (g/L)	NC	20	30	40
Peptone (g/L)	Pep	3	5	7
Beef extract (g/L)	BE	1	3	5
pH	pH	7	8	9
Inoculum size (ml)	IS	1	3	5

2.11. Verification of Plackett-Burman Experiment

In order to validate the obtained results and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates. According to the main effect results, the predicted near optimum and far from optimum levels of the independent variables were examined and compared to the basal condition settings. The enzymes activity and reducing sugar concentration were then estimated as described before (Plackett and Burman; 1946, Yu *et al.*, 1997).

2.12. Reducing Sugar Estimation by High Performance Liquid Chromatography (HPLC)

The concentration of monosaccharides obtained from predicted near optimum conditions were measured by HPLC. The Shimpack SPR-Ca column (Shimadzu, Japan) at 80°C with IR-detector was used. The mobile phase was distilled water at a flow rate of 0.5 ml/min. The samples were filtered with 0.45 µm of cellulose acetate filter and 10 µl of injection volume was added.

3. Results

3.1. Characterization of the Promising Marine Bacterial Isolate

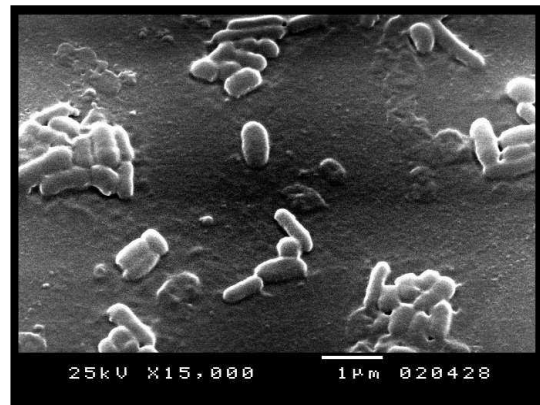


Figure 1. Scanning electron microscopic feature of bacterial isolate WM21 shown as rods.

The most promising marine bacterial isolate (WM21) was submitted to the phenotypic characterization through morphological, physiological and biochemical tests and submitted to genotypic characterization through 16S rDNA technique.

It grew at 20-45°C, pH range (6-10) and at all examined sodium chloride tested concentrations (0-10%) Also catalase and oxidase are positive. Moreover, produce gelatinase, amylase, agarase, lipase (Tween 80), and lysine decarboxylase. In addition to, it was utilizing of glucose.

It has been found that the bacterial isolate WM21 had 96% identical counterpart with respect to 16S rRNA sequence. Sequence of the isolate WM21 was affiliated according to their 16S rDNA to the genus *Pseudoalteromonas*. However, the isolate WM21 showed 96% sequence homology to *Pseudoalteromonas piscicida*.

Table 3. Accession number of the experimental 16S rDNA sequence and similarity percentage to the closest known species.

Accession no.	Most related Species	Similarity (%)
359805839	<i>Pseudoalteromonas piscicida</i>	96

3.2. Chemical Composition of Algal Substrate

The chemical analysis for the algal species; *U. lactuca* was carried out and the content of organic matter included the total carbohydrates, lipids, proteins and ash percentages were detected (Table 4).

Table 4. The chemical composition of different algal substrates.

Composition (%)	<i>U. lactuca</i>
Moisture content	10
Organic matter	77
Carbohydrate	44
Lipid	5
Protein	16
Fibers	12
Ash	23

3.3. Optimization of Saccharification Process by Plackett-Burman Design

Seven factors were examined as independent variables affecting the production of reducing sugars by *P. piscicida*. The symbols and level of these factors were presented in Table 2, while their distribution in nine major trials according to fitted design and their responses were shown in Table 5. Data conducted that the highest concentration of reducing sugar recorded 144 mg/g by trial No.6 with maximization of 41 mg/g comparing to the basal condition 103 mg/g. The main effect data presented in Table 6 as well as the *t*-test results in suggested that the beef extract is the most effective variable that controlled the reducing sugar value by *P. piscicida*. The main effect of each variable, based on the reducing sugar concentration, was estimated as the difference between both averages of measurements made at the high level (+) and the low level (-) and represented graphically in Figure 2. The main effect data presented in Table 6 as well as the *t*-test results suggested that the beef extract and inoculum size are the most effective variables that controlled the reducing sugar produced by *P. piscicida*. According to these results, the lower inoculum size (1ml) and the higher beef extract (5g/L) are nearer to optimum than their opposite

