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Evaluation of the growth medium of a new ice-nucleating-active *Pseudomonas*: A response surface methodology approach

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ABSTRACT

Aims: Attention to ice nucleation proteins has increased for more than two decades. Ice nucleation proteins have been utilized for artificial snow-making known as Snowmax™, cryopreservation of tissues and cells, and cloud condensation nuclei. There is a direct relationship between bacterial growth and ice nucleation activity. Therefore, the optimization of the culture medium seems necessary.
Methodology and results: The effect of different carbon and nitrogen sources on the growth of a new native *Pseudomonas* sp. IRL.INP1 was evaluated by using fractional factorial design, the path of the steepest ascent experiment and central composite design. Ice nucleation activity, biomass and whole-cell protein were identified afterward. The model predicted by response surface methodology indicated that the maximum bacterial growth was observed when sucrose, ammonium sulfate [$(NH_4)_2SO_4$] and manganese (II) (Mn^{2+}) were utilized at 12.46 g/L, 321.97 mg/L and 938.09 µM, respectively. Also, 1.10 g/L biomass and 0.85 µg/µL whole-cell proteins were gained, and the isolate showed ice nucleation activity 31 sec sooner after optimization.
Conclusion, significance and impact of study: Ice nucleation proteins are growth-dependent and the growth condition optimization leads to higher bacterial cells growth. Therefore, best bacterial growth was obtained when proper carbon and nitrogen sources were used, and ice nucleation activity was observed in shorter time. This is the first study concerning ice nucleation activity optimization using different carbon and nitrogen sources.

Keywords: Ice nucleation protein, response surface methodology, *Pseudomonas*

INTRODUCTION

Ice nucleation is an expression for the formation of initial ice particles in water through a phase transition from liquid to the solid phase (Hartmann *et al*., 2013). For pure water, this phase transition happens at –40 °C (Wilson and Haymet, 2009). The term supercooled water describes liquid water at temperatures below 0 °C (Li *et al*., 2011). The supercooled water is stable and ice crystals must aggregate to reach a cluster size for ice formation and subsequently, ice is constructed promptly (Russo *et al*., 2014). This phenomenon was named ice nucleation and was divided into two different groups: homogeneous and heterogeneous ice nucleation. The components of the homogeneous ice nucleation are pure water molecules. In contrast, the heterogeneous ice nucleation is composed of some impurities in the water. Ice nucleators are define as any substances which are made from heterogeneous ice nucleation (Möhler *et al*., 2008; Beydoun *et al*., 2017). The most focus on heterogeneous ice nucleation is about the bacteria are derived from leaves (also known as plant pathogens). Originally, these plant pathogens were considered since they were potent to generate frost to damage crop plants (Lundheim, 2002; Kawahara, 2017). The bacterial ice nucleators are proteinaceous compounds that attach to the cell membrane by phosphatidylinositol (PI) as an anchor. The bacterial ice nucleation proteins are divided into three classes: types I, II and III with threshold temperatures ranges of –5 °C or warmer; –5 °C to –8 °C; and –10 °C or colder, respectively (Hew and Yang, 1992; Polen *et al*., 2016). Various epiphytic Gram-negative bacteria have been found as the ice nucleation protein producers belonging to genera *Pseudomonas*, *Erwinia* and *Xanthomonas* (Joly *et al.*, 2013).

Ice nucleation bacteria are used for snowmaking as an artificial snow maker, also known as Snowmax™ (Hartmann *et al*., 2013). The other promising applications are cryopreservation of tissues and cells in biology research and making bacterial cell-free ice nucleation activator for the food industry. Ice nucleation activators can serve as cloud condensation nuclei (CCN) and affect

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climate. In Arctic regions, paths and aircraft runways are fabricated from seawater at low temperatures and it initiates without ice nucleation in Arctic regions. *Pseudomonas syringae* is applied to decrease supercooling in seawater streams. Thus, a large amount of seawater will freeze and ice construction can occur in various temperatures. Besides, the use of ice nucleation activator as a signal transmitter in immunoassay has been studied (Lorv *et al*., 2014). Owing to the numerous uses of ice nucleation proteins, optimization of bacterial ice nucleation activity (INA) should be taken into consideration.

The published papers relating to INA optimization are limited, and this is the first study concerning INA optimization using different carbon and nitrogen sources by applying response surface methodology. Also, the effects of different metal ions on INA were studied. In this paper, a native ice nucleation bacterium, *Pseudomonas* sp. IRL.INP1 was used and given that INA by *Pseudomonas* sp. IRL.INP1 was growth-dependent, optimization of the growth conditions was considered. Also, *Pseudomonas* sp. IRL.INP1 has great ice nucleation activity belonging to the type I of ice nucleation protein which is even better than the positive control, *P. syringae* pv. *syringae*.

MATERIALS AND METHODS

Bacterial isolates

Pseudomonas syringae pv. *syringae* was received from the National Center for Genetic, and Biological Reserves, Tehran, Iran and applied as positive control in this study. The negative control *Escherichia coli* PTCC 1330 was obtained from Iran Scientific and Industrial Research Organization Tehran, Iran. The native strain *Pseudomonas* sp. IRL.INP1 was received from the Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences. It was previously isolated from the Damavand region (a cold region) in Iran and was identified biochemically and genetically.

Ice nucleation assay by frozen-droplet technique

For INA evaluation, a cooling circulator (LAUDA® Alpha RA 8 Refrigerating Circulator, Berlin, Germany) was applied to determine the freezing point. Thermal conductivity was prepared by coating the surface of the aluminum plate with paraffin and the aluminum plate was chilled at the rate of 1 °C/min. Cell suspension of 10 µL was placed on the aluminum plate and the temperature was gradually lowered. The drops at the given temperature were monitored until the droplets froze. The freezing temperature and time were registered as the temperature and time for observing INA, respectively (Snider *et al*., 2000; Tebyanian *et al*., 2014).

