Polish Journal of Microbiology
2014, Vol. 63, No 4, 433–441

ORIGINAL PAPER

Antiquorum from Freshwater Zoosporic Isolated Fungi: Maximization of Some Ameliorative Factors

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Submitted 28 March 2014, revised 2 August 2014, accepted 4 August 2014

Abstract

Many bacteria detect their critical cell numbers through a mechanism known as quorum sensing (QS). Acyl homoserine lactones (AHLs) mediated QS systems have been shown to operate in important human pathogens. The aim of this study was to evaluate the inhibitory effect generated by zoosporic freshwater fungi on AHLs production. A potent strain of zoosporic fungi, *Leptolegniella keratinophilum,* was isolated after extended screening study. A new cheap medium was used to support the anti-AHLs activities. Statistical strategy depending on two levels factorial model and three levels quadratic model were applied to guide a production maximization process. The three levels Box-Behnken model possessed the ability to optimize the significant nutritional and cultural conditions and to predict the maximum that can be achieved. In the course of study, we reached 88% anti-AHLs activity, while the initial activity was 63%. There was no previous maximization methodology for either the experimental medium, or the fungi. The results suggest that freshwater zoosporic fungi can be potentially used against bacterial infections.

Key words: antiquorum, maximization, zoosporic fungi

Introduction

Quorum sensing is a bacterial communication mechanism that depends on population density, and is mediated by hormone-like compounds called autoinducers. These molecules activate receptors, enabling the transcription of genes that encode information needed to control several biochemical mechanisms associated with bacterial survival and pathogenicity. Some of these processes are biofilm formation, expression of virulence factors, luminescence, pigment production, and mechanisms of resistance to stress conditions, which are of major importance in bacterial pathogenesis (Gobbetti *et al*., 2007; Vattem *et al.*, 2007; Waters and Bassler, 2005). Many species of bacteria use quorum sensing to coordinate gene expression according to the density of their local population (Ahmer, 2004). Acyl homoserine lactones (AHLs) mediated QS systems have been shown to operate in important human pathogens.

The misuse of antibiotics has led to the emergence of serious multiresistant bacteria. This has guided a search for alternative approaches other than those using antibiotics, in order to fight infectious diseases. Quorum sensing inhibition (QSI) is considered a new approach of antimicrobial chemotherapy as anti-QS compounds target genes that are essential for basic metabolism *in vitro*, rather than the microorganism itself (Dong *et al*., 2007, Rasmussen and Givskov, 2006 and Roy *et al*., 2001). Quorum quenching is one of the mechanisms to control the development of drug resistance in microbes (Rajesh and Rai, 2014). It has been found that quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo* (Brackman *et al*., 2011). Quorum sensing inhibitors would help in providing means of controlling and treating some microbial infections without increasing the number of the resistant strains (Costerton *et al*., 1999; Chang *et al*., 2012). In this light, inhibition of quorum sensing has been envisioned to be a new target for developing sustainable anti-infective therapies because impediment in QS will weaken the virulence of invading pathogens making them more susceptible to the applied mode of treatment (Bhardwaj *et al.*, 2013). This led the interest of the scientific community to concentrate on quorum sensing inhibitors. Nature offers a wide range of plants, algae and bacteria capable of inhibiting QS mechanisms. This fact has allowed the development of various studies aimed at finding new options for the treatment

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of infections caused by pathogenic bacteria (Dong *et al.*, 2007; Roy *et al.*, 2001).

Fungi produce a vast range of secondary metabolites. Some of these are high value products with pharmaceutical applications. Freshwater fungi, as a distinctive ecological group, have only recently been studied by a very limited number of research groups, and remain unexplored in comparison with other fungal niche groups. The growth habit and the morphology of freshwater zoosporic fungi attracted the interest of many researchers and led to intensive studies on their isolation and characterization (Barr, 1975, 1980; Czeczuga *et al.*, 1997). Studies of freshwater fungal isolates have led to the discovery of a variety of new bioactive metabolites, suggesting significant untapped potential among these organisms (Jiao, 2006). Despite these limited results, new compounds described from freshwater aquatic fungi display some uncommon features, and some of them represent novel structural types (Robeson *et al.*, 1984). To our knowledge, a little work covering zoosporic fungal metabolites and no work concerned with antiquorum compounds from freshwater aquatic fungi has been published.

