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Optimization of inulinase production from low cost substrates using Plackett–Burman and Taguchi methods

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ABSTRACT

Four marine-derived fungal isolates were screened for the production of inulinase enzyme from low cost substrates under solid state fermentation (SSF), one of them identified as *Aspergillus terreus* showed the highest inulinase activity using artichoke leaves as a solid substrate. Sequential optimization strategy, based on statistical experimental designs was employed to optimize the composition of the medium, including Plackett–Burman and Taguchi's (L9 3^4) orthogonal array designs. Under the optimized conditions, inulinase activity (21.058 U/gds) reached the predicted maximum activity derived from the taguchi methodology, which increased about 4.79-folds the initial production medium. Fructose was produced, as an end product of inulin hydrolysis proving that the enzyme produced was exoinulinase. The marine-derived *A. terreus* is suggested as a new potential candidate for industrial enzymatic production of fructose from low cost substrate containing inulin as an economic source.

1. Introduction

Although microbial inulinase (E.C. 3.2.1.7) production has been reported by many researchers, studies on inulinase production by marine-derived fungi under SSF are relatively less. A marine enzyme is a unique protein molecule with novel properties derived from an organism whose natural habitat comprises saline or brackish water. Apart from marine-derived microorganisms were bacteria (including actinomycetes) and fungi. Properties like high salt tolerance, hyperthermostability, barophilicity, cold adaptivity and ease in large scale cultivation were the key interests of the scientists. These properties may not be expected in terrestrial sources as marine organisms (Ghosh, Saha, Sana, & Mukherjee, 2005).

Inulin occurs as a reserved carbohydrate polymer mainly in the roots and tubers of jerusalem artichoke, chicory, dandelion, burdrock and dahlia (Chen, Chen, Chen, Xu, & Jin, 2011). It consists of linear chains of β-2, 1-linked D-fructofuranose molecules terminated by a glucose residue (Vandamme & Derycke, 1983). Inulin has recently received a great interest for the production of high fructose syrup. D-Fructose is occupying an increasingly important position in the modern world as a sweetener because of its higher sweetening value, its physiological metabolism in human body and its insignificant insulinogenic effects (Pandey et al., 1999).

Although inulin can be converted into fructose by chemical approach, this is associated with some drawbacks. Microbial inulinase yields 95% pure fructose after one step enzymatic hydrolysis of inulin. Thus microbial inulinases are important class of industrial enzymes that have gained much attention in recent times. Inulinases can be produced by many of the microorganisms including strains of *Aspergillus* sp., *Penicillium* sp. and *Kluyveromyces* sp. Owing to the cost of pure inulin, alternate inulin containing raw, inexpensive substrates are preferred for microbial inulinase production (Trivedia, Divechab, & Shaha, 2012).

Microbial inulinases are extensively produced through submerged fermentation (Kalil, Suzan, Maugeri, & Rodrigues, 2001; Silva-Santisteban & Maugeri Filho, 2005). Few studies on the production of inulinase by solid state fermentation have been recently reported (Bender, Mazutti, De Oliveira, Di Luccio, & Treichel, 2006; Selvakumar & Pandey, 1999). Inulinase production by solid state fermentation (SSF) has attracted much attention because of high productivity, simple operation, cost effectiveness and better product recovery (Singhania, Patel, Soccol, & Pandey, 2009). Moreover, the crude fermented products from SSF can be used directly as the enzyme source for biotransformation (Chen et al., 2011).

Statistical methods are increasingly preferred for fermentation optimization because they reduce the total number of experiments needed and provide a better understanding of the interactions among factors on the outcome of the fermentation (Revankar & Lele, 2006). Some of the popular choices in applying statistical designs are Plackett–Burman design and Taguchi's experimental

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design. The Plackett–Burman design provides an efficient way of a large number of variables and identifying the most important ones (Deshmukh & Puranik, 2010). Taguchi's experimental design has gained broad acceptance in fermentation optimization. It specifies orthogonal arrays for combining the various variables and levels in a minimum acceptable number of experimental trials. In addition, Taguchi's approach facilitates the identification of the influence of individual factors and interactive effects of factors on performance with a few well-defined experimental sets (Prasad & Mohan, 2005).

Assessment of fermentation conditions for inulinase production is of relevance since many fermentation parameters may significantly affect the productivity of the enzyme and thus production cost. In this context, the present work was focused to study the optimization of the process parameters for inulinase production on low cost substrate under SSF using Plackett–Burman and Taguchi methods employing a newly isolated marine-derived *Aspergillus terreus*. The hydrolysis products of inulin with crude enzyme were also determined.

