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Original Article

Optimization of Ice Nucleation Activity of a Newly Isolated *Pseudomonas* sp. IRL.INP1 Using Rice Bran Based on Response Surface Methodology

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Abstract

Introduction: Several isolated species from symptomatic frozen leaves and soil produce ice nucleation proteins and have been exploited for their Ice Nucleation Activity (INA). Ice nucleation proteins can be employed as promising substances for biotechnological applications such as artificial snow-making, cryopreservation of tissues, and the condensation of ice nuclei in clouds. Considering INA has a direct relationship with bacterial growth, optimization of the culture medium seems indispensable. In this study, the INA of a newly isolated *Pseudomonas* sp. IRL.INP1 was evaluated.

Materials and Methods: Plackett–Burman was applied for screening several cost-effective carbon and nitrogen sources affecting bacterial growth and INA. The response surface method was employed for medium optimization. Moreover, biomass, whole-cell protein, specific growth rate, and INA were estimated.

Results: Rice bran, ammonium sulfate, temperature, and olive oil had significant effects on the INA of *Pseudomonas* sp. IRL.INP1. Results demonstrated that 5% rice bran, 31 °C, 1.0% olive oil, and 6 g/L ammonium sulfate led to the best INA. The final optical density at 600 nm was 2.3. Also, 1.94 g/L biomass, 1.75 µg/µl whole-cell protein, and 0.26 specific growth rate (day^{-1}) were obtained, and INA was observed after 5 sec at -5 °C.

Conclusions: The present research is the first study using agricultural waste for INA optimization. Since rice bran is a cost-effective agro-waste and a qualified replacement for glucose, it can be utilized as a promising substrate for bacterial growth and INA.

Keywords: *Pseudomonas*, Rice Bran, Olive Oil, Placket-Burman Statistical Design, Response Surface Methodology

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Introduction

Water is an indispensable element for all living cells and a medium for biological reactions. Water does not freeze at 0 °C and remains liquid below 0 °C. This phenomenon is called undercooled or supercooled.[1] Owing to the density fluctuations in water, the water molecules assemble to shape ice crystals. In terms of energy, this procedure is undesirable. Therefore, it inclines to enhance the total energy of the system. Accordingly, these clusters tend to be separated drastically. By additional cooling, ice nuclei formation occurs to a certain dimension (a critical size/volume ratio).[2] Further increase in both number and size results in the reduction in the total energy, which is favorable for the process, and ice formation occurs promptly. This phenomenon happens at about -40 °C for homogeneous ice nucleation and is named 's, upercooling temperature threshold'.[3,4] Each foreign particle or impurity in water attaches water molecules to its surfaces. As a result, water molecules gather to form an ice nucleus. These impurities are called heterogeneous ice nucleation activators such as silver iodide or organic materials, namely Ice Nucleation Proteins (INP).[5] Generally, heterogeneous ice nucleation happens at a temperature greater than homogeneous ice nucleation mostly, between -2 °C and -15 °C. Bacterial nuclei were categorized into three classes: types I, II, and III with respective temperature threshold ranges of -5 °C or greater; -5 °C to -8 °C; and -10 °C or lower.[6,7] Various Gram-negative epiphytic bacteria have been recognized to generate ice nucleation activators, including *Erwinia*, *Xanthomonas*, and *Pseudomonas*.[3] Ice nucleation bacteria have several applications in ecology, agriculture, and biotechnology.[8] The production of artificial snow by *Pseudomonas syringae* has been proved and is known as Snowmax™.[9] In addition, the use of ice nucleation activators as a signal transmitter has been studied. In immunoassay, ice nucleation activators are alternatives for the typical tags, including linked enzymes and fluorescent groups. The other fields of applying ice nucleation proteins are in the food

industries. For this purpose, the preparation of cell-free ice nucleation activators with similar activity is inevitable. Cryopreservation of cells and tissues is another field of INP utilization. In order to control intracellular ice formation, minimizing the super cooling temperature is required for cryopreservation.[6,9] The production of INP is not species-specific, and each strain of the same species generates a special kind of INP with various biotechnological features. For this reason, more strains should be evaluated to detect INP with remarkable properties (for example, INA at higher temperatures). In this study, a new strain of *Pseudomonas* was previously isolated by our research center, and its INA was determined. The new strain was identified biochemically and genetically (16S rRNA sequencing) and was named *Pseudomonas* sp. IRL.INP1. The high INA has a direct relationship with high biomass production. Therefore, some cost-effective carbon sources, including glucose, sucrose, and rice bran as the main byproducts in the process of rice milling, and three nitrogen sources were tested. Also, it has been proven that adding the components of the ice-nucleating site to the culture media is essential for enhancing the efficiency of INA. Therefore, the influences of some ingredients, including olive oil, wheat bran, and sesame oil as the precursors for ice nucleation sites were studied.[10,11] Optimization is an essential method to determine the optimum parameters affecting bacterial growth. Since one-factor-at-a-time experimentation is a time-consuming procedure, Response Surface Methodology (RSM) was applied. RSM is a famous procedure in the optimization of different variables accountable for the production of biomolecules.[12] This method was carried out in several stages. The main nutrients and environmental conditions were screened by the Plackett-Burman design, and the best conditions and concentrations for bacterial growth were determined by Central Composite Design (CCD). Until now, there have been few reports about the optimization of culture conditions for the INA bacteria. Also, this is the first study relating to the optimization of culture conditions for INA bacteria based on the byproduct from cellulosic wastes.