The object of this study was to investigate the ability of freshwater aquatic fungi to produce antiquorum compounds. The aim of this work was also to evaluate and improve the ability of new microbial sources to produce anti-AHLs compounds. The work involved the isolation and identification of the freshwater zoosporic fungi. In addition, a statistical strategy to optimize the medium composition and culture conditions was applied. This work can be considered as a modest contribution to the quest of promoting antimicrobial agents.

Experimental

Material and Methods

Microorganisms. The content of the gut *Tilabia nilotica*, scrap from skin, piece of gills and debris from Nile River were used as samples for isolation of the freshwater aquatic fungi. The samples were placed on plates of glucose-yeast extract agar (GY) containing chloramphenicol (10 mg/l) and penicillin (6 mg/ml) (Min *et al.,* 1994). After incubation at 26°C for five days the growing cultures were examined macroscopically and microscopically. The isolates of the potential antiquorum were selected after screening and subjected for identification. The fungi were identified according to their morphological features, colony appearance, hyphae, sexual and/or asexual reproduction. When the colonies had tubular, variably branched, very poorly septate, hyphae (looking like oomycetes), they were placed in sterile Nile water at 26°C, to develop sporangia and/or sexual structures (Diéguez-Uribeondo *et al.*, 1994).

Pseudomonas aeuroginosa were isolated from clinical specimens using *Pseudomonas* isolation agar medium, Neogen.

The biosensor strain *Chromobacterium violaceum* ATCC 12427 produces a purple pigment, violacein, which is under quorum sensing (AHLs) control (McClean, *et al.*, 1997). The inhibition of violacein production by antiquorum sensing material makes this bacterium an excellent model for the isolation of antiquorum sensing substances from natural products. This strain was grown on Luria-Bertani (LB) medium solidified with 1.5% agar at 37°C for 24 h.

Culture conditions. The potential fungal species were cultivated on five different culture media of different composition to select a suitable medium for subsequent study. Fractions of 50 ml of sterile media placed in 100 ml conical flask after inoculation the flasks were incubated at 25°C. The cultures were examined for the production of antiquarian compounds. For the preparation of all media sterile Nile water was used. Fungal inoculation was made by means of suspension of zoospores. Zoospore suspension was adopted by growing the tested three fungi on sesame seeds water culture in sterilized Petri-dishes, each containing 30 ml distilled Nile water. The Petri-dishes were incubated at 25°C for eight days for zoosporangial discharge and zoospore production. Three ml of zoospore suspension of each species was pipetted under aseptic conditions and was used as inoculum. The flasks were incubated for 15 days at 25°C static and shacked at 100 rpm. After nine days the culture contents were homogenized and those were the test materials. The following culture media were used: Medium I: Peptone yeast extract glucose (PYG) (g/l): K_2HPO_4, 2 g, glucose 20 g, yeast extract 5 g, peptone, 5 g, Medium II: Modified Cornmeal (g/l): corn meal, 50 g, yeast extract, 2. Medium III: Sabouraud's dextrose (g/l): dextrose 20 g and peptone, 10 g. Medium IV (g/l): crushed sesame seeds, 20 g and glucose, 20 g. Medium V (g/l): dry fish waste, 20 g, glucose, 20 g, ammonium chloride, 2 g and K_2HPO_4, 1 g.

Measurement of antiquorum sensing activity. Quantification of violacein production. A single colony of the biosensor strain was transferred to LB medium, allowed to grow at 30°C for 24 hours, and then the culture was adjusted to 0.5 on the corresponding Mc-Farland scale. 1 ml of this suspension were placed in separate tubes and then 1 ml of different dilutions (25, 50 and 100%) of the test material was added. The tubes were further incubated at a temperature of 30°C for 24 hours (Choo *et al.*, 2006).

After the incubation period, the tubes were vortexed to resuspend cells and biofilms and 300 µl of this suspension were placed in 1.5 ml Eppendorf tubes. The cells were lysed with 300 µl of 10% sodium dodecyl sulfate, vortexed for 2 minutes, and then incubated

Table I
Levels of the variable for two level factorial design.