levels. Considerable positive effects of the high levels of substrate concentration and low levels of incubation period are also suggested by Figure 2. On the other hand, variations within the examined levels of pH levels, NaCl and peptone recorded slight effects. While the main effect data presented in Table 6 as well as the *t*-test results suggested that the substrate concentration and incubation period are the most effective variables that controlled amylase activity produced by *P. piscicida*. According to these results, the lower incubation period (24 h) and the higher substrate concentration (5%) are nearer to optimum than their opposite levels. Considerable of the low levels of pH and the high levels of peptone are positive effect also suggested by Figure 3. On the other hand, variations within the examined levels of beef extract, NaCl, and inoculum size recorded slight effects. The interacting effects of beef extract and substrate concentration as described in three-dimensional graph (Figure 4; A), suggest that, within the examined ranges the higher beef extract accompanied by the higher substrate concentration would markedly increase the reducing sugar expressed by the experimental bacterium; *P. piscicida*. However, the interaction beef extract and inoculum size (Figure 4; B) with respect to production of the reducing sugar appeared to be high.

Table 5. Applied Plackett-Burman design for seven cultural variables and the experimental results of reducing sugar produced by *P. piscicida*.

Trial No.	Independent variables							Reducing sugar conc. (mg/g)	Amylase (mm)
	SC	IP	NC	Pep	BE	pH	IS		
1	-	-	-	+	+	+	-	110	21
2	+	-	-	-	-	+	+	92	28
3	-	+	-	-	+	-	+	90	18
4	+	+	-	+	-	-	-	106	29
5	-	-	+	+	-	-	+	80	23
6	+	-	+	-	+	-	-	144	32
7	-	+	+	-	-	+	-	70	15
8	+	+	+	+	+	+	+	116	27
9	0	0	0	0	0	0	0	103	29

Table 6. Statistical analyses of the Plackett-Burman experimental results.

Variable	Reducing sugar conc. (mg/g)		Amylase (mm)	
	Main effect	<i>t</i> -value ¹	Main effect	<i>t</i> -value ¹
Substrate conc. (%)	27	1.943	9.75	2.015
Incubation period (h)	-11	2.015	-3.75	2.015
NaCl (g/L)	3	2.013	0.25	1.943
Peptone (g/L)	4	2.013	1.75	2.131
Beef extract (g/L)	28	2.015	0.75	1.943
pH level	-8	1.943	-2.75	1.943
Inoculum size (ml)	-13	2.013	-0.25	2.015

¹*t*-value significant at the 1% level = 3.70

t-value significant at the 5% level = 2.446

t-value significant at the 10% level = 1.94

t-value significant at the 20% level = 1.372

Standard *t*-values are obtained from Statistical Methods (Snedecor and Cochran 1989).

The interacting effects of substrate concentration and incubation period as described in three-dimensional graph (Figure 5; A) suggest that, within the examined ranges, the

shorter incubation period accompanied by the higher substrate concentration would markedly increase the amylase activity expressed by the experimental bacterium; *P. piscicida* to activity of the amylase appeared to be very slight. However, the interaction of substrate concentration and pH level (Figure 5; B) with respect to activity of the amylase appeared to be very slight.

3.4. Verification of Plackett-Burman Experiment

In order to validate the obtained results and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates. The predicted near optimum and far from optimum levels of the independent variables were examined and compared to the basal condition settings. The Reducing sugar concentration and amylase activity of out using *P. piscicida* observations are shown in Table 7. The applied near optimum condition, resulted in approximately 56 mg/g increase in reducing sugar and 6 mm amylase activity by *P. piscicida* when compared to the basal medium formulation. On the other hand, the condition predicted to be far from optimal recorded

approximately 45 mg/g decreases in reducing sugar and 3 mm amylase activity. These results support the predictions predicted from the applied Plackett-Burman experiment.