2. Materials and methods

2.1. Microorganisms and medium

The marine-derived *A. terreus*, *Aspergillus versicolor*, *Aspergillus parasiticus* and *Penicillium brevicompactum*, previously isolated from decayed wood samples collected from Ismalia, Egypt, were screened for inulinase production in this study. Strains were identified in the National Research Centre, Microbial Culture Collection Unit (MCCU) based on the morphological characterization according to its colonial and microscopic properties comparing with fungal species described by (Kohlmeyer & Kohlmeyer, 1991; Pitt & Hocking, 1985). The strains were maintained on malt extract agar (MEA) medium at 4 °C. The medium contained the following components (g/L): biomalt 20, agar 15, 800 ml sterile sea water and 200 ml distilled water (Höller, König, & Wright, 1999).

The yeast *Kluyveromyces marxianus NRRL 7571*, known as a good producer for inulinase enzyme used in the screening step as a positive control compare with the selected marine fungal isolates.

2.2. Inoculum preparation

Inocula were prepared by incubating the cultures on MEA slants at 30 °C for about 7 days, until sufficient sporulation was observed. The spores were harvested using 15 ml sterilized sea water and 1 ml of the suspension was used for inoculation purpose.

2.3. Solid-state fermentation

In the screening step, six carbon sources were used as substrates for inulinase production. Chicory root, artichoke leaves, banana leaves, garlic peel, orange rinds and sugarcane bagasse were air-dried and cut in small pieces (particle size: 1.0, 1.5 and 2.0 mm). Solid state fermentation was carried out in Erlenmeyer flasks (250 ml) with 3 g of the solid substrates. Moisture was adjusted by the addition of 15 ml of sea water to each flask. Each flask was covered with hydrophobic cotton and autoclaved at 121 °C for 20 min. After cooling, each flask was inoculated with 1 ml spore suspension and incubated at 30 °C for 7 days in a static mode.

During the preliminary screening process, the experiments were carried out for 7 days, and it was found that at the 96 h, the maximum production occurs. Hence, experiments are carried out for 96 h. All the experiments were carried out in duplicate and the average values are reported as mean ± SD calculated using MS Excel.

Table 1
The Plackett–Burman design for Physical variables and nutrient screening in inulinase production by *A. terreus*.

Variable code	Variable	Levels	
		Low (−1)	High (+1)
A	Initial pH	4.5	7.5
B	Incubation temperature (°C)	25	35
C	Wheat bran (%)	1	3
D	Glucose (%)	1	3
E	Sucrose (%)	1	3
F	Yeast extract (%)	0	1
G	$NH_4H_2PO_4$ (%)	0	1
H	Urea (%)	0	1
J	KH_2PO_4 (%)	0.1	0.5
K	Mg^{2+} (mM)	0	5
L	Ca^{2+} (mM)	0	5

2.4. Extraction of Inulinase enzyme

When fermentation was completed, 50 ml of distilled water were added to the fermented matter, and the mixture was mixed thoroughly on a rotary shaker (150 rpm) at room temperature (28 ± 2 °C) for 60 min.

The mixtures were filtered through muslin cloth. After centrifugation of the filtrate at 4 °C for 10 min, the supernatant was collected as the crude enzyme solution (Chen et al., 2011).

2.5. Inulinase activity assay

Inulinase activity was assayed by measuring the amount of reducing sugar fructose released from inulin, using Nelson's method (1944). The reaction mixture containing 0.5 ml of enzyme extract and 0.5 ml of 1% (w/v) inulin in 0.2 M sodium acetate buffer (pH 5). The mixture was incubated at 50 °C for 15 min. The amount of reducing sugars released was measured using Somogyi's copper reagent. Absorbance was read at 520 nm. One unit of inulinase (IU) was defined as the amount of enzyme which librated 1 μmol of fructose per min under the assay conditions. Results of the determination of inulinase activity were presented in units of activity/gram solid substrate (U/gds).

2.6. Screening of the supplemental nutrients using a Plackett–Burman design.

Plackett–Burman design, an efficient technique for medium component optimization (Naveena, Altaf, Bhadriah, & Reddy, 2005), was employed for screening fermentation parameters that significantly influenced inulinase production. Each independent variable was tested at two levels, high and low, which were denoted by (+) and (−), respectively. In the present study, the supplemental nutrients were screened by a Plackett–Burman design for eleven variables at two levels (Table 1). The eleven assigned variables were screened in twelve experimental designs. All experiments were carried out in duplicate and the averages of inulinase activity were taken as response (Table 2).