Materials and Methods

Bacterial Strains

P. syringae pv *syringae* was received from the National Center for Genetic and Biological Reserves, Tehran, Iran, and was used as a positive control. The negative control *Escherichia coli* PTCC 1330 was achieved from the Iran Scientific and Industrial Research Organization, Tehran, Iran. The native strain *Pseudomonas* sp. IRL.INP1 was received from our research center, Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran. It had been isolated from symptomatic frozen leaves and their surrounding soil from the Damavand region (a cold region) in Iran. It was identified biochemically and genetically (16S rRNA) and was named *Pseudomonas* sp. IRL.INP1. Accordingly, it was cultured onto King's medium B, and plates were incubated at 30 °C for 24 h, and cream-colored bacterial colonies with fluorescence were also collected.

One-Factor-at-a-Time Experiments

The one-factor-at-a-time experiment was applied to screen the factors influencing cell growth. The optimum initial pH for bacterial growth was identified by adjusting pH with 1 M NaOH and 1 M HCl at 4 to 12 before sterilization and the optimum temperature was studied by incubating broth cultures at 10, 15, 20, 25, 30, and 35 °C. To evaluate different carbon and nitrogen sources, 1% of each carbon source including glucose, sucrose, and rice bran, and (0.1%) of each nitrogen source including, $(NH_4)_2Cl$, NH_4NO_3, and $(NH_4)_2SO_4$ was added to the basal medium (10 g/L proteose peptone). To enhance INA, after evaluating carbon and nitrogen sources, two kinds of vegetable oils including, olive oil and sesame oil, and agro-industrial waste including, wheat bran at 1% (v/v) was separately added to the basal medium as media supplements. To prepare rice bran and wheat bran, 1% of each of them was suspended in distilled water and autoclaved. Then, the suspension was passed through a cloth, and the liquid phase was filtered and added to the basal medium. In each experiment, 2×10^7 CFU/ml of an overnight culture of isolate in peptone broth was inoculated.[10,12,13]

Plackett-Burman (PB) Design

The screening of the nutrients was performed using the Plackett-Burman design. The Plackett-Burman design determined which selected components by one-factor-at-a-time experiments (rice bran, ammonium sulfate [$(NH_4)_2SO_4$], olive oil, and wheat bran) and environmental parameters (temperature and pH) had significant effects ($p<0.05$) on bacterial growth. The assumption for using the Plackette-Burman experimental design is that there are no interactions between various factors. Based on this, six independent variables in 12 experiment trials were selected for the study. Each variable was used at low and high concentrations, designated as ±1 levels. Also, the concentration levels for this experiment were chosen by one-factor-at-a-time experiments. Plackett-Burman design has been represented on the first-order polynomial model.

$$Y = \beta_0 + \Sigma\beta_i X_i \qquad \text{Eq. 1}$$

Where Y is the response (bacterial growth), β_0 is the model intercept, X_i is the level of the independent variable, β_i and X_i are the linear regression coefficient and the level of the independent variable, respectively. Minitab 17.0™ package software was used for data analysis and experimental design.[13]

Central Composite Design (CCD)

A central composite design was applied to optimize three factors (rice bran, $(NH_4)_2SO_4$, temperature) that were significantly identified by Plackett-Burman. Three independent factors were studied at five levels (-1.68, -1, 0, +1, +1.68) and 20 experimental runs. All tests were carried out in 250 ml Erlenmeyer flasks with 150 ml of media, for 24 h 150 rpm, according to the design. The manner of the system was obtained by the following second-degree polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad \text{Eq. 2}$$

Where Y was the response, β_0 was a constant, and 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.[12]

Measurement of Growth

The growth of *Pseudomonas* sp. IRL.INP1 was characterized by evaluating the optical density at 600 nm (OD600). To determine dry cell biomass, an aliquot (50 ml) of the culture sample was removed from the flask at regular intervals and centrifuged for 15 min at 8,000 rpm and 4 °C. To eliminate any slimy substances on the surface of the cell, the Cell-Free Culture Broth (CFCB) pellet was rinsed with n-hexane. Then the cell suspension was prepared in sterilized deionized water and centrifuged again. An oven at 105 °C was employed for 4 h to dry the bacterial cells, and the growth was judged using dry weight. The specific growth rate (μ) was determined according to the equation $\mu = (\ln x_2 - \ln x_1)/t_2 - t_1$, where x_1 and x_2 are dry cell weight concentrations at the time t_2 and t_1, respectively.[14]

Cellular Protein Estimation

The cell pellet was gained after centrifugation at 4 °C, 8,000 rpm for 15 min. Given that the interfering material must be removed, the cell pellet was washed with distilled water and tetrahydrofuran. The cells were ruptured by adding 1N NaOH and heating at 90 °C in a water bath for 5 min. To determine the whole-cell proteins, the Bradford micro-assay was applied.[15]