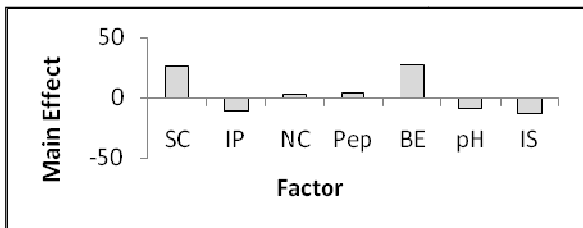


Figure 2. Elucidation of the factors affecting the production of reducing sugars by *P. piscicida*.

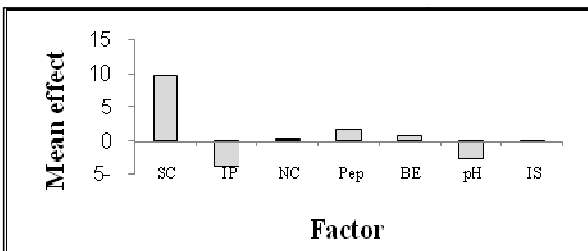


Figure 3. Elucidation of the factors affecting the amylase activity by *P. piscicida*.

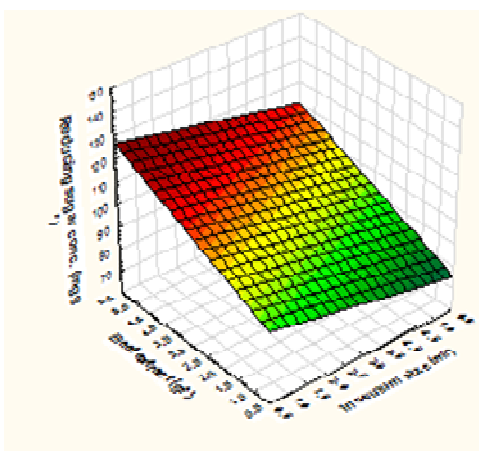
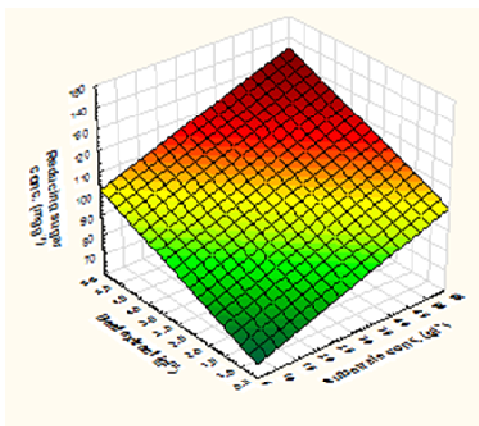


Figure 4. The response 3D Surface Plot analysis show the interaction of the different beef extract with different substrate concentration (A) and different inoculum size (B) in relation to reducing sugar mg/g by *P. piscicida*.

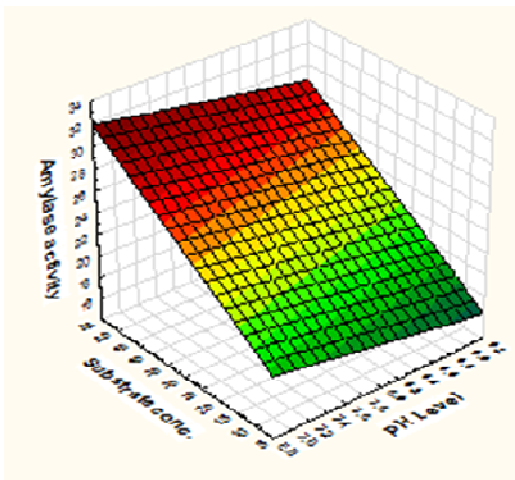
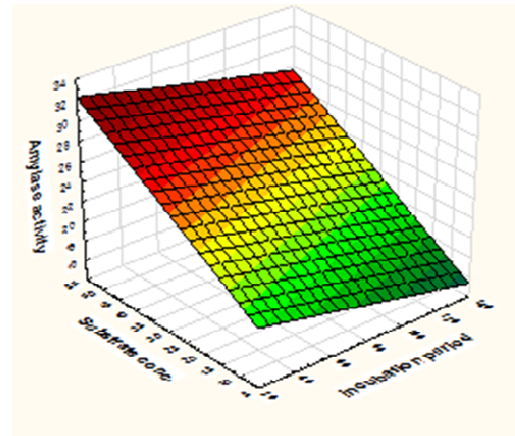


Figure 5. The response 3D Surface Plot analysis show the interaction of the different substrate concentration with different incubation period (upper) and different pH level (lower) in relation to amylase activity by *P. piscicida*.