2.7. Optimization of the supplemental nutrients using Taguchi methodology

The Taguchi methodology was used to investigate the relationship between variables of medium components and to optimize their concentrations for inulinase production by the marine-derived *A. terreus*.

In this study, four factors at three levels of variations (Table 3) were used in the experiments. The factors optimized included the initial pH level, incubation temperature and the concentrations

Table 2
The Plackett–Burman design variables with inulinase activity as response.

Trials	Physical variables		Carbon sources			Nitrogen sources			Energy source	Metal ions		Inulinase activity (U/gds)
	Initial pH	Incubation temp.	Wheat bran	Glucose	Sucrose	Yeast extract	$NH_4H_2PO_4$	Urea	KH_2PO_4	Mg^{2+}	Ca^{2+}	
1	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1	0.443
2	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	1.286
3	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	4.655
4	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	4.433
5	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	0.665
6	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	9.975
7	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	13.300
8	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	0.554
9	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	0.488
10	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	1.108
11	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	6.428
12	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1	10.861

The sign +1 and −1 represent the two different levels (high and low) of the independent variable under investigation.

of KH_2PO_4 and Ca^{2+}, because they have significant impact on inulinase production as screened by Plackett–Burman design. The various combinations of factors and levels were in accordance with Taguchi's L9 orthogonal array. The factor level combinations for all the experiments are shown in (Table 3). A verification test was also performed to check the optimum condition. An analysis of variance (ANOVA) for the obtained results was investigated. For designing the experiments, analysis of variance and optimization of process, Design-Expert® 8 software from Stat-Ease, Inc., was used.

2.8. Determination of hydrolysis products

The hydrolysis products of inulin with crude enzyme were determined by paper chromatogram (Wehaidy, 2012). The paper chromatogram spotted with reaction products after 15 min incubation was developed using (butanol:acetone:water) (4:5:1, v/v/v) as irrigating solvent. After 48 h. the hydrolysis products (sugars) were visualized by heating the plates at 105 °C for 15 min after spraying with detection solution containing (0.91 ml aniline, 1.66 g phthalic acid, 48 ml diethyl ether, 48 ml butanol and 4 ml water). Glucose, fructose and sucrose were used as standards.

3. Results and discussion

3.1. Survey of low cost substrate and marine-derived strain favouring inulinase production

Although inulinase was initially isolated from plants, it is difficult to obtain high production (Kumar, Kunamneni, Prabhakar, & Ellaiah, 2005). In the last decades, a large number of microorganisms such as bacteria, yeast and filamentous fungi (*Penicillium* sp., *Fusarium* sp., *Aspergillus* sp.) were used for inulinase production (Kango, 2008; Naidoo, Ayyachamy, Permaul, & Singh, 2009; Singh & Bhermi, 2008; Souza-Motta, Cavalcanti, Porto, Moreira, & Lima filho, 2005). In order to select the best solid substrate (inuline source) and the fungal strain for inulinase production, six agro industrial wastes (low cost substrates) were checked for their effect on inulinase production by four marine-derived fungal isolates, *A. terreus*, *A. versicolor*, *A. parasiticus* and *P. brevicompactum*, locally isolated from decayed wood samples collected from Ismalia, Egypt.

Results documented in (Table 4) showed that, all the tested strains produced the enzyme on all the substrates but with different activities. Among the six different solid substrates, artichoke leaves were found to be the most suitable substrate for SSF by *A. terreus* giving maximum inulinase production of 4.433 ± 0.121 U/gds, greater than that obtained with *K. marxianus NRRL 7571*(positive control) on the same waste. Other substrates were found to be poor inducers for inulinase production by *A. terreus*. Significant amount of inulinase was also produced on SSF of orange rinds and sugar cane bagasse with *A. versicolor* and *A. parasiticus*, respectively. *P. brevicompactum* showed the highest production with artichoke leaves, banana leaves and sugar cane bagasse than the other substrates.

There for, the marine fungal isolate *A. terreus* was the most promising one for inulinase enzyme production and the present work will focused to study the optimization of the SSF using artichoke leaves. Also the type of the produced enzyme (endo- or exo-) will be determined.

3.2. Time course of fermentation

The time courses of the inulinase production by the selected fungus *A. terreus* during SSF of artichoke leaves (basal medium)

Table 3
Factors and their levels which were studied by the orthogonal array of L9 (3^4) design for optimization of inulinase production from *A. terreus* by the Taguchi method.