Medium optimization by one-factor-at-a-time experiments

The one-factor-at-a-time method was used for evaluating the factors affecting cell growth, thereby enhancing INA. The pH of medium culture was adjusted from 5 to 8 with 1 M HCl and 1 M NaOH before sterilization and the optimum temperature was evaluated by incubating the cultures at 18, 20, 25, 30 and 35 °C, respectively. The different carbon sources at 1% and nitrogen sources at 0.01% concentrations were added to the basal medium (10 g/L proteose peptone broth). The carbon sources such as glucose, sucrose, fructose, galactose, maltose, lactose, glycerol, xylose, sorbitol and cellulose were used. The nitrogen sources including ammonium nitrate (NH_4NO_3), ammonium sulfate [$(NH_4)_2SO_4$], asparagine, glycine and ammonium chloride [$(NH_4)_2Cl$] were studied. Different chloride ions (Merck®) at different concentrations from 200 to 800 µM were evaluated as media supplements including $CaCl_2$ (Ca^{2+}), $NiCl_2$ (Ni^{2+}), $ZnCl_2$ (Zn^{2+}), $MgCl_2$ (Mg^{2+}), $CuCl_2$ (Cu^{2+}), $MnCl_2$ (Mn^{2+}), $PbCl_2$ (Pb^{2+}) and $FeCl_3$ (Fe^{3+}). In each experiment, 2×10^7 CFU/mL of an overnight culture of *Pseudomonas* sp. IRL.INP1 in proteose peptone broth was inoculated (Raza *et al*., 2012; Sirajunnisa *et al*., 2016; Khomarlou *et al*., 2018).

Fractional factorial design (FFD)

After evaluating the temperature and pH based on one factor-at-a-time experiment, the temperature and pH were set at 30 °C and pH 7, respectively. Based on one factor-at-a-time experiment, the carbon and nitrogen sources including sucrose and ammonium sulfate [$(NH_4)_2SO_4$], respectively, and three ions including Ca^{2+}, Mn^{2+} and Ni^{2+}, which had the highest impact on microbial growth (OD_{600}) were selected. Then, FFD was employed to screen the most substantial factors. The 5 main factors, including Ca^{2+}, Mn^{2+}, Ni^{2+}, sucrose and ammonium sulfate [$(NH_4)_2SO_4$] were designated as X_1, X_2, X_3, X_4 and X_5, respectively. Each factor was tested at two levels (–1 for low level and +1 for high level) and a center point (0). An FFD was regressed by Minitab statistical software (V 17.0) to generate the first-order polynomial Equation (1).

$$Y = \beta_0 + \Sigma\beta_i X_i \quad (1)$$

Where Y is the response (bacterial growth), β_0 is the model intercept, β_i is the linear coefficient and X_i is the level of the independent variable (Raza *et al*., 2012; Heidari *et al*., 2019).

Path of steepest ascent experiment

The influential factors (sucrose, ammonium sulfate and Mn^{2+}) were selected by FFD and they were more optimized by applying the steepest ascent design to lead the experimenter close towards the vicinity of the optimum of the surface. The direction of the steepest ascent is the direction in which Y enhances most quickly.

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This process is stopped until the response does not increase anymore. This spot would be in the vicinity of the optimum and can be employed as a middle spot for further optimization by central composite design. In this study, the increasing concentrations of sucrose, ammonium sulfate and Mn^{2+} were used. The concentrations increased until the point in which the response does not increase anymore (Fung *et al*., 2008; Raza *et al*., 2012).

Central composite design

A central composite design was carried out to optimize the three variables selected by FFD, which significantly influenced the bacterial growth. The three independent variables (sucrose, ammonium sulfate and Mn^{2+}) were judged at five levels (−1.68, −1, 0, +1 and +1.68) by 20 experiments. The experiments were carried out in 300 mL Erlenmeyer flasks with 150 mL of media, under agitating state 30 °C for 24 h, prepared according to the CCD design. The obtained response could be expressed by a second-degree polynomial equation as Equation (2).

$$Y = \beta_0 + \sum_{i=1}^{n}\beta_i X_i + \sum_{i<j}^{n}\beta_{ij} X_i X_j + \sum_{j=1}^{n}\beta_{jj} X_j^2 \quad i = 1, 2, ..., k \qquad (2)$$

Where Y is the predicted response, β_0 is the intercept, X_i and X_j are the none-coded independent factors influencing the response variable Y, β_i is the linear effect of X_i, β_{ij} is the second-order interaction and β_{jj} is the quadratic coefficient. In this study, three variables were included. Therefore, n took the value 3. By substituting the value 3 for n in Equation (2), Equation (3) was obtained.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \qquad (3)$$

Where Y was the predicted response, β_0 was a constant, X_1, X_2, and X_3 were input variables, β_1, β_2 and β_3 were the linear coefficients, β_{12}, β_{13} and β_{23} were the second-order interactive coefficients and β_{11}, β_{22} and β_{33} were quadratic coefficients (Raza *et al*., 2012; Sirajunnisa *et al*., 2016). To validate the model, four additional experiments were carried out using the concentrations predicted by the software in shake flasks under the optimum medium compositions. Experimental designs and data analysis were obtained by Minitab statistical software (V 17.0).

Biomass and cellular protein determination

An aliquot (50 mL) of the culture sample was collected from the flask and centrifuged at 4 °C, 8,000 rpm for 15 min. An oven was employed at 105 °C for 4 h for drying the bacterial cells, then the weight of the cells was measured. To estimate cellular protein, the cell pellet was washed with distilled water and tetrahydrofuran. The cells were ruptured by adding 1 N NaOH and were heated at 90 °C in a water-bath for 5 min. The Bradford micro-assay was applied for whole-cell proteins determination. After adding a Bradford reagent, the dye of the protein solution was changed to violet. The absorbance of the solution was obtained at 592 nm by a UV-VIS spectrophotometer. The quantity of protein was determined by comparison the absorbance of the samples with a calibration curve of BSA (Raza *et al*., 2007; Kalapat *et al*., 2009; Kumar *et al*., 2016).

RESULTS

One-factor-at-a-time experiments

In the beginning, INA of the native isolate, *Pseudomonas* sp. IRL.INP1 was compared with the positive control *P. syringae* pv. *syringae*. *Pseudomonas* sp. IRL.INP1 was qualified for INA even better than positive control *P. syringae* pv. *syringae* (Table 1). The optimal condition for bacterial growth were pH 7, at 30 °C (Table 2). Then, the effect of several carbon and nitrogen sources on INA by *Pseudomonas* sp. IRL.INP1 were studied. The results demonstrated that maximum growth was achieved by sucrose as the carbon source, followed by glucose and sorbitol. Also, the best nitrogen source was ammonium sulfate (Table 3). Since we were seeking a cost-effective nitrogen source and concerning that ammonium sulfate is a suitable nitrogen source for most bacteria, proteose peptone broth was selected as a basal medium with the additional of ammonium sulfate. Due to the effect of metal ions on INA, several ions including Ca^{2+}, Ni^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Pb^{2+} and Fe^{3+} at different concentrations from 200 to 800 µM were investigated. From Table 4, it shows higher concentration (> 500 µM) of metal ions (Zn^{2+}, Mg^{2+}, Cu^{2+}, Pb^{2+} and Fe^{3+}) exhibit inhibitory effect for the bacterial growth. However, it does not inhibit the growth of *Pseudomonas* sp. IRL.INP1 when Ca^{2+}, Ni^{2+} and Mn^{2+} was added. Before optimization, the biomass and whole-cell proteins of inoculated proteose peptone broth as the basal medium were evaluated, and 0.5 g/L biomass and

Table 1: The effect of different temperatures on the ice nucleation activity and the time for observing ice nucleation activity before the optimization.