Table 7. Verification of the Plackett-Burman experimental results that carried out using *P. piscicida*.

Response (Average)	Basal medium	Near optimum medium	Far from optimum medium
Reducing sugar conc. (mg/g)	102.3±0.007	158±0.005	57±0.005
Amylase (mm)	29.3±0.003	35±0.005	26.3±0.005

3.5. The Reducing Sugar Analysis by HPLC

The monosaccharides and their concentration were estimated in near optimum medium using HPLC. Glucose and galactose were detected at 144 mg/g and 24 mg/g respectively (Figure 6).

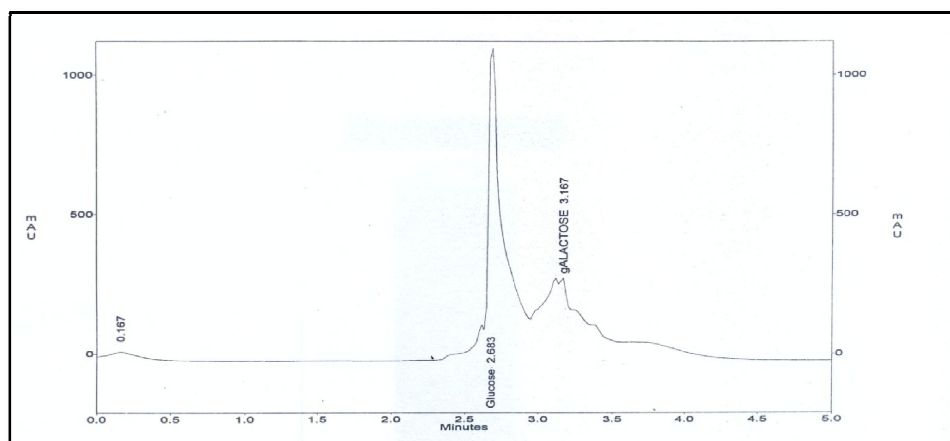


Figure 6. HPLC chromatogram showing glucose and galactose concentrations produced from *U. lactuca* hydrolysis by *P. piscicida* at retention time 2.68 and 3.17 min, respectively.

4. Discussion

Macroalgae are gaining some attention as an alternative renewable source of biomass for the production of bioethanol (Borowitzka, 1992). Some algae were represented to have high contents of carbohydrate that can be used as substrate for bioethanol production (Singh and Olsen, 2011). They also contain a low concentration of lignin (Wi *et al.*, 2009) or no lignin at all (Ge *et al.*, 2011). Therefore, the biomass of marine algae can be converted economically into simple sugars and then to bioethanol (Chynoweth, 2002; Sluiter, 2006; John *et al.*, 2011).

The green macroalgae; *Ulva lactuca* is appropriate substrate that contain 44% carbohydrates.

The marine bacterium WM21 exhibited promising enzymatic hydrolysis of polysaccharides. So, it was selected to be identified using phenotypic and genotypic analyses, respectively. It was gram-negative rod, grew at 20–45°C with pH range (6–10) and sodium chloride concentrations (2–10%). Also, catalase and oxidase were positive. Moreover, it produced gelatinase, amylase, agarase, lipase (Tween 80), and lysine decarboxylase. In addition to, it was utilizing of glucose. This isolate genotypically was *Pseudoalteromonas piscicida* with similarity 96%.

Actually, some workers isolated *Pseudoalteromonas sp.* and *P. piscicida* from different marine resources. For examples, Nelson and Ghiorse (1999) isolated *P. piscicida* from the diseased eggs of two damselfish (Pomacentridae) species; *Amphiprion clarkii* and *Amblyglyphidodon curacao*. As well as, Lau *et al.* (2005) isolated a Gram-negative, non-spore-forming, short rod-shaped bacterium (UST010723-006T) from the surface of the sponge *Mycale* adherents in Hong Kong waters. Cells of UST010723-006 did not have flagella and were non-motile. Colonies were pale orange in color, 2–4 mm in diameter, convex with a smooth surface and an entire translucent margin. Gas bubbles were observed in the colonies and also in the agar matrix underneath and adjacent to the colonies. UST010723-006 was heterotrophic, strictly aerobic and required NaCl for growth (2–6%). It grew at pH 5–10 and