Trial number	Factors								Response enzyme Activity (U/gds)	
	Coded levels				Actual levels					
	A: Initial pH	B: incubation Temp. (◦C)	C: Initial concent. of KH_2PO_4 (%)	D: Initial concent. of Ca^{2+} (mM)	A: Initial pH	B: incubation Temp. (◦C)	C: Initial concent.of KH_2PO_4 (%)	D: Initial concent. of Ca^{2+} (mM)	Experimental (actual)	Predicted
1	3	3	2	1	8.5	40	0.5	5	3.547	4.029
2	3	1	3	2	8.5	30	0.8	6	21.058	20.374
3	1	1	1	1	6.5	30	0.3	5	8.246	8.729
4	2	3	1	2	7.5	40	0.3	6	2.66	1.975
5	2	2	3	1	7.5	35	0.8	5	17.955	18.438
6	1	2	2	2	6.5	35	0.5	6	9.532	8.847
7	1	3	3	3	6.5	40	0.8	7	1.995	2.197
8	2	1	2	3	7.5	30	0.5	7	13.300	13.502
9	3	2	1	3	8.5	35	0.3	7	15.295	15.497

Table 4
Effect of different low cost substrates on inulinase production by locally isolated marine-derived fungi under solid-state fermentation.

Fungal isolates	Inulinase activity (U/gds) with different solid substrates					
	Chicory roots	Artichoke leaves	Banana leaves	Garlic wastes	Orange rinds	Sugar cane bagasse
Aspergillus terreus	0.133 ± 0.063	4.433 ± 0.121	0.366 ± 0.423	0.022 ± 0.031	0.776 ± 0.470	1.108 ± 0.521
Aspergillus versicolor	0.177 ± 0.125	0.178 ± 0.125	0.205 ± 0.031	0.399 ± 0.067	1.917 ± 0.016	1.319 ± 0.141
Aspergillus parasiticus	0.199 ± 0.141	0.177 ± 0.125	0.321 ± 0.173	0.587 ± 0.329	1.341 ± 0.862	1.773 ± 0.627
Penicillium brevicompactum	0.886 ± 0.626	1.241 ± 0.877	1.219 ± 0.157	0.665 ± 0.156	0.853 ± 0.047	1.217 ± 0.052
Kluyveromyces marxianus NRRL 7571	0.222 ± 0.157	1.773 ± 1.254	1.352 ± 0.188	0.886 ± 0.376	0.443 ± 0.313	0.088 ± 0.012

* *Kluyveromyces marxianus* NRRL 7571: used in the screening step as a positive control for inulinase production.

without any additives were monitored for 240 h. Results indicated that, the high level of inulinase attained within 96 h. The inulinase production reached about (5.680 ± 0.183 U/gds) after 96 h of solid-state fermentation, and after that, the activity decreased with increasing the time of fermentation. The observed decline in inulinase activity after 96 h of incubation could be a result of protease degradation, decrease in nutrient availability in the medium and catabolic repression of the enzyme (Kango, 2008; Wang & Zhou, 2006). This was in agreement with the previous studies on *A. niger* ATCC 20611 (Dinarvand et al., 2012) indicated the maximum inulinase production obtained after 96 h of incubation at 30 °C. Hence, all the experiments of optimization were carried out for 96 h.

The obtained results compared well with the results for other producer microorganisms where the inulinase activity generally was reported to peak much earlier in the fermentation process. For example, (Kumar et al., 2005) observed the highest inulinase activity after 72 h of incubation.

3.3. Screening of significant nutrients using a Plackett–Burman design

Plackett–Burman design is a powerful technique for screening important variables. In the present study, it was used to analyze the effect of eleven variables, including fermentation conditions and medium constitution on inulinase production (Table 1). In the experiment design, each row represents an experiment and each column represents an independent variable (Table 2). The yield of inulinase, determined for each experiment design, was shown in Table 2. Where we found a wide variation in the activity from 0.443 to 13.300 U/gds. This variation reflects the importance of medium optimization to attain higher productivity.