Ice Nucleation Assay

The frozen-droplet technique is the approach by which INA is monitored. In this procedure, a freezing plate (an aluminum plate) was applied, and for providing thermal conductivity, paraffin was utilized on top of the plate. 10-μl of the samples was placed on the plate. A cooling circulator (LAUDA® L000650 Model Alpha RA 8 Stainless Steel Refrigerated Circulating Water Bath, Berlin, Germany) gradually provided a low temperature. Subsequently, the number of solidified droplets was measured at 1 °C periods. Distilled water droplets and *Escherichia coli* PTCC 1330 were used as the negative controls and *P. syringae* pv *syringae* was employed as the positive control. The experiment was carried out three times for each strain, and the mean temperature threshold was registered.[16]

Results

One-Factor-at-a-Time Experiments

In the present study, the maximum growth was obtained at 30 °C and pH 7 (Data not shown). Three carbon sources (rice bran, glucose, and sucrose) were used at 1% concentration of which rice bran resulted in more bacterial growth, biomass, and total protein production and subsequently had the most effect on INA (Table 1). Among three nitrogen sources evaluated, $(NH_4)_2SO_4$ influenced cell growth, biomass, and total protein more than the others (Table 1). Given that the present study aimed at finding the cheap nitrogen source for the large-scale bacterial growth and total protein, and, since preparing nutritional starvation for nitrogen has been proved for better INA, $(NH_4)_2SO_4$ was selected at a minimum concentration as the nitrogen source. By using the one-factor-at-a-time experimentation, the basal medium supplemented with wheat bran and olive oil led to a higher INA than the medium without them, but they slightly affected bacterial growth (Table 1).

Plackett-Burman Design

Before the optimization process using the CCD, the Plackett-Burman design was applied to select significant parameters. Using the one-factor-at-a-time experiment, six parameters, including rice bran, $(NH_4)_2SO_4$, temperature, pH, olive oil, and wheat bran which had the most effects on bacterial growth and INA, were selected. The designed tests were precisely carried out, and the results indicated the significant model in the analysis of variance (ANOVA). The R^2 value was 0.962 indicating 96.2% of the variability in the response could be described by the model. Adjusted R^2 was 0.917 proving the significance of the model. The signification of every coefficient was obtained by *p*-values. The X_1, X_2, and X_3 were rice bran, $(NH_4)_2SO_4$, and temperature, respectively, and had positive and significant effects on bacterial growth, and X_4, X_5, and X_6 that were pH, olive oil, and wheat bran, respectively, were not significant. The *p*-value of 0.002 indicates the significance of the model. The actual and predicted values using the Plackett-Burman design have been presented in Table 2. Furthermore, it explains how the results (Mean of OD600) were close to the predicted values. The equation for Plackett-Burman is shown below:

$$Y = -0.468 + 0.03167X_1 + 0.03833X_2 + 0.07167X_3 - 0.0333X_4 - 0.0067X_5 + 0.0020X_6 \quad \text{Eq.3}$$

Table 1. The Effect of Different Carbon, Nitrogen Sources, and Supplements on the Bacterial Growth (OD600), Dry Cell Weight, the Whole-cell Proteins, Specific Growth Rate (μ) and Ice Nucleation Activity by *Pseudomonas* sp. IRL.INP1. The data is presented as the mean value ± SD of three replicates for the bacterial growth (OD600)

Effect of Different Carbon and Nitrogen Sources					
Carbon and Nitrogen Sources	**OD600**	**Biomass (g/L)**	**whole-cell Proteins (μg/μl)**	**Specific Growth Rate (day^{-1})**	**The Mean of the Time for Observing INA (sec)**
Proteose peptone	0.45 ± 0.02	0.35	0.30	0.02 ± 0.01	72
Glucose	0.64 ± 0.03	0.42	0.35	0.03 ± 0.03	54
Sucrose	0.77 ± 0.10	0.61	0.51	0.04 ± 0.01	48
Rice bran	1.58 ± 0.11	1.1	0.94	0.18 ± 0.02	27
$(NH_4)_2Cl$	0.26 ± 0.02	0.05	0.03	---------[1]	118
NH_4NO_3	0.32 ± 0.01	0.14	0.11	---------[1]	100
$(NH_4)_2SO_4$	0.55 ± 0.02	0.43	0.48	0.03 ± 0.01	61
Supplements					
Olive oil	0.48 ± 0.03	0.32	0.30	0.02 ± 0.02	61
Sesame oil	0.34 ± 0.04	0.24	0.17	0.01 ± 0.01	82
Wheat bran	0.40 ± 0.01	0.25	0.20	0.01 ± 0.03	75

[1] Low specific growth rates.