between 12 and 44°C. Phylogenetic analysis of the 16S rRNA gene sequence placed UST010723-006T within the genus *Pseudoalteromonas* of the c-subclass of the Proteobacteria. These data supported the affiliation of UST010723-006T to the genus *Pseudoalteromonas*. The closest relatives were *Pseudoalteromonas luteviolacea*, *P. phenolica*, *P. rubra* and *P. ruthenica* with similarity values ranging from 95.4 to 96.8%. Molecular evidence, together with phenotypic characteristics, suggests that UST010723-006T constitutes a novel species within the genus *Pseudoalteromonas*. The name *Pseudoalteromonas spongiae* sp. is proposed for this bacterium.

Tao *et al.* (2008) examined the ability of *P. piscicida* and/or closely related species for hydrolytic enzymes secretion especially; amylase and agarase. They isolated marine bacteria producing extracellular α -amylase from seawater and identified as member of *Pseudoalterimonas* species. Matsumoto *et al.* (2003) inoculated *Pseudoalterimonas undina* NKMB 0074 into suspensions containing the green microalgae NKG 120701 cells and increasingly reduced suspended sugars with incubation time. Terrestrial amylase and glucoamylase were inactive in saline suspension. They concluded that the marine amylase is necessary in saline conditions for successful saccharification of marine microalgae.

In a complementary step of optimization saccharification, the concentrations of medium components were simultaneously investigated using Plackett-Burman experiment design. Occasionally, seven factors were examined as independent variables affecting the production of reducing sugars by *P. piscicida*. Data conducted that the highest concentration of reducing sugar recorded 144 mg/g by trial No.6 with maximization of 41 mg/g comparing to the basal condition (103 mg/g) in trail No. 9. And when applied the near optimum media we reached to reducing sugar production about 158 mg/g.

By comparing these data (158 mg/g equals 7.9 mg/ml) with the reducing sugar ratio in other investigations, it was observed that our yield is was much more than that achieved by Malek *et al.* (1988) which was (5.8 mg/ml) obtained with a

Cytophaga sp., using sugar cane bagasse as growth and hydrolysis substrates. While, Sunarti *et al.* (2010) obtained lower hydrolytic activity produced from cellulose fraction by isolate C4-4, which liberated (3.5 mg/ml) of total sugar. Yanagisawaa *et al.* (2011) used successive saccharification with an enzyme that was effectively used to obtain high concentrations of glucose from these seaweeds. They hypothesized that agar weed contained both galactan and glucan. For this reason, it was possible to obtain a high concentration of ethanol from agar weed using combined saccharification, which is the acid hydrolysis of galactan to produce galactose followed by the enzymatic hydrolysis of glucan to obtain glucose. Begum and Alimon (2011) pretreated three lignocellulosic substrates viz. sugarcane bagasse, sawdust and water hyacinth with alkali and enzyme and studied their effect on bioconversion agricultural and industrial wastes to chemical feedstock. They found that the maximum degree of conversion of substrate by *Aspergillus oryzae* ITCC (0.415%) and improved specific substrate consumption (0.99 g substrate/g dry biomass) was exhibited in sugarcane bagasse after alkali treatment at 96 h. they observed that alkali-treatment and enzyme-treatment, water hyacinth was the best for cellulase induction and showed maximum endoglucanase activity of 11.42 U/ml. Reducing sugar yield ranged from 1.12 mg/ml for enzyme treated sawdust at 48 h to 7.53 mg/ml for alkali treated sugarcane bagasse at 96 h. Alkali-treated sugarcane bagasse gave the highest saccharification rate of 9.03% after 96 h. The most resistant substrate was sawdust which produced 5.92% saccharification by alkaline treatment.

However, the current study supports the following points:

1. Marine macroalgae (*U. lactuca*) can be used as promising substrates for the bioethanol production.
2. *Pseudoalteromonas piscicida* have ability to hydrolysis of *U. lactuca* substrate for production of reducing sugars.
3. Optimization of saccharification process enhanced the reducing sugars production.

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