According to these results, a medium of the following composition is expected to be near optimum: initial pH 7.5, incubation temperature 35 °C, 0.5% KH_2PO_4, 5 mM Mg^{2+}, 1% glucose, 1%sucrose, 1% $NH_4H_2PO_4$ by employing 3 g of artichoke leaves (moistened with 15 ml of tris-maleate buffer, 0.1 M, pH 7.5) as the solid substrate in 250 ml Erlenmeyer flasks for 96 h incubation period. The enzyme activity measurement on this medium was 13.300 U/gds. This result presented about 2.34-folds increase in the enzyme activity, when compared to (5.679 U/gds), the results obtained in basal production medium containing only the solid substrate and sea water after 96 h incubation time.

The analysis of variance (ANOVA) for the experiment design showed that, A, B, D, E, F, H, J, K, L are significant model terms. But the factor C is not significant and removed. The first order model equation developed by PB design showed the dependence of *A. terreus* inulinase production on the medium constituents: Eq. (1).

$$R1(\text{inulinase activity U/gds}) = +4.52 + 3.23 * A + 0.51 * B - 0.72 * D - 0.47 * E - 0.92 * F + 0.020 * G - 1.22 * H + 2.19 * J + 0.45 * K + 0.95 * L$$

The analysis of the regression coefficients of the 11 variables indicated that, initial pH, KH_2PO_4, Ca^{2+}, incubation temperature and Mg^{2+} had positive effect on inulinase production. Urea, yeast extract, glucose, sucrose and $NH_4H_2PO_4$ were contributed negatively (Fig. 1).

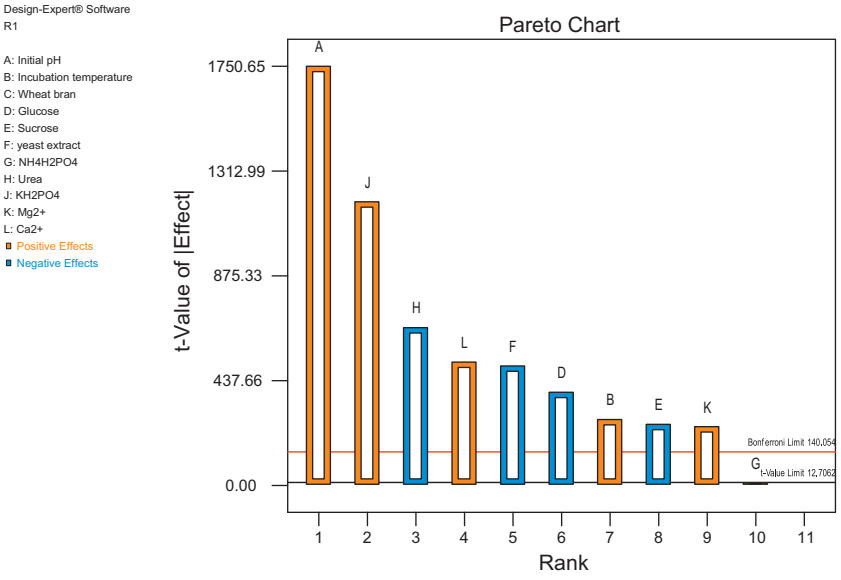

Fig. 1. Pareto chart showed the effect of each variable on the enzyme activity (U/gds) produced by *A. terreus*.

Table 5
Analysis of variance (ANOVA) for the experimental results of the Taguchi method.

Source	Sum of squares	df	Mean square	*F* value	*p*-Value Prob > *F*
Model	383.73	6	63.96	57.41	0.0172 significant
A-initial pH	71.22	2	35.61	31.96	0.0303
B-incubation temperature	264.37	2	132.19	118.66	0.0084
C:Initial concent.of KH_2PO_4 (%)	48.15	2	24.07	21.61	0.0442
Residual	2.23	2	1.11		
Cor Total	385.96	8			

Std. Dev. = 1.06; Mean = 10.40; C.V.% = 10.15; PRESS = 45.12; *R*-squared = 0.9942; Adj *R*-squared= 0.9769; Pred *R*-squared = 0.8831; adeq precision = 19.766 (the factor, D-Ca^{2+} removed where, *p*-value Prob > *F* = 1.0000).

The obtained results showed that, varying the initial cultivation pH of the medium between pH 4.5 and 7.5 had high effect on inulinase production by the marine-derived *A. terreus*. Maximal enzyme activities were obtained only when the initial pH of the culture medium was adjusted to 7.5. This is a logic observation because the culture used in the present study was isolated from marine source where the pH lied in the neutral to alkaline range. On the other hand (Dilipkumar, Rajasimman, & Rajamohan, 2011) indicated that KH_2PO_4 was found to be one of the most significant nutrient components affecting inulinase production by *Streptomyces* sp. in solid-state fermentation (SSF).