Table 2. Plackett-Burman Design for Factors Selected from One-factor-at-a-time Experiments

Runs	Rice Bran (%)	Ammonium Sulfate (g/L)	Temperature (◦C)	pH	Olive Oil (%)	Wheat Bran (g/L)	Mean of OD600[1]	Predicted Value[1]
1	5	5	28	7	1	50	1.73	1.75
2	3	5	30	7	0.5	70	1.86	1.87
3	5	3	30	6	0.5	50	1.84	1.85
4	5	5	28	7	0.5	50	1.77	1.75
5	5	5	30	6	1	70	1.98	1.97
6	3	3	28	7	1	70	1.63	1.65
7	3	3	30	7	1	50	1.79	1.75
8	3	5	30	6	1	50	1.84	1.86
9	3	5	28	6	0.5	70	1.78	1.76
10	5	3	30	7	0.5	70	1.85	1.86
11	5	3	28	6	1	70	1.75	1.75
12	3	3	28	6	0.5	50	1.64	1.65

[1] Values are rounded.

Where Y is the response, X_1, X_2, X_3, X_4, X_5, and X_6 are the coded values of rice bran, $(NH_4)_2SO_4$, temperature, pH, olive oil, and wheat bran, respectively.

Optimization by CCD

Based on the results obtained from Plackett-Burman, temperature, rice bran, and ammonium sulfate were chosen for CCD. Optimization was carried out applying a standard CCD with fixed central points of rice bran (5% w/v), ammonium sulfate (5 g/L), and temperature at 30 °C. The design matrix and response have been presented in Table 3. The regression analyses (Table 4) demonstrated that the quadratic model was significant (p = 0.000), and the model can truly represent the data in the test region. The sufficiency of the second-order terms is supported by the coefficient of regression. By employing multiple regression analyses to test the data of bacterial growth, the test variables, and response were correlated by the following second-order polynomial equation:

$$Y_1 = -55.67 + 1.687X_1 + 3.395X_2 + 0.226X_3 - 0.1183X_1X_1 - 0.05654X_2X_2 - 0.0635X_3X_3 - 0.0044X_1X_2 - 0.0588X_1X_3 + 0.0269X_2X_3 \quad \text{Eq. 4}$$

Where Y_1 is the predicted response at OD600, X_1, X_2, and X_3 are rice bran, temperature, and ammonium sulfate, respectively. X_1 (rice bran), X_2 (temperature), X_3 (ammonium sulfate) displayed significant linear effects (p<0.05) on the growth of *Pseudomonas* sp. IRL.INP1. Also, the quadratic effects of all factors were significant. The coefficient judgments of all the quadratic terms ($x_1^2, x_2^2,$ and x_3^2) had negative signs, indicating that the parabola would open downward, reaching the highest point of the model. Furthermore, the temperature indicated the strongest effect on the growth of *Pseudomonas* sp. IRL.INP1. The R^2 value of 0.94 and adjusted R^2 of 0.89 implies a high level of correlation between the predicted and observed values for cell growth. Lack of fit demonstrates an adequate precision response to noise (deviation) ratio. Statistically, a non-significant lack of fit (p>0.05) implies that the responses are sufficient for the model (Table 4). Three-dimensional response surface plots show regression equations and the interactions between the response and variables illustrate the location of maximum levels of each variable for the greatest bacterial growth (Figure 1a, 1b, and 1c). The optimal concentrations obtained for rice bran, temperature, and ammonium sulfate from this study were 5.0%, 31.2 °C, approximately 31 °C, and 6 g/L, respectively. The maximum cell growth was predicted 2.36 at OD600. To validate the model, three experiments in the agitated and optimized medium were carried out and the mean value was 2.20 at OD600 corresponding to the predicted response, and INA was observed after 8.1 sec at -5 °C.

Table 3. CCD Matrix with Responses

Run	Rice Bran (%)	Temperature (°C)	Ammonium Sulfate (g/L)	Mean of OD600[1]	Predicted Value [1]
1	6(+1)	28(−1)	6(+1)	1.71	1.68
2	6(+1)	32(+1)	6(+1)	2.11	2.23
3	4(−1)	28(−1)	4(−1)	1.51	1.4
4	4(−1)	32(+1)	4(-1)	1.73	1.77
5	4(−1)	32(+1)	6(+1)	2.14	2.21
6	6.68(+1.68)	30(0)	5(0)	2.06	2.03
7	3.31(−1.68)	30(0)	5(0)	1.76	1.77
8	6(+1)	32(+1)	4(−1)	2.01	2.03
9	5(0)	30(0)	5(0)	2.31	2.24
10	4(−1)	28(−1)	6(+1)	1.63	1.62
11	5(0)	30(0)	5(0)	2.25	2.24
12	5(0)	30(0)	5(0)	2.11	2.24
13	5(0)	30(0)	5(0)	2.30	2.24
14	5(0)	30(0)	5(0)	2.21	2.24
15	5(0)	26.63 (−1.68)	5(0)	1.08	1.21
16	5(0)	30(0)	5(0)	2.24	2.24
17	5(0)	33.36(+1.68)	5(0)	2.13	1.99
18	5(0)	30(0)	6.68(+1.68)	2.31	2.23
19	6(+1)	28(−1)	4(−1)	1.75	1.69
20	5(0)	30(0)	3.31(−1.68)	1.82	1.88

[1] Values are rounded.