Strains	Time for observing ice nucleation activity at a given temperature		
	–5 °C	–7 °C	–9 °C
Pseudomonas sp. IRL.INP1	51 sec	20 sec	8 sec
Pseudomonas. syringae pv. *syringae* (Positive control)	–	23 sec	11 sec
Escherichia coli PTCC 1330 (Negative control)	–	–	–

(–) Not having ice nucleation activity.

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Table 2: The effect of different temperature and pH on the growth of *Pseudomonas* sp. IRL.INP1 (OD_{600}) after 24 h.

	Temperature (°C)				
	18	20	25	30	35
OD_{600}	0.22	0.56	0.82	1.26	1.10
	pH				
	5	6	7	8	
OD_{600}	0.8	1.45	1.51	1.301	

Table 3: The effect of different carbon and nitrogen compounds on the growth of *Pseudomonas* sp. IRL.INP1 after 24 h.

Carbon sources	OD_{600}	Nitrogen sources	OD_{600}
Glucose	0.57 ± 0.09	NH_4NO_3	0.30 ± 0.03
Sucrose	0.72 ± 0.12	$(NH_4)_2SO_4$	0.55 ± 0.02
Fructose	0.32 ± 0.05	Asparagine	0.30 ± 0.04
Galactose	0.31 ± 0.07	Glycine	0.34 ± 0.05
Maltose	0.24 ± 0.06	$(NH_4)_2Cl$	0.12 ± 0.05
Lactose	0.22 ± 0.01		
Glycerol	0.51 ± 0.18		
Xylose	0.28 ± 0.03		
Sorbitol	0.56 ± 0.09		
Cellulose	0.41 ± 0.05		

Data is presented as the mean value ± SD of three replicates.

Table 4: The effect of different metal ions on OD_{600} of *Pseudomonas* sp. IRL.INP1 after 24 h in basal medium containing 10 g/L proteose peptone broth.

Metal ion	Control	200 µM	300 µM	400 µM	500 µM	600 µM	700 µM	800 µM
Cu^{2+}	0.37 ± 0.03	0.35 ± 0.02	0.33 ± 0.03	0.30 ± 0.05	0.41 ± 0.02	0.38 ± 0.02	0.30 ± 0.09	0.20 ± 0.03
Ni^{2+}	0.33 ± 0.01	0.30 ± 0.03	0.32 ± 0.02	0.35 ± 0.01	0.40 ± 0.02	0.42 ± 0.02	0.48 ± 0.05	0.50 ± 0.01
Pb^{2+}	0.34 ± 0.01	0.31 ± 0.02	0.30 ± 0.03	0.34 ± 0.05	0.41 ± 0.03	0.40 ± 0.04	0.35 ± 0.05	0.20 ± 0.01
Mg^{2+}	0.34 ± 0.01	0.33 ± 0.03	0.30 ± 0.01	0.30 ± 0.03	0.31 ± 0.03	0.20 ± 0.05	0.26 ± 0.02	0.15 ± 0.01
Fe^{3+}	0.31 ± 0.02	0.30 ± 0.05	0.36 ± 0.02	0.34 ± 0.01	0.30 ± 0.03	0.20 ± 0.01	0.12 ± 0.01	0.10 ± 0.02
Ca^{2+}	0.30 ± 0.02	0.41 ± 0.04	0.50 ± 0.01	0.52 ± 0.04	0.50 ± 0.01	0.48 ± 0.04	0.44 ± 0.05	0.48 ± 0.01
Zn^{2+}	0.27 ± 0.03	0.34 ± 0.02	0.41 ± 0.03	0.30 ± 0.01	0.34 ± 0.02	0.25 ± 0.02	0.17 ± 0.03	0.10 ± 0.05
Mn^{2+}	0.35 ± 0.04	0.35 ± 0.01	0.38 ± 0.04	0.40 ± 0.01	0.46 ± 0.05	0.50 ± 0.02	0.55 ± 0.05	0.53 ± 0.01

The OD_{600} data is presented as the mean value ± SD of three replicates.

0.25 µg/µL whole-cell proteins were gained.

Fractional factorial design (FFD)

The screening of significant nutrients was carried out using a fractional factorial design. By using one factor-at-a-time experiment, sucrose, ammonium sulfate and metal ions, including Ca^{2+}, Mn^{2+} and Ni^{2+} were chosen for further optimization (Table 5). Temperature (30°C), pH (7) and the time of incubation (24 h) were kept constant, and the main factors were designated as Ca^{2+} (X_1), Mn^{2+} (X_2), Ni^{2+} (X_3), sucrose (X_4) and ammonium sulfate(X_5). Except for Ni^{2+} and Ca^{2+}, all the other selected variables represented positive effects on bacterial growth. Additionally, the effect of ammonium sulfate (P = 0.016), sucrose (P = 0.000) and Mn^{2+} (P = 0.010) were significant, while the other two variables (Ni^{2+} and Ca^{2+}) presented non-significant influence at 95% confidence interval (Table 6). The second-order polynomial equation was obtained as Equation (4).

$$Y = 0.22 - 0.000167 X_1 + 0.000487 X_2 - 0.000063 X_3 + 0.1625 X_4 + 0.000900 X_5 - 0.0850 Ct\,Pt \quad (4)$$

Results showed that the model was significant (P = 0.000). The R-squared (R^2) of the model was 0.9138, representing that 91.38% of the variability in the response could be described by the model. The adjusted R^2 of the model was 87.06% (Table 6).

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Table 5: Fractional factorial design and responses after 24 h.