The obtained results also agreed with (Gouda, 2002) who indicated that, the metal ions, Ca^{2+} was found to be more effective in inulinase production by *A. fumigatus*. This effect may due to the formation of complex with ionized inulinase resulting in changing solubility and behaviour at the conformation of protein to a less stable form by interaction with enzyme surface charge which could markedly affect the ionization of some amino acid residues (Masomian, Rahman, Salleh, & Basri, 2010).

There for, the optimum combination of the variables (initial pH, KH_2PO_4, Ca^{2+} and incubation temperature) which had the highest significant influence on inulinase production was further analyzed by a Taguchi method for the best solid state fermentation bringing maximum inulinase activity. Other variables with less significant effect were not included in the next optimization experiment, but instead were used in all trials at their (−1) level and (+1) level, for the negatively contributing variables and the positively contributing variables, respectively.

3.4. Further optimization of the nutrients using Taguchi methodology

Based on the results of Plackett–Burman design, four variables including initial pH, incubation temperature, KH_2PO_4 and Ca^{2+}, which significantly influenced inulinase production, were further investigated for their optimum combination using Taguchi's ($L9\,3^{\hat{4}}$) orthogonal array design. The design and results of the experiments together with the predicted activity from the regression equation for the combinations are shown in (Table 3). It showed that the actual response values agree well with the predicted response values.

Final equation in terms of coded factors: Eq. (2).

$$\text{Inulinase enzyme activity} = +10.40 - 3.8 * A[1] + 0.91 * A[2] + 3.80 * B[1] + 3.86 * B[2] - 1.66 * C[1] - 1.61 * C[2]$$

The results of experiments performed in this section showed that the maximum average yield of inulinase was 21.058 U/gds, which occurred when experiment's conditions were as follows: the initial pH 8.5, incubation temperature 30 °C and the concentration of KH_2PO_4 and Ca^{2+} were 0.8% and 6 mM, respectively added to 3 g of artichoke leaves (low cost solid substrate containing inulin) moistened with 15 ml buffer (0.1 M, Tris–maleate pH 8.5). Also the other variables which not included in the Taguchi experimental design were included at the concentrations of, 1% glucose, 1% sucrose, 1% $NH_4H_2PO_4$ and 5 mM Mg^{2+} which give maximum production with Plackett–Burman design.

Analysis of variance (ANOVA) of main effects of factors indicated that, the model *F*-value of 57.41 implies the model is significant. Values of "Prob > *F*" less than 0.0500 indicate model terms are significant, in this case the factors, A-initial pH, B-incubation temperature, C:Initial concentration of KH_2PO_4 (%) are significant model terms.Values greater than 0.1000 indicate the model terms are not significant as the factor D-Ca^{2+}. Results also showed that, The "Pred *R*-Squared" of 0.8831 is in reasonable agreement with the "Adj *R*-Squared" of 0.9769. An adequate precision value greater than 4 is desirable. The adequate precision value of 19.766

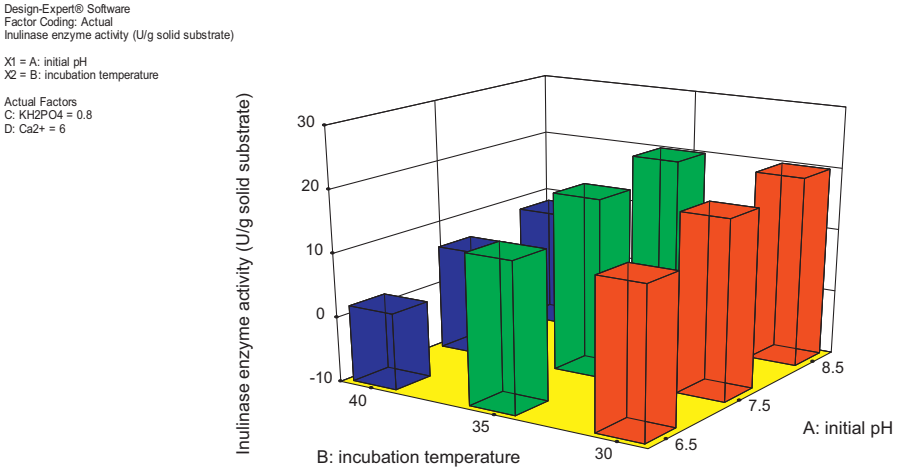

Fig. 2. 3D-surface plot showing the effect of initial pH and incubation temperature on inulinase activity.

