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Effects of different nitrogen sources on invertase production by *Aspergillus niger*

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Abstract: Investigation various nitrogen sources effects on the production of invertase by *Aspergillus niger* was researched in this study. Invertase is a precious enzyme used in many industries like food, pharmacy, confectionery, invert syrup production. Taguchi design of experiment (DOE) was preferred to optimize the cultivation conditions. L16 (4^3) orthogonal array was selected in the current study including nitrogen source, initial pH of the medium and incubation time at four levels for statistical optimization. The data showed that optimized version of invertase production was achieved using proteose peptone, 5.5 initial pH and 3 days for incubation time. Bacto peptone had higher enzyme activity than casein and yeast extract. pH of the medium was found as the most efficient factor among nitrogen source and incubation time. Besides, percentage contribution of the nitrogen source and incubation time were indicated at similar rates (9 and 10%, respectively). The highest enzyme activity was defined as 45.87 U/ml, which was found to be closer to the predicted result (46.33 U/ml). As a conclusion, proteose peptone increased the invertase activity and use of Taguchi DOE supported quick and effective optimization.

Keywords: Invertase, *Aspergillus niger*, optimization, Taguchi DOE

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1. Introduction

Invertase (EC 3.2.1.26, β-fructofuranosidase) is a valuable enzyme used in various industries that acts on the hydrolysation of sucrose and related glycosides into reducing sugars (fructose and glucose) at equal concentration. This mixture is named "invert sugar" (Kotwal and Shankar 2009; Nadeem et al. 2015; Tasar 2017). Invert sugar has higher sweetener capacity and has been widely used in many industries such as beverage, confectionary, soft-centered candy production, non-crystallizing creams (Canli et al. 2011; Kotwal and Shankar 2009; Taskin et al. 2013). Invertase enzyme is also used for the production of invert sugar for honeybees (Das et al. 2016; Shafiq et al. 2001).

Invertase is produced by *Saccharomyces cerevisiae* or *S. carlbergensis* commercially (Aranda et al. 2006). Furthermore, filamentous fungi and bacteria have been studied for invertase production (Rubio and Navarro 2006). This enzyme is inducible in filamentous fungi, however, it is structurally synthesized by the yeasts (Rubio et al. 2002). *Aspergillus niger* has been extensively used in biotechnological applications. *A. niger* has GRAS (Generally Regarded As Safe) status that was approved by US Food and Drug Administration (FDA) (Abarca et al. 2004). *A. niger* has license to produce 19 different food enzymes. This microorganism is also preferred as an efficient and excellent producer of many valuable products either in submerged or in solid state fermentation (Li et al. 2020).

Optimization of the process parameters is one of the main parts of microbial fermentation. The target product is affected from the composition of the medium. Besides, different carbon and nitrogen sources, mineral salts and other medium components strongly affect the production of the desired product. Classical and statistical optimization tools are preferred due to enhance the product yield. Both of these two optimization techniques have positive and negative aspects (Abdel-Rahman et al. 2020; Tasar 2022). Classical one variable at a time method is a simple and basic technique for optimization; on the other hand, it needs more time and labour when compared with the statistical optimization tools. The statistical optimization tools allow quick screening and effective production of the desired product (Farid et al. 2013; Tasar 2020). Taguchi design of experiment (DOE) presents a robustness optimization with less time and labour (Rao et al. 2008).

The purpose of the current study was to find out the effects of different nitrogen sources on the production of invertase enzyme by *A. niger* OZ-3 using Taguchi DOE methodology. Medium composition and fermentation conditions alter the yield of enzyme; hence the optimization of the medium ingredients and some environmental conditions were studied.

2. Materials and Method

All the reagents were purchased from Sigma Chemical Co. (USA).