Table 4. 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	1.89784	0.210871	18.93	0.0001*
Linear	3	0.96024	0.32008	28.73	0.0001*
X_1	1	0.08455	0.084546	7.59	0.020*
X_2	1	0.72927	0.729274	65.45	0.0001*
X_3	1	0.14642	0.146419	13.14	0.005*
Square	3	0.88626	0.29542	26.51	0.0001*
X_1^2	1	0.20176	0.20176	18.11	0.002*
X_2^2	1	0.73709	0.737087	66.16	0.0001*
X_3^2	1	0.05815	0.058149	5.22	0.045*
2-way interaction	3	0.05134	0.017113	1.54	0.265[NS]
X_1X_2	1	0.00061	0.000613	0.05	0.819 [NS]
X_1X_3	1	0.02761	0.027613	2.48	0.147 [NS]
X_2X_3	1	0.02311	0.023113	2.07	0.18 [NS]
Error	10	0.11142	0.011142		
Lack-of-fit	5	0.08508	0.017017	3.23	0.112 [NS]
Pure error	5	0.02633	0.005267		
Total	19	2.00925			

* <0.05; NS: Non-significant.

Evaluation of the Effect of Olive Oil on Ice Nucleation Activity (INA)

To evaluate the effect of olive oil, different percentages of olive oil (Figure 2) were tested with the optimized concentration of rice bran (5.0% w/v), ammonium sulfate (6 g/L), and temperature (31 °C) obtained from the CCD. Given that olive oil had a very slight effect on bacterial growth, the influence of olive oil on INA was evaluated. The result showed that the combination of optimized values of 5.0% rice bran, 31 °C temperature, 6 g/L ammonium sulfate with 1.0% olive oil led to the INA after 5 sec at -5 °C (Figure 3). The final optical density at 600 nm was 2.3. Also, 1.94 g/L biomass, 1.75 µg/µl whole-cell protein, and 0.26 specific growth rate (day^{-1}) were obtained. It was indicated that INA was modified by adding olive oil to the culture at 1.0%. Inhibition of growth and decrease in INA occurred when olive oil was added at concentrations higher than 1.0%.

Discussion

The focus of the present research was on the media conditions of *Pseudomonas* sp. IRL.INP1 to reach the maximum bacterial growth and ice nucleation activity. The time-course experiment indicated that INA was growth-dependent.[17] Therefore, the high growth of *Pseudomonas* sp. IRL.INP1 resulted in the high production of total protein and subsequently increasing in ice nucleation protein. The higher production of INP causes INA at higher temperatures in a few seconds. In this study, maximum growth was obtained at 30 °C and pH 7. It was demonstrated that recombinant *Halomonas elongata* cells expressing *inaZ* gene of *P. syringae* release the ice nuclei into the growth medium at 24 °C in the late exponential phase.[18,19] This result is almost near to the findings of Blondeaux and Cochet, showing that 24 °C was the optimum temperature for *P. syringae*.[10,20] The research on several snow molds indicated that maximum growth occurred between -5 and 30 °C.[21] A new mutant of *Pseudomonads*, *P. syringae* SO754, which is originated from the parent strain *P. syringae* SO7 could maintain its INA at room temperature, throughout the recovery, fermentation, drying steps, and during the storage without the freezing method.[22] The pH affects