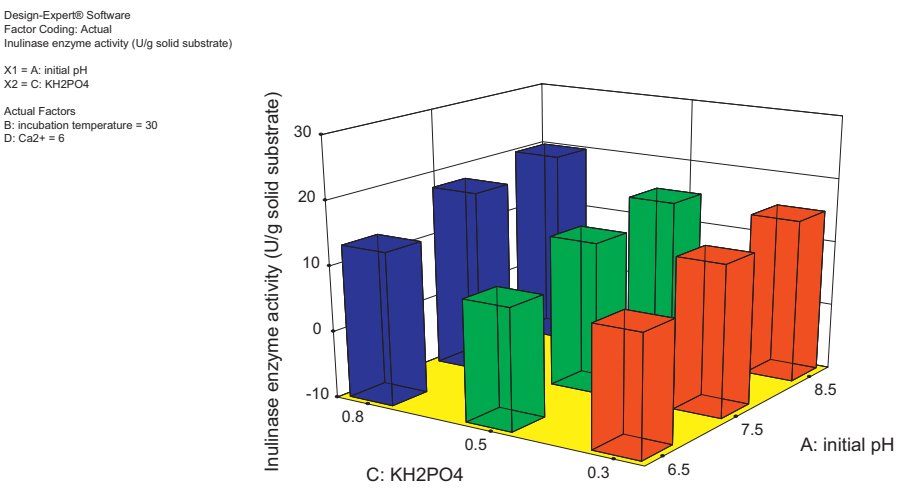

Fig. 3. 3D-surface plot showing the effect of initial pH and KH_2PO_4 concentration on inulinase activity.

indicates an adequate signal and suggests that the model can be used to navigate the design space (Table 5).

The interaction effects of variables on inulinase production were studied by plotting 3D surface plot against any two independent variables, as in Figs. 2–4. The results illustrated graphically in these figures indicated that the dependency of inulinase enzyme on initial pH level and the incubation temperature followed by the concentration of KH_2PO_4 as an energy source. The inulinase activity increased with increasing the initial pH level to 7.5 and 8.5, where the neutral to alkaline range was observed to be most favourable conditions for inulinase production by this strain than the acidic conditions. This to some extend agreed with (Naidoo et al., 2009) who found that the maximal inulinase activity of *Xanthomonas campestris pv. phaseoli* KM24 mutan was obtained when the initial pH of the culture medium was adjusted to 7.0. Previous studies indicated that Geofungi growing in marine environments appear to be conditioned to their environment (Raghukumar & Raghukumar, 1998). The optimum temperature favouring inulinase production is 30 °C, also the activity increased at 35 °C but decreased greatly at the temperature 40 °C.

3.5. Validation of the experimental model

In order to determine accuracy of the model and to verify the optimization results, experiments were repeated in triplicates under optimized culture conditions i.e. the initial pH of the medium 8.5, incubation temperature 30 °C and the concentration of KH_2PO_4 and Ca^{2+} were 0.8% and 6 mM, respectively. Under these conditions, 21.058 ± 0.84 U/gds of inulinase was obtained. This value of enzyme yield corresponds very well to the values predicted by the model (20.37 U/gds), which proved the validity of the model. After statistical optimization, inulinase yield was increased to 4.79-folds when compared to (4.433 U/gds), the results obtained in the first basal production medium, without any optimization.

3.6. Hydrolysis products of inulin

The paper chromatogram procedure employed to characterize the mode of action of the enzyme produced by the marine-derived fungus *A. terreus* and particularly, if it is of the endo- or exo-type. Analysis of the products of inulin hydrolysis after 15 min, showed the presence of fructose (Fig. 5). Release of fructose as an end product of inulin hydrolysis states that inulinase of *A. terreus* was an exoacting enzyme. These results agreed with (Trivedia et al., 2012) who reported the production of fructose as an end product of inulin hydrolysis by a newly isolated *Aspergillus tubingensis* CR16 inulinase enzyme. (Ertan & Ekinci, 2002) also reported that the inulinases of *A. alternata* and *A. niger* hydrolyse inulin through an exo-type reaction producing fructose. Fructose is an