2.1. Microorganism and medium

The microorganism *A. niger* OZ-3 (GenBank accession number JX110160) was isolated and determined before (Taskin et al., 2013). The fungus was grown in potato dextrose agar (PDA) slants at 4˚C and subcultured monthly. The minimal medium composition was designated as following (g/l): 20 sucrose, 1 KH_2PO_4, 3 nitrogen sources. 100 ml of basal medium was placed to each 250-ml flasks and incubated at 200 rpm and 30 ˚C for all runs. The pH was adjusted to the desired value using 1 N NaOH or 1 N HCl and the flasks were sterilized in autoclave at 121 ˚C for 20 min.

2.2. Enzyme assay

Extracellular invertase activity was measured in culture filtrate using a previous method that was described by Ge and Zhang (Ge & Zhang, 2005) with a few modification. The culture filtrate was used for enzyme source due to measure the reducing sugar released from sucrose. 100 µl of enzyme source was placed to the test tubes and 900 µl of 0.1 M sodium acetate buffer (pH 5.5) containing 2% of sucrose (w/v) was added. The glass test tubes were incubated at 55 ˚C for 15 min. After this step, the reducing sugar was measured using the DNS method (Miller, 1959). The preparation of DNS reagent was reported in detail before (Canli et al., 2011). 1 ml of DNS reagent was added in each test tubes and they were allowed to incubate in boiling water (90 ˚C) for 15 min and the total volume of the tubes were raised to 15 ml with distilled water. The reducing sugar released from sucrose was determined at 550 nm absorbance. For the control, a blank was prepared using distilled water instead of enzyme source. One invertase unit was determined as the enzyme catalysing the release of 1 µmol reducing sugar from sucrose per min.

2.3. Taguchi DOE and ANOVA test

Taguchi DOE methodology was preferred in the current study for its advantages on optimization. For this purpose, nitrogen source, pH of the medium and incubation time factors were selected at four levels using Taguchi L16 (4^3) orthogonal array (Table 1).

Table 1. Optimization factors and preferred levels.

Levels	Factors		
	Nitrogen sources	**pH**	**Time (h)**
1	Bacto peptone	4.0	24
2	Casein peptone	4.5	48
3	Proteose peptone	5.0	72
4	Yeast extract	5.5	96

Taguchi DOE benefits the signal/noise (S/N) ratio to interpret the situation of the target value with three different characteristics following, the larger-the better, the nominal-the better and the smaller-the better (Tan et al. 2005). It was aimed to optimize and enhance the enzyme activity in the current study, hence, the larger-the better criterion was preferred using the following equation:

$S/N = -10 \log_{10} (1/n \sum_{i=1}^{n} 1/Yi^2)$.

The *n* symbol is for the definition of the number of repetitions for the setup conditions, Y*i* defines the situation of the *i*th experiment (Jean and Tzeng 2003). Analysis of variance (ANOVA) test was calculated to perform the quality characteristics which were derived from the selected factors. Minitab® 19.0 version Statistical Software was used for both Taguchi methodology and ANOVA test.

3. Results

3.1. Taguchi DOE L16 orthogonal array results

Taguchi L16 (4^3) orthogonal array showed that the selected factors caused variation on the enzyme activity. Effects of nitrogen sources, initial pH of the medium and incubation time were defined. The data illustrated that invertase activity was highly reliant with the selected factors (Table 2). The maximum enzyme activity was existed from the 4th experimental set while the minimum activity was determined from the 13th run.