Variables	Unit	Levels	
		−1	+1
Dry fish waste	g/l	10	30
Glucose	g/l	10	30
CSL	ml/100 ml	1.58	5.55
K2HPO4	g/l	0.5	1
Ino. size	ml	3	5
pH		5	7
Time	days	5	9
Temperature	°C	20	30
Inoculum size	ml	3	7

Table II
The level of variables chosen for the Box-Behnken design

Variables	Symbol	Unit	Levels		
			−1	0	+1
Glucose	X_1	g/l	30	35	40
CSL	X_2	ml/100 ml	5.5	6.5	7.5
Time	X_3	Days	8	9	10

at room temperature for 5 minutes. Violacein was extracted quantitatively adding 800 µl of a mixture of butanol/water 1:1, stirred for 5 seconds and then centrifuged at 13000 rpm for 5 minutes. Once centrifuged, the violacein, which was present at the upper layer, was carefully removed and its absorbance measured at 585 nm (Blosser and Gray, 2000). Violacein concentration was normalized to the concentration obtained for the control (no test material) (Jesús *et al*., 2011). Percentage of violacein inhibition = (control $OD_{585\,nm}$ – – test OD_{585} nm / control $OD_{585\,nm}$) × 100

Optimization strategy – factorial design. Two level factorial design was used to screen nine factors to select the most significant ones (Antony, 2003).

Number of runs = 2^{9-4}. Each variable is represented at two levels, high and low, which are denoted by (+1) and (−1), respectively (Table I).

Optimization strategy-Box-Behnken model. Three level quadratic design requires an experiment number according to the following equation: N = k2 + k + cp. Where, (k) is the factor number and (cp) is the replicate number of the central point (Souza Anderson, 2005). For the three-level three-factorial Box-Behnken experimental design, a total of 17 experimental runs, shown in Table II were applied. The model is of the following form:

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

Where Y is the predicted response, β_0 model constant x_1, x_2, and x_3 independent variables; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are cross product coefficients and β_{11}, β_{22} and β_{33} are the quadratic coefficients (Box and Behnken, 1960).

Results and Discussion

Microorganisms. After the screening study the most, anti-AHLs, the potent fungal strain was identified as *Leptolegniella keratinophilum* according the description by Barr (1973, 1975); Booth and Barrett (1971); Canter and Ingold (1984); Fuller and Jaworski (1987); Karling (1968, 1977) Sparrow (1950, 1973); Youatt *et al*., (1971). Thereafter, this strain was selected to be used throughout the subsequent experiments.

Culture medium. Five different media, I, II, II, IV and V were tested for the production of the ant quorum. The results have proved that Medium V was the best one to produce anti-AHLs compound by the fungus, since the AHLs inhibition activity was of 68%. These results may be achieved because of the medium composition, which included natural material like dry fish waste (DFW) that may be rich in nutrient and growth factors (Fig. 1)

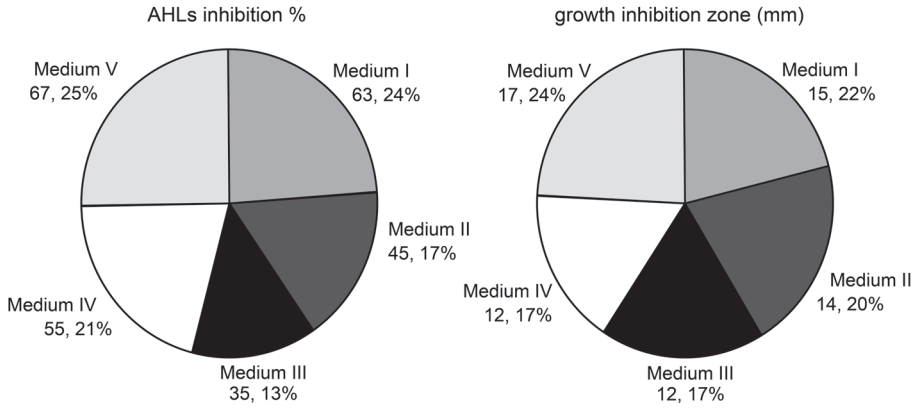

Fig. 1. Comparison of the antiquorum activities for *Leptolegniella keratinophilum* using different media. Growth inhibition zone is the measure for the growth inhibition for *P. aeroginosa.* Medium V (g/l): Dry fish waste 20, glucose 20, amm. chloride, 2 and K_2HPO_4, 1. Test materials at 100% dilution were used.