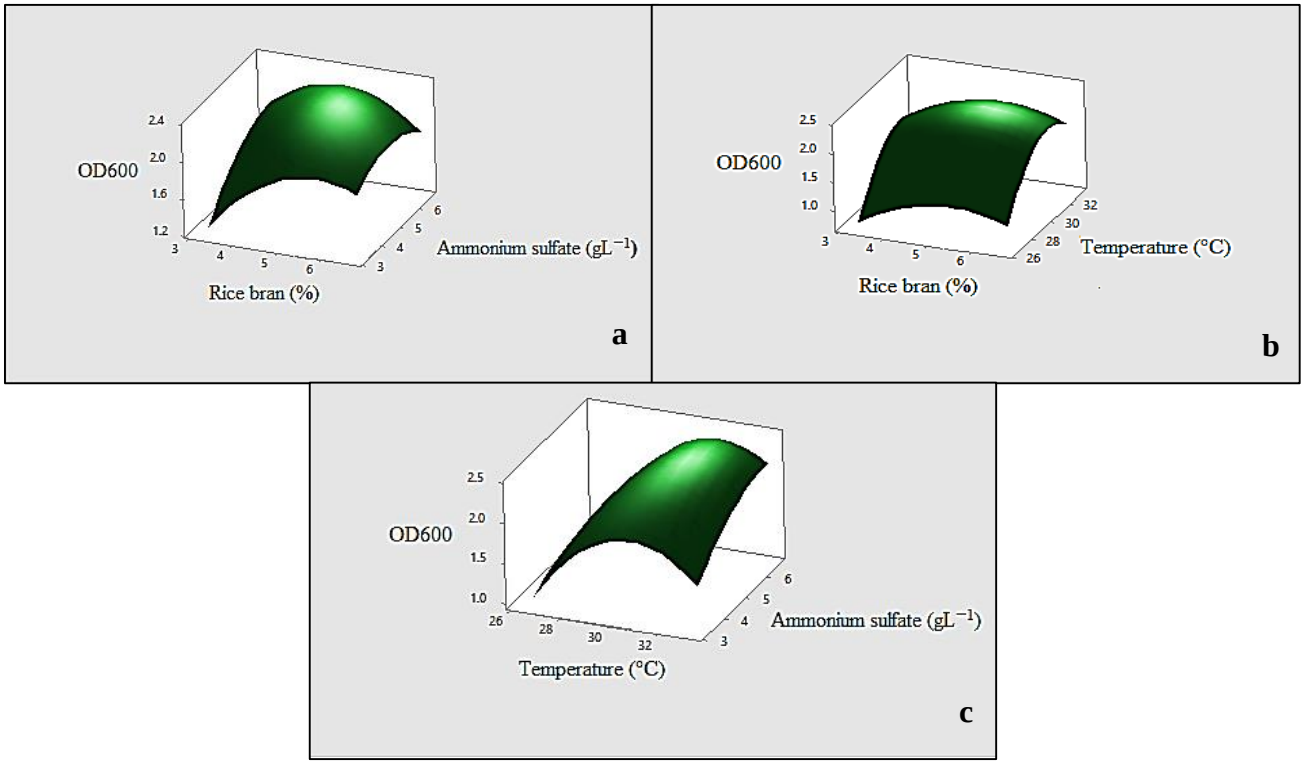

Figure 1. Response Surfaces for Bacterial Growth (OD600) of *Pseudomonas* sp. IRL.INP1. The surface plot of OD600 vs ammonium sulfate (g/L) and rice bran (%), the temperature was kept at 30 °C **(a)**; the surface plot of OD600 vs temperature (°C) and rice bran (%), ammonium sulfate was kept at 5 g/L **(b)**; the surface plot of OD600 vs ammonium sulfate (g/L) and temperature (°C), rice bran (%) was kept at 5% **(c)**.

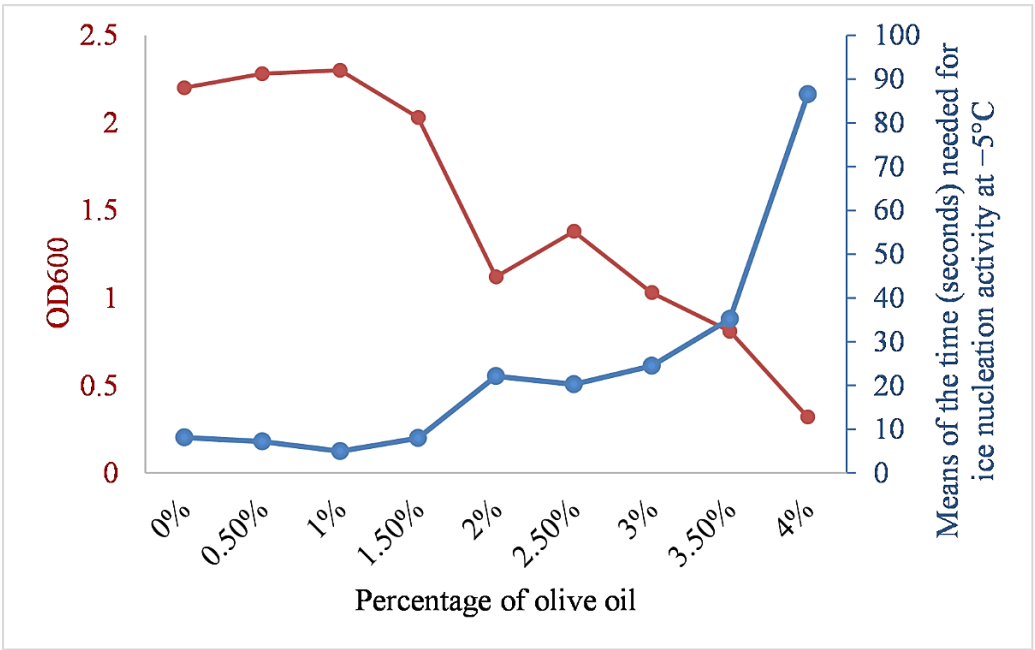

Figure 2. Evaluating the Effects of Different Percentages of Olive Oil with the Optimized Concentration of Rice Bran (5% w/v), Ammonium Sulfate (6 g/L), and Temperature at (31 °C) on Bacterial Growth (OD600) and INA. The less time (seconds) for observing INA was desired.