Runs	Ca^{2+} (µM)	Mn^{2+} (µM)	Ni^{2+} (µM)	Sucrose (g/L)	Ammonium sulfate (mg/L)	OD_{600}	Predicted OD_{600}*
1	400 (−1)	700 (−1)	800 (−1)	3 (−1)	200 (+1)	0.86	0.87
2	700 (+1)	700 (−1)	1000 (+1)	3 (−1)	200 (+1)	0.80	0.81
3	700 (+1)	900 (+1)	1000 (+1)	3 (−1)	100 (-1)	0.85	0.82
4	700 (+1)	700 (−1)	800 (−1)	3 (−1)	100 (-1)	0.79	0.73
5	400 (−1)	900 (+1)	800 (−1)	3 (−1)	100 (-1)	0.90	0.88
6	400 (−1)	900 (+1)	1000 (+1)	3(−1)	200 (+1)	0.98	0.96
7	700 (+1)	700 (−1)	800 (−1)	5 (+1)	200 (+1)	1.18	1.15
8	400 (−1)	700 (−1)	800 (−1)	5 (+1)	100 (-1)	1.10	1.11
9	700 (+1)	900 (+1)	800 (−1)	3 (−1)	200 (+1)	0.91	0.92
10	400 (−1)	900 (+1)	1000 (+1)	5 (+1)	100 (-1)	1.26	1.19
11	400 (−1)	700 (−1)	1000 (+1)	3 (−1)	100 (-1)	0.65	0.77
12	700 (+1)	700 (−1)	1000 (+1)	5 (+1)	100 (-1)	1.03	1.05
13	550 (0)	800 (0)	900 (0)	4 (0)	150 (0)	0.89	0.92
14	700 (+1)	900 (+1)	1000 (+1)	5 (+1)	200 (+1)	1.18	1.23
15	550 (0)	800 (0)	900 (0)	4 (0)	150 (0)	0.86	0.92
16	400 (−1)	900 (+1)	800 (−1)	5 (+1)	200 (+1)	1.25	1.3
17	550 (0)	800 (0)	900 (0)	4 (0)	150 (0)	1.01	0.92
18	700 (+1)	900 (+1)	800 (−1)	5 (+1)	100 (-1)	1.10	1.16
19	400 (−1)	700 (−1)	1000 (+1)	5 (+1)	200 (+1)	1.24	1.19

OD values are rounded into 2 decimal places.

Table 6: ANOVA analysis for the fractional factorial design.

Source	DF	Adj SS	Adj MS	*F*-value	*P*-value
Model	6	0.521803	0.086967	21.19	0.000
Linear	5	0.50355	0.10071	24.54	0.000
X_1*	1	0.01	0.01	2.44	0.145
X_2*	1	0.038025	0.038025	9.26	0.01
X_3*	1	0.000625	0.000625	0.15	0.703
X_4*	1	0.4225	0.4225	102.94	0.000
X_5*	1	0.0324	0.0324	7.89	0.016
Curvature	1	0.018253	0.018253	4.45	0.057
Error	12	0.04925	0.004104		
Lack-of-Fit	10	0.03665	0.003665	0.58	0.772
Pure Error	2	0.0126	0.0063		
Total	18	0.571053			
Model summary					
R-squared (R^2)				91.38%	
Adjusted R^2				87.06%	

*X_1, X_2, X_3, X_4 and X_5 are Ca^{2+}, Mn^{2+}, Ni^{2+}, sucrose and ammonium sulfate, respectively.

Path of steepest ascent experiment

The steepest ascent test was carried out to find the best point with the highest response. The method was originated from the maximum point of FFD (Table 5) (run 16: +1 for high levels of FFD, which were 5 g/L of sucrose, 200 mg/L ammonium sulfate and 900 µM of Mn^{2+}) and the concentrations of sucrose, ammonium sulfate and Mn^{2+} were increased from the concentrations above the maximum point of FFD (Table 7). As a result, optical density (OD_{600}) was enhanced at each step of the steepest ascent till run 3 that the highest growth was obtained (Table 7) and a reduction was observed afterward. The highest OD_{600} was reached at 1.35, with the combination of sucrose (11 g/L), ammonium sulfate (350 mg/L) and Mn^{2+} (960 µM). Therefore, run 3 was chosen for the CCD experiment.

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Table 7: Experimental design and results of the steepest ascent path experiment after 24 h of the increasing concentrations of sucrose, ammonium sulfate and Mn^{2+} and their effects on the optical density at 600 nm.

Run	Sucrose (g/L)	Ammonium sulfate (mg/L)	Mn^{2+} (µM)	OD_{600}
1	7	250	910	1.31
2	9	300	935	1.27
3	11	350	960	1.35
4	13	400	985	1.30
5	15	450	1010	0.93

Central composite design (CCD)

The concentrations of the three variables were fixed at the center of the results which were gained from the steepest ascent path experiment. The respective high and low levels with the coded levels of the three factors are shown in Table 8. A second-order polynomial equation by multiple regression analysis was obtained as shown below Equation (5).

$$Y = -15.8 - 0.178X_1 + 0.00393X_2 + 0.0379X_3 - 0.01162X_1X_1 - 0.000021X_2X_2 - 0.000026X_3X_3 - 0.000187X_1X_2 + 0.000563X_1X_3 + 0.000012X_2X_3 \quad (5)$$

Where Y is the bacterial growth, X_1, X_2 and X_3 are sucrose, ammonium sulfate and Mn^{2+}, respectively. The values for R^2 and adjusted R^2 were 93.09% and 86.86%, respectively, indicating the sample variance of 93.09% was allocated to the variables, and only 6.91% of the total variance could not be described by the model. The ANOVA for the model (Table 9) indicates that the regression was statistically significant (P = 0.000) at the 95% confidence level. Statistically, an insignificant lack of fit (P = 0.544) indicates that the model is sufficient for prediction within the range of variables selected. The coefficient values of Equation (5) shows that the linear coefficients (X_1, X_2 and X_3), quadratic term coefficients (X_1^2 and X_2^2) and X_1X_3 were significant. A visual analysis of the interactions among variables is provided by three-dimensional response surface plots. Maximum levels of all variables for the highest bacterial growth and pairwise interactions of the two variables are illustrated by these plots (Figure 1). The optimal concentration of sucrose, ammonium sulfate and Mn^{2+} were predicted at 12.46 g/L, 321.97 mg/L and 938.09 µM, respectively. The maximum bacterial growth was estimated to be 1.46 at OD_{600}. To validate the model, four additional experiments were carried out in shake flasks under the optimum medium compositions. The mean value gained at OD_{600} was 1.41 (Table 10), which was close to the predicted response. Also, the 1.10 g/L biomass and 0.85 µg/µL whole-cell proteins were gained.