Table III
Matrix for the two levels factorial design

Run	Levels of variables								AHLs inhibition (%)
	DFW	Glucose	CSL	K_2HPO_4	Inoculum size	pH	Time	Temp.	
1	–1	1	1	–1	–1	–1	1	1	81
2	1	–1	1	1	1	–1	–1	–1	77
3	1	1	1	1	1	1	1	1	86
4	–1	1	1	1	–1	–1	–1	–1	75
5	1	1	–1	–1	–1	–1	–1	–1	72
6	–1	–1	1	–1	1	1	–1	1	71
7	–1	–1	1	1	1	1	1	–1	79
8	1	1	–1	–1	1	–1	–1	1	77
9	–1	–1	1	1	–1	1	1	1	79
10	–1	–1	–1	–1	–1	–1	–1	1	68
11	1	–1	–1	1	1	1	–1	1	69
12	1	1	1	–1	1	1	–1	–1	80
13	–1	–1	1	–1	–1	1	–1	–1	79
14	1	1	–1	1	1	–1	1	–1	80
15	1	–1	1	1	–1	–1	–1	1	75
16	1	1	1	–1	–1	1	–1	1	77
17	1	–1	–1	1	–1	1	–1	–1	78
18	–1	1	–1	1	–1	1	–1	1	75
19	–1	–1	–1	1	1	–1	1	1	74
20	1	–1	–1	–1	1	1	1	–1	72
21	1	–1	–1	–1	–1	1	1	1	68
22	–1	1	–1	–1	1	1	1	1	70
23	–1	1	1	–1	1	–1	1	–1	80
24	1	–1	1	–1	1	–1	1	1	81
25	1	1	1	1	–1	1	1	–1	85
26	–1	1	1	1	1	–1	–1	1	76
27	–1	–1	–1	1	–1	–1	1	–1	68
28	1	1	–1	1	–1	–1	1	1	71
29	–1	–1	–1	–1	1	–1	–1	–1	63
30	1	–1	1	–1	–1	–1	1	–1	76
31	–1	1	–1	–1	–1	1	1	–1	76
32	–1	1	–1	1	1	1	–1	–1	69

Carbon and nitrogen sources. The carbon sources, fructose, maltose, mannose, lactose, sucrose, starch, mannitol and soluble cellulose, were used to replace the glucose in the medium (Fig. 2). Any one of these carbon sources was not able to compete with glucose in its effect. All the utilized carbon sources were found to reduce the percent of the AHLs inhibition, since glucose is a fast and easily metabolized carbon source.

Ammonium nitrate, ammonium citrate, sodium nitrate, sodium nitrite and corn steep liquor (CSL) were added instead of ammonium chloride in medium V. Upon addition of corn steep liquor instead of ammonium chloride the AHLs inhibition activity was increased to to 2% in comparison with ammonium in the case of ammonium chloride.

Test materials concentration. Different concentrations of the test material were tested for their activities (Fig. 3). The results indicated that the inhibition is directly proportional to the test material concentration. For *P. aeuroginosa* growth inhibition was initiated at 50% of test material concentration. Further confirmation by polynomial model for this analysis proved the proportional relation (Fig. 3). Test material at concentration 100% was used through all subsequent experiments.

Optimization strategy. Two levels factorial model. According to this model 32 runs were performed to screen the most significant factors (Table III). The p value "Prob > F" less than 0.05 indicates model terms are significant. These factors have confidence levels above 95% (Table IV). In this case four factors, DFW,