INA by the influence on cell growth. Previous studies determined that pH 6.5 was the optimum pH for three classes of INP A, B, and C.[23,24] INP gene from *Fusarium acuminatum* was expressed in *E.coli* and subsequently purified and identified as the second type of INP with the optimum pH 5.5, relative stability, and activity between pH 5 and 9.5, up to 45 °C.[25] The studies relating to the optimization of INA are limited, and this is the first study using rice bran for INA

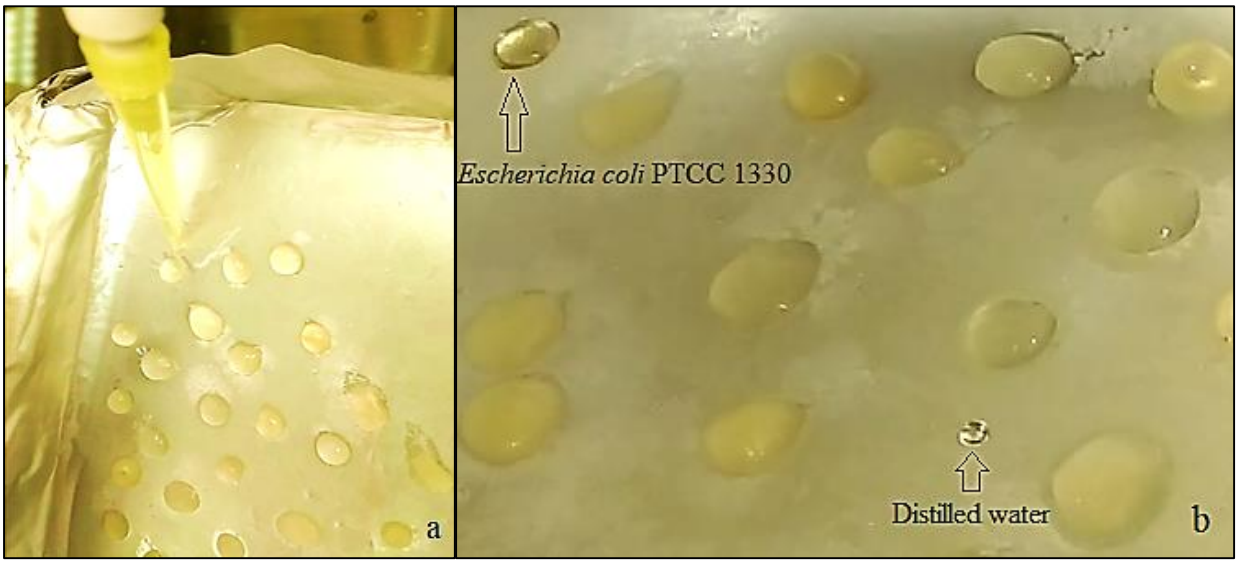

Figure 3. Ice Nucleation Assay by the Frozen-droplet Technique. Lauda's Alpha recirculating refrigerated water bath was used. **(a)** All droplets of *Pseudomonas* sp. IRL.INP1 froze after growing in the optimized medium at -5 °C in 5 sec; **(b)** The negative control and distilled water did not freeze at the given temperature.

optimization. Main nutrients such as nitrogen and carbon sources are essential ingredients for bacterial growth, thereby enhancing INA. In order to use a medium for industrial purposes, being cost-effective is an indispensable criterion, and the use of agro-industrial waste such as rice bran is the appropriate choice for bacterial growth. Three carbon sources (rice bran, glucose, and sucrose) were used at 1% concentrations of which rice bran resulted in more bacterial growth, biomass, and total protein production and subsequently had the most effect on INA. Glucose is the most abundant sugar in rice bran causes bacteria to utilize it by the glycolysis pathway.[26] Blondeaux et al. demonstrated that glucose and peptone with a concentration range of 50-80 g/L and 28 g/L, respectively, are preferable for biomass production and INA of *P. syringae* and adding olive oil or traces of silicone oil to the culture medium had a positive influence upon the expression of INA.[20] Previous studies indicated that higher levels of carbon sources affect INA.[5,27] Since rice bran is one of the local agricultural wastes of Iranian rice mills and a source of carbohydrates, saccharifying enzymes, and nutrients;[28] it can be utilized as a promising carbon source. Although the studies using agro-wastes as carbon sources for INA optimization are few, several studies used rice bran as an ingredient for other biotechnological purposes. Choi et al. and Devi et al. used rice bran hydrolysate as one of the substrates for generating polyhydroxyalkanoate, PHA, and exopolysaccharide.[26,29] Gao et al. applied rice bran as a carbon source for enzyme production such as carboxy methycellulase.[30] Since rice bran is an energy and growth enhancer, increasing the concentration of rice bran makes it a promising carbon source for biotechnological approaches.

In this study, $(NH_4)_2SO_4$ influenced cell growth, biomass, and total protein. Mainly, organic nitrogen sources are more compatible with bacterial cells than inorganic ones and stimulate cell growth owing to their proteins, amino acids, and vitamin contents.[12] Wu et al. used Tryptic Soy Broth (TSB) as a nitrogen source to judge the capability of *Pseudomonas borealis* DL7 for INA.[31] The INA of *Pseudomonas fluorescens* strain F26-4C in the nutrient broth with 2.5% glycerol was studied by Castrillo et al.[32] In contrast, nutritional starvation for nitrogen sources was shown to lead to highly active nuclei.[33] *Fusarium acuminatum* SRSF 616 was studied in nutrient limitation for phosphorus, nitrogen, sulfur, iron, and low temperature to enhance fungal ice nucleation.[34,35] Given that the present study aimed at finding the cheap nitrogen source for large-scale bacterial growth and total protein, and since preparing nutritional starvation for nitrogen has been proved for better INA, $(NH_4)_2SO_4$ was selected at a minimum concentration as the nitrogen source. Considering that ammonium is an efficient nitrogen source for most bacteria, the effects of molar ammonium concentrations on several model bacteria namely, *E. coli*, *Corynebacterium glutamicum*, and *Bacillus subtilis*, were studied. The mentioned bacteria were highly resistant to ammonium, and it was postulated that weakened growth was due to the high osmolarity rather than the toxicity of $(NH_4)_2SO_4$.[36]