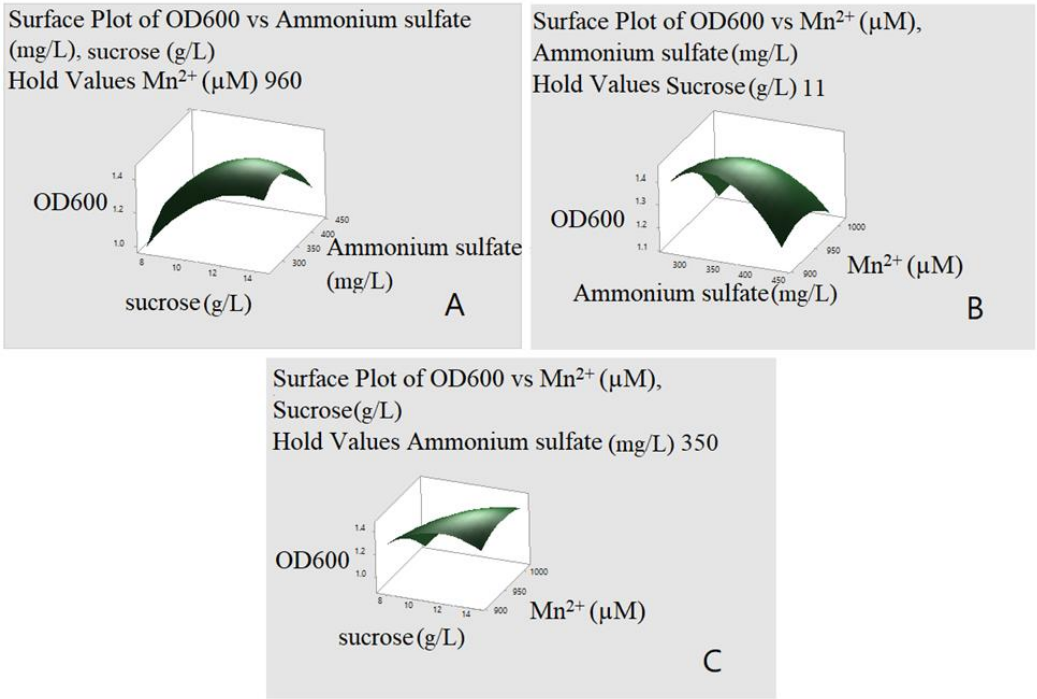

Figure 1: Response surface 3D plots of mutual interactions of (A) sucrose and ammonium sulfate, (B) ammonium sulfate and Mn^{2+} and (C) sucrose and Mn^{2+} towards bacterial growth.

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Table 8: Central composite design arrangements and responses after 24 h.

Runs	Sucrose (g/L)	Ammonium sulfate (mg/L)	Mn^{2+} (µM)	OD_{600}	Predicted OD_{600}*
1	13 (+1)	300 (−1)	990 (+1)	1.35	1.39
2	13 (+1)	400 (+1)	990 (+1)	1.30	1.33
3	9 (−1)	300 (−1)	930 (−1)	1.33	1.32
4	9 (−1)	400 (+1)	930 (−1)	1.28	1.26
5	9 (−1)	400 (+1)	990 (+1)	1.12	1.14
6	14.36 (+1.68)	350 (0)	960 (0)	1.45	1.42
7	7.63 (−1.68)	350 (0)	960 (0)	1.13	1.15
8	13 (+1)	400 (+1)	930 (−1)	1.32	1.32
9	11 (0)	350 (0)	960 (0)	1.36	1.42
10	9 (−1)	300 (−1)	990 (+1)	1.10	1.12
11	11 (0)	350 (0)	960 (0)	1.43	1.42
12	11 (0)	350 (0)	960 (0)	1.40	1.42
13	11 (0)	350 (0)	960 (0)	1.37	1.42
14	11 (0)	350 (0)	960(0)	1.43	1.42
15	11 (0)	265 (−1.68)	960 (0)	1.34	1.32
16	11 (0)	350 (0)	960 (0)	1.47	1.42
17	11 (0)	434 (+1.68)	960 (0)	1.21	1.22
18	11 (0)	350 (0)	1010.45 (+1.68)	1.31	1.27
19	13 (+1)	300 (−1)	930 (−1)	1.45	1.46
20	11 (0)	350 (0)	909.55 (−1.68)	1.40	1.43

*OD_{600} values are rounded into 2 decimal places.

Table 9: Analysis of variance of predictive equation for the bacterial growth by *Pseudomonas* sp. IRL.INP1.

Source	DF	Adj SS	Adj MS	*F*-value	*P*-value
Model	9	0.220032	0.024448	14.96	0.000**
Linear	3	0.138678	0.046226	28.28	0.000**
X_1	1	0.093197	0.093197	57.02	0.000**
X_2	1	0.013453	0.013453	8.23	0.017*
X_3	1	0.032028	0.032028	19.6	0.001**
Square	3	0.066616	0.022205	13.59	0.001**
X_1^2	1	0.031117	0.031117	19.04	0.001**
X_2^2	1	0.038625	0.038625	23.63	0.001**
X_3^2	1	0.007949	0.007949	4.86	0.052 NS
2-way interaction	3	0.014738	0.004913	3.01	0.081 NS
X_1X_2	1	0.002813	0.002813	1.72	0.219 NS
X_1X_3	1	0.009113	0.009113	5.58	0.04*
X_2X_3	1	0.002812	0.002812	1.72	0.219 NS
Error	10	0.016343	0.001634		
Lack-of-fit	5	0.007743	0.001549	0.9	0.544 NS
Pure error	5	0.0086	0.00172		
Total	19	0.236375			

*<0.05; **<0.01; NS: non-significant.

Ice nucleation assay by frozen-droplet technique

INA was tested for the isolate after growing in the optimized culture medium containing sucrose, ammonium sulfate and Mn^{2+} at 12.46 g/L, 321.97 mg/L and 938.09 µM, respectively. The isolate exhibited type I of ice nucleation protein with threshold temperature ranges of −5 °C or warmer. Also, INA was observed after 20 sec which was 31 sec sooner compared to before optimization (51 sec) (Figure 2).

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Table 10: The predicted optimal concentration of sucrose, ammonium sulfate and Mn^{2+} by Minitab statistical software (V 17.0) and the response of the predicted bacterial growth and the mean of actual values of four additional experiments in shake flasks under the optimum medium compositions to validate the model predicted by software.