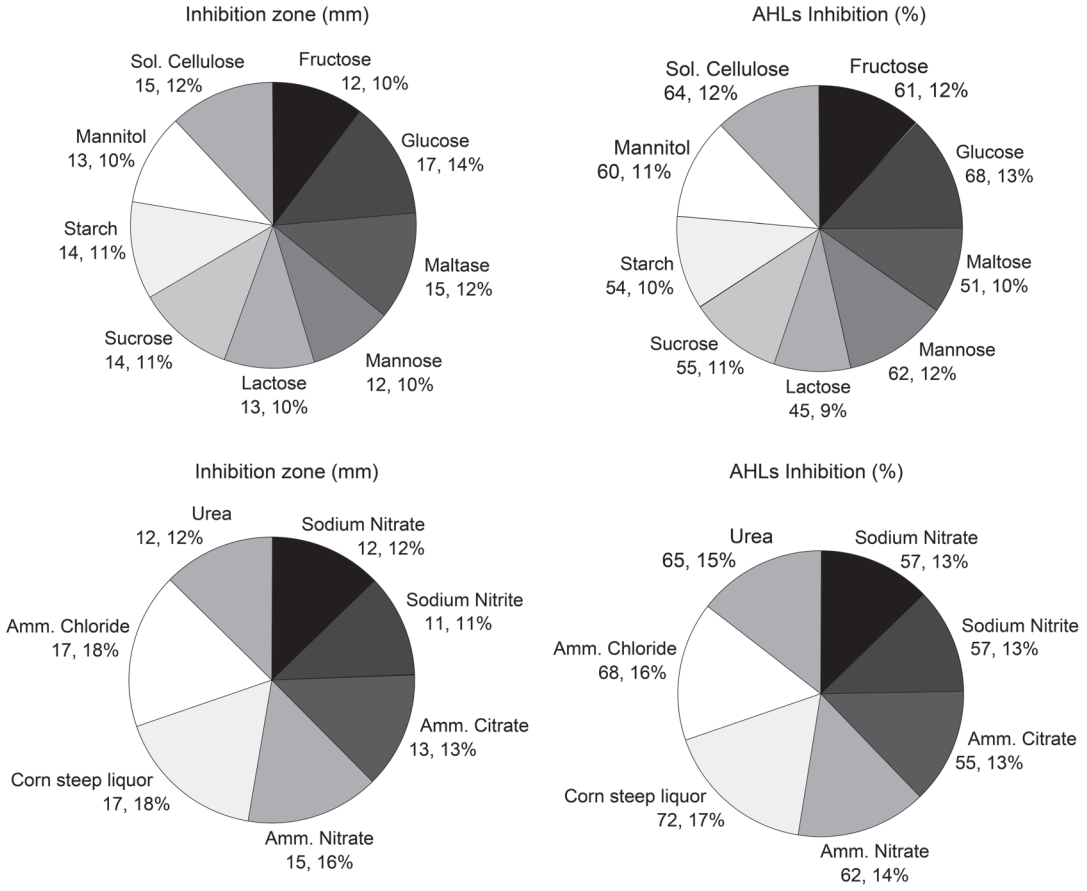

Fig. 2. Distribution of the effect of carbon (A) and nitrogen sources (B) on the anti-AHLs activities and on growth inhibition of *P. aerginosa.* The distribution was expressed as the AHLs inhibition (%) and the percent of participation of each medium in the total. Growth inhibition zone is the measure for the growth inhibition for *P. aeroginosa*, Test materials at 100% dilutions were used.

glucose, CSL and time are significant model terms. Furthermore, the effects and the Pareto chart for the model variable in Figure 4 confirm the significance of these factors. Consequently, the most significant three factors, namely, glucose, CSL and time will be subjected to the second step of the optimization process, three levels quadratic deign, Box-Behenken model to determine the optimum conditions.

Table IV
Analysis of variance for the two levels factorial model

Source	Sum of Squares	df	Mean Square	F Value	p-value prob > F	Confidence level (%)
Model	634.28	9	70.48	6.38	0.0002	99.98
A-DFW	52.53	1	52.53	4.75	0.0402*	95.98
B-Glucose	87.78	1	87.78	7.94	0.0100*	99.00
C-CSL	357.78	1	357.78	32.37	< 0.0001*	99.99
D-K_2HPO_4	19.53	1	19.53	1.77	0.1974	80.26
E-Incol. size	0.03	1	0.03	0.00282	0.9581	4.19
F-pH	11.28	1	11.28	1.02	0.3234	67.66
G-Time	63.28	1	63.28	5.72	0.0257*	97.43
H-Temp	3.78	1	3.78	0.34	0.5646	43.54
FH	38.28	1	38.28	3.46	0.0762	92.38
Residual	243.19	22	11.05			
Cor Total	877.47	31				

* Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, G are significant model terms

Table V
Matrix of Box-Behnken model

Run	Levels of variables			AHLs Inhibition (%)
	Glucose	CSL	Time	
1	0	0	0	84.90
2	0	1	–1	82.65
3	1	–1	0	81.91
4	0	0	0	84.9
5	1	0	–1	79.34
6	–1	1	0	81.99
7	0	0	0	84.00
8	0	–1	1	87.00
9	–1	–1	0	82.11
10	1	1	0	83.00
11	0	0	0	84.90
12	–1	0	–1	82.33
13	0	0	0	84.90
14	0	1	1	86.70
15	1	0	1	81.97
16	0	–1	–1	82.33
17	–1	0	1	88.23