In the present study, using one-factor-at-a-time experimentation, the basal medium supplemented with wheat bran and olive oil led to a higher INA than the medium without them, but they slightly affected bacterial growth. Wheat bran is composed of sugars, cellulose, some proteins, and a high proportion of phytic acid (1% of dry weight). It was demonstrated that the higher INA was gained using phytic acid or phytate after

pre-culture in a standard medium without K_2HPO_4 and KH_2PO_4. Phytase degrades phytic acid and phytate to provide myoinositol, which is necessary for the nucleating site (a glycosylphosphatidylinositol anchor into the outer membrane). Therefore, wheat bran at a concentration between 34 g/L to 85 g/L caused higher INA.[10,11,20] Cochet et al. mentioned that inorganic phosphate-starved pre-culture and culture media containing the precursors of myoinositol (phytate, phytic acid, and wheat bran) enhanced INA. Furthermore, the effect of wheat bran as a cheap agro-food byproduct resulted in the number of types I of ice nucleation protein structure to 1000-fold.[37] INA has an inverse relationship with membrane fluidity. The increased membrane fluidity causes the disaggregation in ice-nucleating sites, thereby decreasing ice nucleation activity.[38,39] In order to reduce the membrane fluidity, vegetable oils at 1% (v/v) were used. Between the two kinds of vegetable oils, olive oil had more influence on INA than sesame oil. The differences between these two oils could be due to the variation of their fatty acids, which are integrated into the bacterial membrane resulting in the various aggregations of the ice nucleation proteins. Vegetable oils compose of saturated and unsaturated fatty acids. Poly-unsaturated fatty acids are fluid, whereas mono-unsaturated, such as vegetable oils and saturated fatty acids, are described as low fluidity. The hydrophobic environment during bacterial growth has a profound effect on the INA. Therefore, olive oil provides qualified conditions for assembling the ice nucleation sites. Increased INA represented that membrane fluidity may be regulated throughout the growth. Two other suggestions are put forward to describe the effect of the olive oil: stabilization of the proteinaceous structure of the outer membrane and the alteration of membrane fluidity owing to the change of the culture medium's water activity (aw).[40,41]

In this study, before optimization, the biomass and whole-cell proteins of inoculated rice bran were evaluated, and 1.1 g/L biomass and 0.94 µg/µl whole-cell proteins were gained. After optimization, the optimum concentrations of rice bran (5.0%) and ammonium sulfate (6 g/L) as carbon and nitrogen sources, respectively, which was supplemented with 1% olive oil at 31 °C enhanced the bacterial growth of 2.3 at OD600, and INA was observed after 5 sec at -5 °C. In addition, 1.94 g/L biomass, 1.75 µg/µl whole-cell protein, and 0.26 specific growth rate (day^{-1}) were obtained. Kvíderová et al. evaluated the effect of bacterial biomass on INA. With the higher bacterial concentration, the high INA of the snow alga *Chloromonas nivalis* was proved.[42] The short time for the observation of INA proves the high ice activity, and it shows the capability of INA.[42-44] This study intended 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, applying a relatively cost-effective culture medium that promotes bacterial growth was needed. Therefore, several simple carbon and nitrogen sources and rice bran as an agricultural waste were investigated. It is also recommended to employ other sources of carbon and nitrogen obtained from food and agricultural wastes such as molasses and whey to provide a cost-effective culture medium.

Conclusion

Pseudomonas sp. IRL.INP1 is a novel bacterium with high ice nucleation activity and a proper choice for biotechnological uses. This is the first report in regards to the use of the agricultural waste medium for the optimization of ice nucleation activity. In this study, the screening of different factors affecting bacterial growth was carried out by the Plackett-Burman design. Based on this, temperature, ammonium sulfate, and rice bran were chosen for CCD. After optimization by CCD, different percentages of olive oil were utilized to reach the optimum percentage of olive oil, which had the highest effect on INA. Accordingly, 5% rice bran and 6 g/L ammonium sulfate supplemented by 1% olive oil at 31 °C enhance the bacterial growth of 2.3 at OD600, and INA was observed after 5 sec at -5 °C. Also, 1.94 g/L biomass, 1.75 µg/µl whole-cell protein, and 0.26 specific growth rate (day^{-1}) were obtained.

Authors' Contributions

Study concept and design by NS and AML; Analysis and interpretation of data by NS, AML, and SM. Drafting of the manuscript and critical revision of the manuscript for important intellectual content by NS, AML, SM, and AK.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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