Multiple Response Prediction	
Variable	Setting
Predicted sucrose (g/L)	12.46
Predicted ammonium sulfate (mg/L)	321.97
Predicted Mn^{2+} (µM)	938.09
Response	
Predicted Response (OD_{600})	1.46
The mean actual values of four additional experiments	1.41

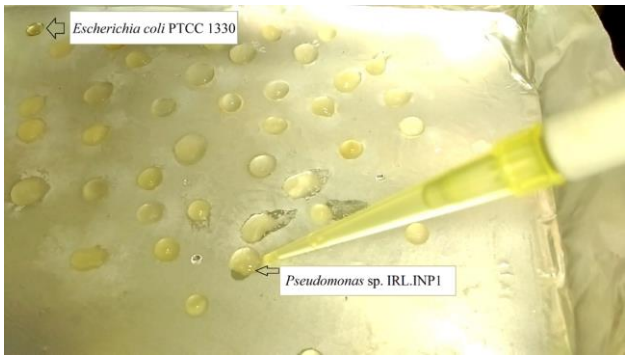

Figure 2: Ice nucleation assay by the frozen-droplet technique. LAUDA® Alpha RA 8 Refrigerating Circulator was used to evaluate ice nucleation activity for each sample. All droplets of *Pseudomonas* sp. IRL.INP1 froze after growing in the optimized medium at –5 °C in 20 sec. The negative control (*Escherichia coli* PTCC 1330) has not frozen at the given temperature.

DISCUSSION

It is demonstrated that INA is growth-dependent and cell concentration affects ice nucleation efficiency. It was demonstrated that INA in *Erwinia herbicola* and *P. syringae* were associated with bacterial cell concentration, the age of the bacteria and the culture condition (Möhler *et al.*, 2008). The temperature has a profound impact on ice nuclei, especially type I. In the present study, the maximum bacterial growth was obtained after 24 h at 30 °C and pH 7. Temperature was almost in agreement with the findings by Mortazavi *et al*. (2008) which was 35 °C for their experiments (Mortazavi *et al*., 2008; Zolfaghar *et al*., 2019). Snider *et al*. (2000) studied the growth temperature of several different snow molds and reported that the growth temperature was from –5 to 4 °C and the highest threshold ice nucleation temperature was at –7 °C. Isolates of *Pseudomonas borealis* were identified after the ice-affinity selection of soil samples collected in summer. *P. borealis* DL7 was further characterized and was shown its INA after growing at 22 °C, but considerably more activity at 4 °C or 8 °C (Wu *et al*., 2009). The differences between our results and the mentioned findings might be due to different types of ice nucleation proteins or different strains used. pH is a substantial factor affecting cell growth and INA. pH acid affects living bacteria and non-living bacteria, such as the bacteria that have turned into dry ice with ice nucleation features (Zonouri *et al*., 2015).

The impacts of different carbon and nitrogen sources on INA were studied. Based on the fractional factorial design, sucrose, ammonium sulfate and Mn^{2+} had a significant influence on bacterial growth. Blondeaux and Cochet (1994) studied the impact of glucose as a low-cost media component supplemented with wheat bran. Wheat bran contains the phosphatidylinositol precursor, thereby exposing the protein on the cell membrane. Results demonstrated that the presence of the additional glucose was indispensable for the growth and ice-nucleating activity of *P. syringae.* They reported that the optimal substrate concentrations for INA and bacterial growth were 50–80 g/L of glucose and 28 g/L of peptone. They also proved that the maximum biomass production and INA by *P. syringae* are correlated to the C/N ratio (optimal ratio: 10) rather than the substrate concentration (Blondeaux and Cochet, 1994; Kafshgari *et al.*, 2019). Carbon limitation reduced nucleation activity of *P. fluorescens*, *Xanthomonas* spp. and to some extent *Erwinia* spp. Nitrogen restriction affected all groups greatly and reduced their INA, except for *P. fluorescens* and *P. syringae* which were only slightly affected (Nejad *et al.*, 2006). In our study, the maximum growth was gained by using sucrose. Sucrose is a common sugar produced naturally in plants and it is compatible with the strain isolated from plants. To take glucose for central metabolism, *P. syringae* requires to produce both sucrose-utilizing levansucrase and plant cell wall-degrading glycosyl hydrolase (Srivastava *et al.*, 2012). *Pseudomonas syringae* pv. *glycinea* PG4180 invades the leaf tissue through stomata or open scars, leading to the bacterial disease on soybean plants. Therefore, the bacteria encounter a sucrose-rich milieu. To utilize sucrose, levansucrase is produced to liberate glucose (Mehmood *et al*., 2015).

Our results showed that the qualified nitrogen source was ammonium sulfate. Wu *et al*. (2009) evaluated the capability of *P. borealis* DL7 for INA after cultivating in tryptic soy broth (TSB) as a nitrogen source. de Araujo *et al*. (2019) demonstrated that nutritional deprivation resulted in the rapid induction of ice nuclei. Considering

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that ammonium is an efficient nitrogen source for most bacteria, the impacts of molar ammonium concentrations on several model bacteria specifically, *Corynebacterium glutamicum*, *E. coli* and *Bacillus subtilis* were investigated. The studied bacteria were highly resistant to ammonium. Results demonstrated impaired growth was due to the enhanced osmolarity rather than the toxicity of ammonium sulfate (Müller *et al*., 2006). Ammonium is an efficient nitrogen source for most bacteria and previous studies proved that the media with a limited level of nitrogen coupled with a temperature shifting to 4 °C for 12 h increases the INA protein expression (Šantl-Temkiv *et al*., 2015). Therefore, the selection of ammonium sulfate as a nitrogen source due to its inorganic nature is proper.

In the present study, based on the *P-value* calculated by fractional factorial design (Table 6), Mn^{2+} had the most effect on bacterial growth, thereby enhancing INA. To maximize INA, components that corporate in the assembly of the ice-nucleating site such as Mn^{2+} ions and inositol should be added to the culture medium (Lagriffoul *et al.*, 2010). Blondeaux *et al*. (1999) used a culture medium that included glucose and meat peptone supplemented with a salt solution including calcium carbonate, zinc sulfate hexahydrate, iron (III) chloride hexahydrate, manganese (II) chloride, copper (II) chloride dihydrate, calcium chloride hexahydrate, phosphoric acid, magnesium oxide and sodium molybdate dehydrate to prepare the growth condition for *P. syringae* and to evaluate the influence of vegetable oils on the ice-nucleating activity of *P. syringae*.