Optimization strategy. Three levels Quadratic model. The methodology based on the Box-Behnken design, which was used to optimize nutritional/cultural requirement for production of anti-AHLs, 17 experimental runs with different combinations of three factors were carried out (Table V). The variables used for the factorial analysis were glucose, CSL and time named X_1, X_2, and X_3 in this design, respectively. The experimental responses for the 17 runs shown in Table V demonstrated that there was a considerable variation in the AHLs inhibition depending on the three independent variables in the medium. The maximum AHLs inhibition (88.23%) was achieved in run number 17, while the minimum AHLs inhibition (79.34%) was observed in run number 5. Statistical testing of the model was done for analysis of variance (ANOVA). ANOVA of the model in Table VI showed that the second model is well adjusted to the experimental data. The fit of the model can be checked by the determination coefficient (R^2) and correlation coefficient (R). The determination coefficient (R^2) implies that the sample variation of 99% for the anti-AHLs activities is attributed to the independent variables, and only about 1% of the total variation cannot be explained by the model. The corresponding P value, along with the parameter estimate, are given in Table VI. The P values are used as a tool to check the significance of each of the coefficients, which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The smaller the P values, the bigger the significance of the corresponding coefficient (Li *et al.*, 2007). This implied that the first- and second-order main effects of glucose, CSL and

Table VI
Statistical analysis of the Quadratic three levels Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	68.85	9	7.65	3.99	0.0408*
A-Glucose	8.90	1	8.90	4.65	0.0681*
B-CSL	0.12	1	0.12	0.06	0.8077
C-Time	37.20	1	37.20	19.41	0.0031*
AB	0.37	1	0.37	0.19	0.6753
AC	2.67	1	2.67	1.39	0.2762
BC	0.10	1	0.10	0.05	0.8292
A^2	18.30	1	18.30	9.55	0.0176*
B^2	0.62	1	0.62	0.32	0.5885
C^2	0.47	1	0.47	0.24	0.6372
Residual	13.42	7	1.92		
Lack of Fit	12.77	3	4.26	26.27	0.0043*
Pure Error	0.65		4	0.162	
Cor Total	82.26		16		
*significant variable					
Std. Dev.	1.3843913		R-Squared	0.836914	
Mean	83.715294		Adj R-Squared	0.627232	
C.V. %	1.6536898		Pred R-Squared	–1.49565	
PRESS	205.2969		Adeq Precision	6.048807	