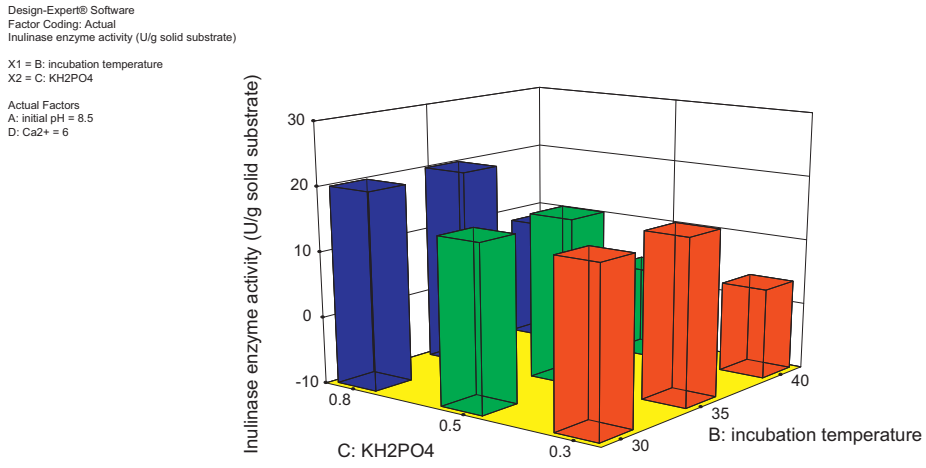

Fig. 4. 3D-surface plot showing the effect of incubation temperature and KH_2PO_4 concentration on inulinase activity.

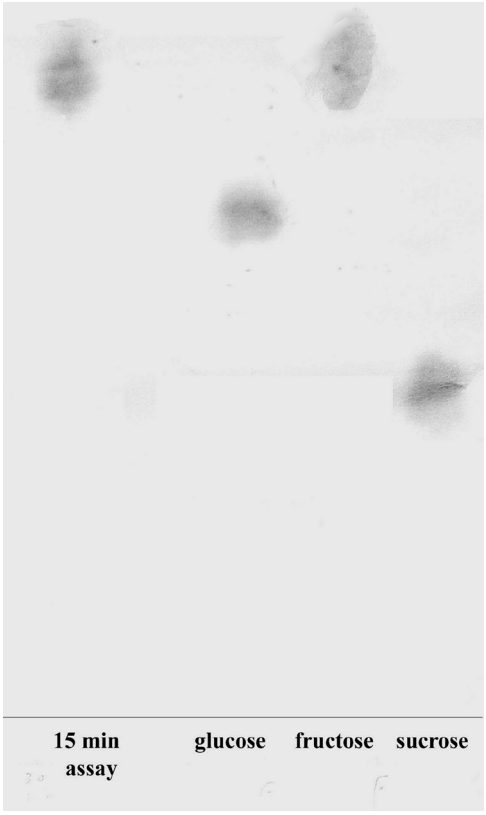

Fig. 5. Paper chromatogram of hydrolyzed products of inulin after 15 min incubation with the *A. terreus* inulinase enzyme. Glucose, fructose and sucrose were used as standards.

important ingredient in food and pharmaceutical industry (Gill, Manhas, & Singh, 2006). Fructose is considered as a safe alternative to sucrose because it has beneficial effects in diabetic patients, increases iron absorption in children, high solubility, low viscosity, higher sweetening capacity and thus can be used as a low calorie sweetener (Pandey et al., 1999).

4. Conclusions

The marine-derived, alkalophilic and salt tolerant fungus *A. terreus*, isolated from marine decayed wood, showed the ability to produce maximal yield of inulinase enzyme on low cost substrate (artichoke leaves) under SSF. After the optimization by using Plackett–Burman and Taguchi methods, the optimal medium composition for the inulinase production by *A. terreus* during the solid-state fermentation was found to be, initial pH 8.5, incubation temperature 30°C, 0.8% KH_2PO_4 and 6 mM Ca^{2+} in the presence of 1% glucose and 1%sucrose as additional carbon sources, 1% $NH_4H_2PO_4$ as nitrogen source and 5 mM Mg^{2+} by employing 3 g of artichoke leaves (moistened with 15 ml of tris-maleate buffer, 0.1 M, pH 8.5). Under the optimized conditions, the maximum yield of inulinase achieved was 21.058 U/gds, which increased about 4.79-folds the initial production medium. In the present work, obtained results indicated the scope for economic production of inulinase by a new marine-derived source using solid-state process and the applied statistical tools proved to be efficient for optimizing inulinase enzyme production in stead of the commonly used methods, Box–Behnken design. Fructose producing capacity of the *A. terreus* inulinase enzyme, make it an attractive source for industrial application. However, further studies on its Properties were also evaluated to predict its end applications which may provide other unexplored information regarding this enzyme.

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