In this study, before optimization, the biomass and whole-cell proteins of inoculated proteose peptone broth as the basal medium were evaluated, and 0.5 g/L biomass and 0.25 µg/µL whole-cell proteins were gained. After optimization, the optimum concentrations of sucrose, ammonium sulfate and Mn^{2+} at 12.46 g/L, 321.97 mg/L and 938.09 µM, respectively, led to 1.10 g/L biomass and 0.85 µg/µL whole-cell proteins and INA was observed after 20 sec. Song *et al.* (2004) studied the effect of mustard seeds, which were added in the medium for the ice nucleation active bacterium, *Xanthomonas ampelina* TS206. It was shown that the activity of the bacteria enhanced by 37.5% and the biomass is increased by 36.2% (Song *et al.*, 2004). Bassi and Margaritis (1996) evaluated the effect of different concentrations of sucrose on cell concentration and INA of *P. syringae* pv. *syringae* during batch cultivation at 25 °C and pH 7.0. The maximum biomass and INA were achieved with 5 g/L sucrose and the final biomass was 1.2 g/L (Bassi and Margaritis, 1996). Cochet *et al*. (1994) used some commercial culture media for the optimization of bacterial growth. Among the commercial culture media, the biomass production in malt extract and meat peptone was 0.76 g/L and 5.69 g/L, respectively, and the best INA was achieved by using meat peptone with the highest biomass production. Kvíderová *et al*. (2013) showed the effect of bacterial biomass on INA. With the higher bacterial concentration, the high INA of the snow alga *Chloromonas nivalis* was confirmed (Kvíderová *et al*., 2013). The short time for the observation of INA is evidence of high ice activity, and it presents the efficiency of INA (Wisniewski *et al*., 1997; Murray *et al*., 2012).

This study aims to optimize the growth conditions and ice nucleation activity of a native species with a high INA for industrial purposes, including snowmaking and cloud condensation nuclei (CCN) which affect climate. For this purpose, finding a relatively cost-effective culture medium that supports bacterial growth is required, and in this study, several simple carbon and nitrogen sources were investigated. It is also recommended to use other sources of carbon and nitrogen obtained from food and agricultural wastes such as whey and molasses to provide a cost-effective culture medium.

CONCLUSION

Pseudomonas sp. IRL.INP1 is a newly isolated strain with high ice nucleation activity. In this study, there is a direct relationship between bacterial growth and ice nucleation activity. *Pseudomonas* sp. IRL.INP1 shows significantly higher ice nucleation activity after growing in the optimized medium containing sucrose, ammonium sulfate and Mn^{2+} at 12.46 g/L, 321.97mg/L and 938.09 µM, respectively. By using these optimize concentrations, 1.10 g/L biomass and 0.85 µg/µL whole-cell proteins were gained, and the isolate showed ice nucleation activity 31 seconds sooner after optimization.

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REFERENCES

Bassi, A. S. and Margaritis, A. (1996). Cell growth kinetics and ice nucleation activity of *Pseudomonas syringae*. *The Canadian Journal of Chemical Engineering* **74(2), 301-307.**

Beydoun, H., Polen, M. and Sullivan, R. C. (2017). A new multicomponent heterogeneous ice nucleation model and its application to Snomax bacterial particles and a Snomax-illite mineral particle mixture. *Atmospheric Chemistry and Physics* **17(22), 13545-13557.**

Blondeaux, A. and Cochet, N. (1994). High-level expression of the ice-nucleating activity of *Pseudomonas syringae* in relation to its growth characteristics. *Applied Microbiology and Biotechnology* **42(1), 116-120.**

Blondeaux, A., Hamel, J., Widehem, P. and Cochet, N. (1999). Influence of water activity on the ice-nucleating activity of *Pseudomonas syringae*. *Journal of Industrial Microbiology and Biotechnology* **23(6), 514-519.**

Cochet, N., Blanc, C., Luquet, M. P., Bouabdillah, D. and Clausse, D. (1994). Ice nucleation by bacteria: Production and activity. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **83(3), 187-191.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

de Araujo, G. G., Rodrigues, F., Gonçalves, F. L. T. and Galante, D. (2019). Survival and ice nucleation activity of *Pseudomonas syringae* strains exposed to simulated high-altitude atmospheric conditions. *Scientific Reports* **9(1), 7768.**

Fung, W. Y., Woo, Y. P. and Liong, M. T. (2008). Optimization of growth of *Lactobacillus acidophilus* FTCC 0291 and evaluation of growth characteristics in soy whey medium: A response surface methodology approach. *Journal of Agricultural and Food Chemistry* **56(17), 7910-7918.**

Hartmann, S., Augustin, S., Clauss, T., Wex, H., Šantl-Temkiv, T., Voigtländer, J., Niedermeier, D. and Stratmann, F. (2013). Immersion freezing of ice nucleation active protein complexes. *Atmospheric Chemistry and Physics* **13(11), 5751-5766.**

Heidari, M. F., Arab, S. S., Noroozi-Aghideh, A., Tebyanian, H. and Latifi, A. M. (2019). Evaluation of the substitutions in 212, 342 and 215 amino acid positions in binding site of organophosphorus acid anhydrolase using the molecular docking and laboratory analysis. *Bratislava Medical Journal* **120(2), 139-143.**

Hew, C. L. and Yang, D. S. (1992). Protein interaction with ice. *European Journal of Biochemistry* **203(1-2), 33-42.**

Joly, M., Attard, E., Sancelme, M., Deguillaume, L., Guilbaud, C., Morris, C. E., Amato, P. and Delort, A. (2013). Ice nucleation activity of bacteria isolated from cloud water. *Atmospheric Environment* **70, 392-400.**

Kafshgari, H. S., Yazdanian, M., Ranjbar, R., Tahmasebi, E., Mirsaeed, S. R. G., Tebyanian, H., Ebrahimzadeh, M. A. and Goli, H. R. (2019). The effect of *Citrullus colocynthis* extracts on *Streptococcus mutans*, *Candida albicans*, normal gingival fibroblast and breast cancer cells. *Journal of Biological Research* **92(1), 8201.**

Kalapat, N., Watthanachote, L. and Nipithakul, T. (2009). Extraction and characterization of proteins from skim rubber. *Agriculture and Natural Resources* **43(5), 319-325.**

Kawahara, H. (2017). Cryoprotectants and ice-binding proteins. *In*: Psychrophiles: From Biodiversity to Biotechnology. Margesin, R. (ed.). Springer, Cham. **pp. 237-257.**

Khomarlou, N., Aberoomand-Azar, P., Lashgari, A. P., Tebyanian, H., Hakakian, A., Ranjbar, R. and Ayatollahi, S. A. (2018). Essential oil composition and *in vitro* antibacterial activity of *Chenopodium album* subsp. *striatum*. *Acta Biologica Hungarica* **69(2), 144-155.**

Kumar, G., Srivastava, R., Sharma, J. G., Singh, R. and Singh, V. (2016). Molasses-based growth and lipid production by *Chlorella pyrenoidosa*: A potential feedstock for biodiesel. *International Journal of Green Energy* **13(3), 320-327.**