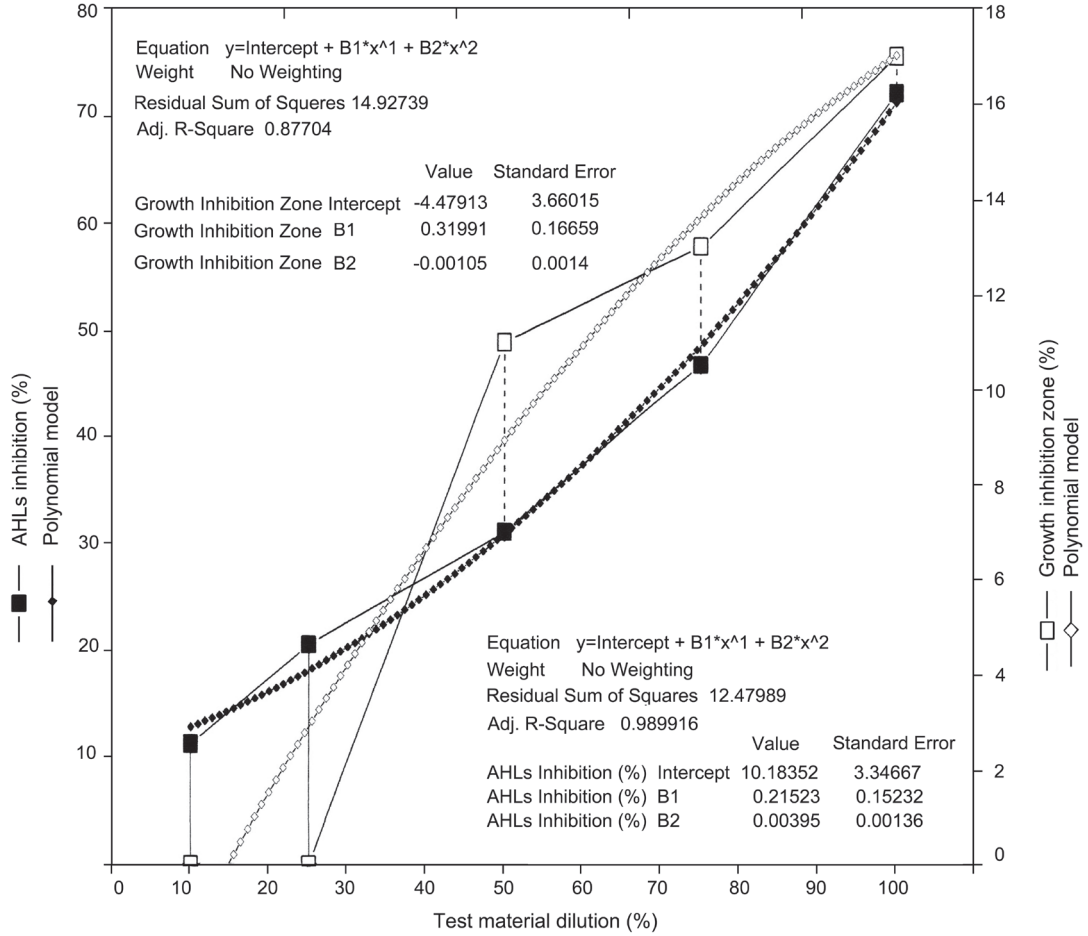

Fig. 3. The relation of test material concentrations to AHLs and growth inhibition

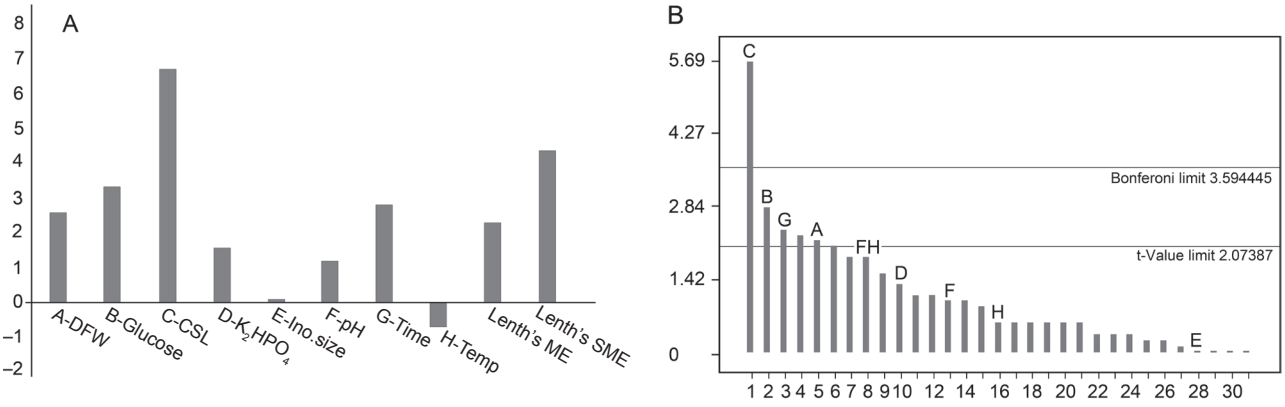

Fig. 4. Standardized effects (a) and Pareto chart (b) for the variables of two level model.

time are highly significant as is evident from their P values. However, interaction effect between glucose x CSL, glucose x time, CSL x time, glucose x glucose and time x time was not significant ($P > 0.05$) to the model. The final equation in terms of coded factors was as follows:

$$\text{AHLs inhibition (\%)} = +84.72 - 1.06 * X_1 + 0.12 * X_2 + \\ + 2.16 * X_3 + 0.30 * X_1X_2 * X_2 - 0.82 * X_1 * X_3 - 0.16 * X_2 \\ * X_3 - 2.09 * X_1^2 - 0.38 * X_2^2 + 0.33 * X_3^2$$

Figure 5 depicts the plot of the combined effect of CSL and glucose, CSL and time and time and CSL. As indicated the anti-AHLs activities gradually increase when glucose and CSL are near to their middle values. The activity increase when the time is at its highest value and glucose at its middle value. With increase of time to its high value and CSL at its middle value the AHLs inhibition reached a maximum.