Table 2. Taguchi L16 orthogonal array and invertase activity

Exp.	Factors			Results
No.	Nitrogen source	pH	Time (d)	Invertase (U/ml)
1	Bactopeptone,	4.0	1	23,67
2	Bactopeptone	4.5	2	28,79
3	Bactopeptone	5.0	3	31,65
4	Bactopeptone	5.5	4	43,37
5	Casein	4.0	2	26,22
6	Casein	4.5	1	21,45
7	Casein	5.0	4	33,57
8	Casein	5.5	3	38,95
9	Proteose peptone	4.0	3	29,33
10	Proteose peptone	4.5	4	33,11
11	Proteose peptone	5.0	1	34,21
12	Proteose peptone	5.5	2	38,36
13	Yeast extract	4.0	4	21,37
14	Yeast extract	4.5	3	31,85
15	Yeast extract	5.0	2	29,67
16	Yeast extract	5.5	1	33,53

Table 3 illustrated the response table for means that indicates the individual effects of the factors. Delta value demonstrates the alteration of the highest and the lowest results for each factor. Initial pH of the medium had the highest delta rank while the nitrogen source had the lowest value.

Table 3. Response table for means

Level	Nitrogen source	pH	Time
1	31.87	25.15	28.22
2	30.05	28.80	30.76
3	33.75	32.28	32.95
4	29.11	38.55	32.85
Delta	4.65	13.40	4.73
Rank	3	1	2

Taguchi DOE uses the main effects plot graphs (Fig. 1). This illustration showed the best conditions for selected factors at the peak levels. The optimum levels for each factor were determined as proteose peptone, 5.5 pH value and 3 days for incubation time, respectively. At the last step of the optimization, Taguchi DOE used these optimal levels to predict the highest enzyme activity. The prediction experiment was done according to the suggested levels above and the maximum invertase activity was found as 45.87 U/ml which was near to the predicted value (46.33 U/ml).

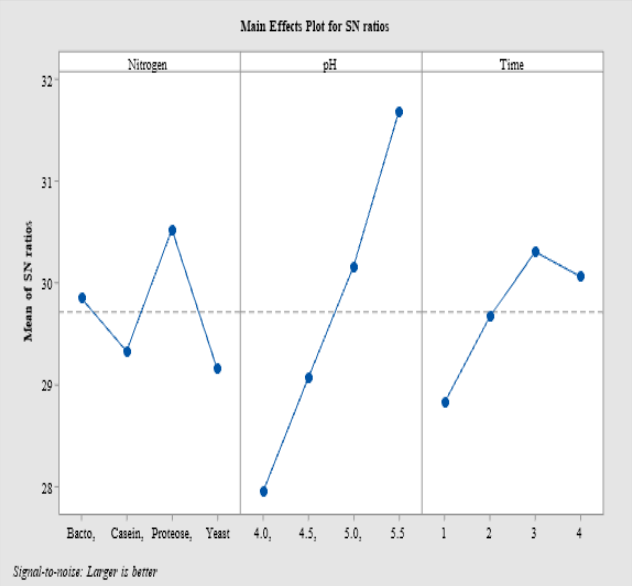

Fig. 1 Main effects plot for S/N ratios

3.2. ANOVA (Analysis of variance)

ANOVA test was performed to calculate the experimental data to evaluate the distinction for the results owing to each factor (Table 4). The P value represented the probability that was calculated from an F-distribution with the degrees of freedom (DF). The highest F value with the lowest P value were obtained from pH of the medium, on the contrary the lowest F value with the highest P value was determined by the nitrogen source. These results were related about the percentage contribution of the factors (Fig. 2).

Table 4. ANOVA table for means

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Nitrogen source	3	50,73	50,73	16,91	1,4	0,30
pH	3	390,43	390,43	130,14	11,4	0,01
Time	3	59,55	59,55	19,85	1,7	0,25
Residual Error	6	67,95	67,95	11,32		
Total	15	568,65				

DF: Degree of freedom; Seq SS: Sequential sum of square; Adj SS: Adjusted sum of square; Adj MS: Adjusted mean of squares; F: F value; P: P value.