Kvíderová, J., Hájek, J. and Worland, R. M. (2013). The ice nucleation activity of extremophilic algae. *CryoLetters* **34(2), 137-148.**

Lagriffoul, A., Boudenne, J., Absi, R., Ballet, J., Berjeaud, J., Chevalier, S., Creppy, E. E., Gilli, E., Gadonna, J., Gadonna-Widehem, P., Morris, C. E. and Sylvie, Z. (2010). Bacterial-based additives for the production of artificial snow: What are the risks to human health? *Science of the Total Environment* **408(7), 1659-1666.**

Li, T., Donadio, D., Russo, G. and Galli, G. (2011). Homogeneous ice nucleation from supercooled water. *Physical Chemistry Chemical Physics* **13(44), 19807-19813.**

Lorv, J. S., Rose, D. R. and Glick, B. R. (2014). Bacterial ice crystal controlling proteins. *Scientifica* **2014, 976895.**

Lundheim, R. (2002). Physiological and ecological significance of biological ice nucleators. *Philosophical Transactions of the Royal Society B: Biological Sciences* **357(1423), 937-943.**

Mehmood, A., Abdallah, K., Khandekar, S., Zhurina, D., Srivastava, A., Al-Karablieh, N., Alfaro-Espinoza, G., Pletzer, D. and Ullrich, M. S. (2015). Expression of extra-cellular levansucrase in *Pseudomonas syringae* is controlled by the *in planta* fitness-promoting metabolic repressor HexR. *BMC Microbiology* **15, 48.**

Möhler, O., Georgakopoulos, D. G., Morris, C. E., Benz, S., Ebert, V., Hunsmann, S., Saathoff, H., Schnaiter, M. and Wagner, R. (2008). Heterogeneous ice nucleation activity of bacteria: New laboratory experiments at simulated cloud conditions. *Biogeosciences* **5(5), 1425-1435.**

Mortazavi, R., Hayes, C. T. and Ariya, P. A. (2008). Ice nucleation activity of bacteria isolated from snow compared with organic and inorganic substrates. *Environmental Chemistry* **5(6), 373-381.**

Müller, T., Walter, B., Wirtz, A. and Burkovski, A. (2006). Ammonium toxicity in bacteria. *Current Microbiology* **52(5), 400-406.**

Murray, B. J., O'sullivan, D., Atkinson, J. D. and Webb, M. E. (2012). Ice nucleation by particles immersed in supercooled cloud droplets. *Chemical Society Reviews* **41(19), 6519-6554.**

Nejad, P., Granhall, U. and Ramstedt, M. (2006). Factors influencing pathogenic ice-nucleation active (INA) bacteria isolated from *Salix* plants, soil and litter. *Journal of Agricultural Technology* **1, 207-222.**

Polen, M., Lawlis, E. and Sullivan, R. C. (2016). The unstable ice nucleation properties of Snomax® bacterial particles. *Journal of Geophysical Research: Atmospheres* **121(19), 11,666-611,678.**

Raza, W., Yang, W., Jun, Y., Shakoor, F., Huang, Q. and Shen, Q. (2012). Optimization and characterization of a polysaccharide produced by *Pseudomonas fluorescens* WR-1 and its antioxidant activity. *Carbohydrate Polymers* **90(2), 921-929.**

Raza, Z. A., Khan, M. S. and Khalid, Z. M. (2007). Physicochemical and surface-active properties of biosurfactant produced using molasses by a *Pseudomonas aeruginosa* mutant. *Journal of*

Environmental Science and Health Part A **42(1), 73-80.**

Russo, J., Romano, F. and Tanaka, H. (2014). New metastable form of ice and its role in the homogeneous crystallization of water. *Nature Materials* **13(7), 733-739.**

Šantl-Temkiv, T., Sahyoun, M., Finster, K., Hartmann, S., Augustin-Bauditz, S., Stratmann, F., Wex, H., Clauss, T., Nielsen, N. W., Sørensen, J. H., Korsholm, U. S., Wick, L. Y. and Karlson, U. G. (2015). Characterization of airborne ice-nucleation-active bacteria and bacterial fragments. *Atmospheric Environment* **109, 105-117.**

Sirajunnisa, A. R., Vijayagopal, V., Sivaprakash, B., Viruthagiri, T. and Surendhiran, D. (2016). Optimization, kinetics and antioxidant activity of exopolysaccharide produced from rhizosphere isolate, *Pseudomonas fluorescens* CrN6. *Carbohydrate Polymers* **135, 35-43.**

Snider, C. S., Hsiang, T., Zhao, G. and Griffith, M. (2000). Role of ice nucleation and antifreeze activities in pathogenesis and growth of snow molds. *Phytopathology* **90(4), 354-361.**

Song, L., Chen, Q. and Ji, R. (2004). Effect of food-originated compounds on the ice-nucleation active bacteria. *Food and Fermentation Industries* **1, 53-56.**

Srivastava, A., Al-Karablieh, N., Khandekar, S., Sharmin, A., Weingart, H. and Ullrich, M. S. (2012). Genomic distribution and divergence of levansucrase-coding genes in *Pseudomonas syringae*. *Genes* **3(1), 115-137.**

Tebyanian, H., Mirhosseiny, S. H., Kheirkhah, B., Hassanshahian, M. and Farhadian, H. (2014). Isolation and identification of *Mycoplasma synoviae* from suspected ostriches by polymerase chain reaction, in Kerman Province, Iran. *Jundishapur Journal of Microbiology* **7(9), e19262.**

Wilson, P. W. and Haymet, A. D. J. (2009). Effect of solutes on the heterogeneous nucleation temperature of supercooled water: An experimental determination. *Physical Chemistry Chemical Physics* **11(15), 2679-2682.**

Wisniewski, M., Lindow, S. E. and Ashworth, E. N. (1997). Observations of ice nucleation and propagation in plants using infrared video thermography. *Plant Physiology* **113(2), 327-334.**

Wu, Z., Qin, L. and Walker, V. K. (2009). Characterization and recombinant expression of a divergent ice nucleation protein from '*Pseudomonas borealis*'. *Microbiology* **155(4), 1164-1169.**

Zolfaghar, M., Amoozegar, M. A., Khajeh, K., Babavalian, H. and Tebyanian, H. (2019). Isolation and screening of extracellular anticancer enzymes from halophilic and halotolerant bacteria from different saline environments in Iran. *Molecular Biology Reports* **46(3), 3275-3286.**

Zonouri, S. S., Fatehinia, M., Nuritabar, S. and Manuchehri, S. (2015). Characterization of ice nucleation bacteria and their applications. *Cumhuriyet Science Journal* **36(3), 1726-1732.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538