Different mechanisms have been proposed to explain the interference of quorum sensing-depending processes by natural products. Some of these mechanisms are the inhibition of signal molecule biosynthesis

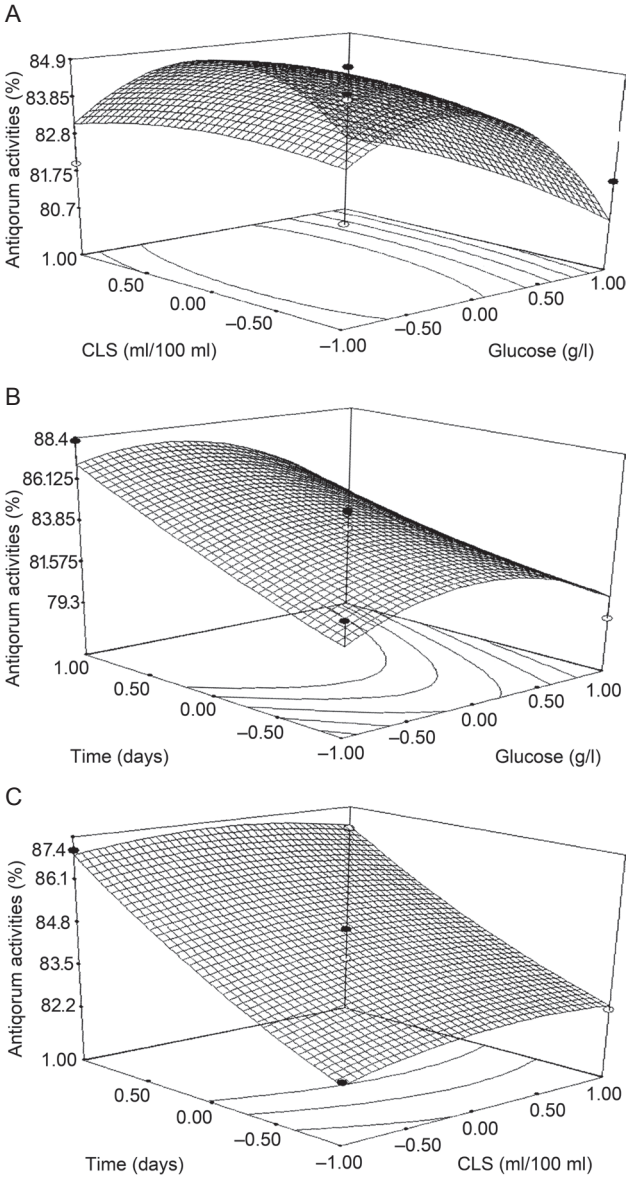

Fig. 5. The combined effect of the three variables glucose, CSL and Time.

CSL: corn steep liquor. A: CSL-Glucose, B: Time-Glucose, C: Time-CSL

(Vattem, *et al.*, 2007 ; Hentzer and Givskov, 2003) or AHL signal reception (Vattem *et al.*, 2007; Hentzer and Givskov, 2003) and the enzymatic inactivation and biodegradation of quorum sensing molecules (Defoirdt *et al.*, 2004). However, the mechanisms through which AHL-activated QS systems are inhibited are still not known for sure.

Validity of the model. The model was verified after applying the optimum values for the parameters. In comparison with predicted values where the AHLs inhibition was about 88%, the experimental value reached about 88%. Therefore, the optimum process conditions obtained through a statistical method fractional factorial design were successfully determined to maximize the inhibtion activities. The results from the second order polynomial model indicated that the optimum process conditions for maximum AHLs inhibition were, glucose 35 g/l, CSL 7.5 ml/100 ml after 10 days.

Conclusion. The results of this work indicated antiquorum activity for zoosporic fungal strain isolated from fresh water environment. This strain was identified as *Leptolegniella keratinophilum*. The fungus was successfully grown on a relatively cheap medium based on dry fish wastes. A statistical study was used to improve the antiquorum activities which led to an obvious increase from about 65% to 88 %. These results indicated for the first time the importance of antiquorum sensing activity of zoosporic fungi. The observed phenomenon needs further investigations.

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