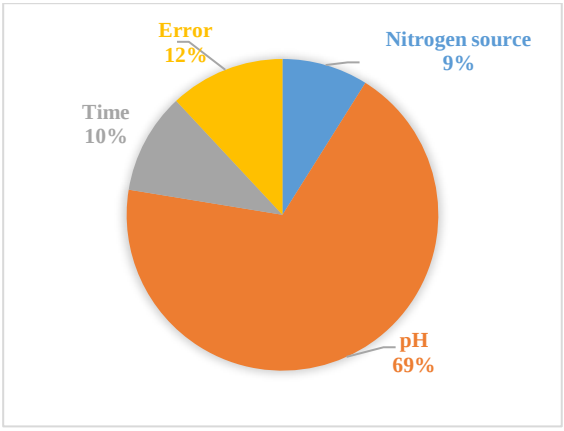

Fig. 2. The percentage contribution of each factor

4. Discussion

Invertase enzyme activity is strongly related with the medium components, the kind of the producer-microorganism and environmental conditions. The results showed that proteose peptone, pH 5.5 and 3 days for incubation time were the best choices for invertase production by *A. niger* OZ-3. Bacto peptone, casein and yeast extract had lower enzyme production. In a previous study about optimization of invertase production by *S. cerevisiae*, urea achieved higher enzyme activity than the nutrient broth and yeast extract. Besides, the optimal pH (6.0) was found closer to the current study (pH 5.5) (Ikram-Ul-Haq et al. 2005).

Medium composition has a great role in enzyme production. In a prior paper, nutrient broth, peptone and yeast extract effects on invertase production by *S. cerevisiae* were studied and the optimal nitrogen source was determined as the peptone at pH 6.0 (Shafiq et al., 2001). On the other hand, another prior studies revealed similar pH value to the current study for different *A. niger* strains for invertase production (Boddy et al. 1993; Laothanachareon et al. 2022). Besides, the same pH value was found to be optimal for invertase production by *Cryptococcus laurentii.* They found the enzyme to be thermostable up to 60˚C at pH 5.5 (Das et al., 2016).

Waste materials are preferred as substrate for microbial fermentation. Either recycling of the waste materials or low-cost production are important parameters for an effective fermentation. Various studies have been done using waste or low-cost materials as substrate for invertase production.

Use of sugar beet, enhanced the economic value of invertase production by *Rhodotorula glutinis*. The optimal conditions were determined for pH and incubation time as 4.5 and 3 days, respectively (Tasar 2017). In a prior study, about invertase production by *A. niger*, the peptone was reported to be enhancer for thermostable invertase from *A. niger* IBK1 strain. The other optimal conditions were determined as pH 5.0 and 120 h at 35˚C (Oyedeji et al. 2017). The pH value and nitrogen source were found to be like the current study; however, the incubation time was found longer from this study. It may be caused by use of a waste substrate (pineapple peel) and different strain.

The response table illustrated the difference between the highest and the lowest enzyme activities for the factors (Table 3). Initial pH of the medium was found to be the most effective factor on invertase production. The percentage contribution of each factor varied depending on the individual effects of the factors (Fig. 2). Initial pH of the medium was found to be the most efficient factor. However, nitrogen source and incubation time factors were found to be closer to each other.

5. Conclusion

Optimization process is the main part of an effective microbial fermentation. Use of classical and statistical optimization tools causes variety on the product yield. Taguchi DOE is one of the most preferred statistical optimization tools for biotechnological applications. In the current study, Taguchi DOE was used in the investigation of the most suitable conditions for invertase production by *A. niger* OZ-3 strain. Effects of bacto peptone, casein, proteose peptone and yeast extract were studied in detail. Initial pH of the medium and incubation time factors were also determined. The results showed that the optimal nitrogen source was proteose peptone at pH 5.5 and 3 days for incubation time. As a conclusion, an enhanced invertase production was achieved in the current study using Taguchi DOE as the statistical optimization tool.

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Authors' contributions: ÖCT: Methodology, investigation, conceptualization, writing, editing and supervision. GET: Data analysis, software, reading-editing and supervision.

Conflict of interest disclosure:

The author declares that she has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate

This study does not contain any studies with human participants or animals performed by the author.

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