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Evaluation of an Edible İnsect (*Locusta migratoria*) as a Substrate for Microbial β-fructofuranosidase Production

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Highlights:

- Edible insect was used as substrate
- Optimization of enzyme production was achieved using Taguchi DOE
- An oleaginous yeast was utilized for β-fructofuranosidase production

Keywords:

- *Locusta migratoria*
- Insect
- β-fructofuranosidase
- Taguchi DOE

ABSTRACT:

Rising population in the world causes reduction on present food resources. Investigators have been looking for sustainable, nutritional and alternative food-stocks. Insects have been consumed as snacks or as food supplement in many countries, but they are still not attractive food resource worldwide. In the current study, β-fructofuranosidase enzyme production by *Galactomyces geotrichum* TS61 (GenBank accession: MN749818) strain was investigated using an edible insect (*Locusta migratoria*) as substrate. β-fructofuranosidase is a valuable enzyme in food industry. Taguchi L16 design of experiment (DOE) was employed to achieve an effective statistical optimization process, including three factors (concentration of locust powder, concentration of sucrose and initial pH) with four levels. The optimized conditions were determined as 40 g/L locust powder, 30 g/L sucrose and 6.0 pH. The analysis of variance results showed that locust powder had more effect on the enzyme production than sucrose and pH. At the end of the optimization process, approximately 4-fold higher β-fructofuranosidase production (40.91 U/mL) was obtained when compared with unoptimized experimental run (9.91 U/mL). Consequently, powdered insects may serve as an effective supplement for valuable enzyme production in food industry.

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INTRODUCTION

Limitless consumption of the natural and/or traditional food sources has been increasing parallel with rapidly rising population all around the world. Investigators have started to investigate alternative food sources to meet the requests. The Food and Agriculture Organization (FAO) of the United Nations predicted the world population approximately 10 billion by the year of 2050. In this context, food availability and cultivation will become more critical (Van Huis et al., 2013; Brogan et al., 2021). The basics of the human dietary include animal and plant originated foods. Animal farming is a traditional nutrition method for humanhood (Bessa et al., 2020; Jantzen da Silva Lucas et al., 2020; García-Gutiérrez et al., 2021). Belong the animal originated food sources exist a huge part of human dietary, mainly meat products of some animals like cow, chicken, sheep, goat, horse, pork, duck etc. However, adulteration of the processed meat products causes commercial frauds to harm the consumers in order to gain more (Zhang et al., 2022). Entomophagy is an alternative and common nutrition method all over the world more than 2000 insect species especially in Asia and Africa (Rumpold and Schlüter, 2013). Insects have many valuable nutrients in their natural structure, like high protein and lipid and less carbohydrate contents; besides, most of commercial natural-originated food colorant agents and supplements are obtained from the insects. Hence, integration of the insects to common foods as food supplements becomes more significant. Belonging to their natural growing habitats, edible insects seem more hygienic from the marine originated animals (Mitsuhashi, 2010; Hamerman, 2016; Brogan et al., 2021). Edible insects are going to be consumed growing up day-by-day, not only as snacks, but also as an ingredient for the other foods (Acosta-Estrada et al., 2021). Although the natural food colouring agents are less stable and have high production cost than the synthetics, in the European Union and the United States, synthetic colourants have restricted for healthy nutrition (Azeredo, 2008; Borges et al., 2012). Like carminic acid (*Dactylopius coccus*), cochineal colour (*Coccus cacti*) and shellac (*Coccus lacca*), insect originated food additives have been widely used in food industry (Arimoto-Kobayashi et al., 2005; Campana et al., 2015). As a food ingredient, a substitute for cereal flour production, to enrich the nutritional value of the flour, insects are used due to their high content of proteins (from 40 to 75 g /100 g on dry weight depending on the species and the stage of the life cycle), lipids and fibers. In this context, consumption of the edible insects may be beneficial for human health (Verkerk et al., 2007; Hamerman 2016; Severini et al., 2018; Jantzen da Silva Lucas et al., 2020; Acosta-Estrada et al., 2021; Çabuk 2021). However, unless stated otherwise, there has been limited data about the influence of insect-derived substrates for enzyme production, hence, it was aimed to investigate the usability of powdered insects for β-fructofuranosidase production in the current study. The insects have been considered as alternative feed stocks due to their high protein ingredient.

L. migratoria is an edible insect, including satisfying essential amino acids and lipids (11.42 g /100 g) with high protein content (71.20 g /100 g) for human nutrition (Purschke et al., 2018; Brogan et al., 2021; Çabuk 2021). Additionally, it has an exoskeleton and the exoskeleton of the insects includes chitin, a kind of carbohydrate, depending on the dry weight of the insects approximately 10% (Belluco et al., 2013). According to these valuable contents, *L. migratoria* has a great nutritional composition both of human dietary and other applications in food industry. *L. migratoria* lives in wild form in the nature and furthermore, it has been cultured for animal feeding as alive bait like in Türkiye.

β-fructofuranosidase (EC 3.2.1.26, namely 'invertase') is a glycoprotein that catalyses the hydrolysis of sucrose into invert sugar at pH 4.5 optimal and stability at 50˚C, depending on its high degree of sweetness of fructose without any crystallization (Kotwal and Shankar, 2009; Kulshrestha et al., 2013). The crystallization causes more sugar content; hence, this enzyme has a significant role for

the formation of the invert syrup (Uma et al., 2010). The reaction exists equimolar concentration of glucose and fructose. The noncrystallizable sugar syrup from sucrose is an important ingredient for the production of soft-centered candies, fondants, etc. (Kulshrestha et al., 2013; Canli Tasar, 2017). Microbially production of β-fructofuranosidase using filamentous fungi and bacteria were reported before, in addition, this enzyme is synthesized constitutively by yeasts; however, it has an inducible form in filamentous fungi (Rubio and Navaroo, 2006). This enzyme naturally exists in plants and helps the osmoregulation, development and defence system, and the commercial producer of this enzyme is *Saccharomyces cerevisiae*, commonly named as baker's yeast. (Kulshrestha et al., 2013).

G. geotrichum has a dimorphic fungal structure with yeast-mold transition and the teleomorph of *Geotrichum candidum* (Yan et al., 2007; Qiao et al., 2017; Altun et al., 2020). Both of these two species are phylogenetically situated on the borderline between the typical yeasts and moulds, moreover, they are closer to the ascomycetes yeast-like fungi phylogenetically (Marcellino et al., 2001; Yan et al., 2007). *G. geotrichum* is not considered as pathogen microorganism and exists in fermented beverages, soil and dairy products (Altun et al., 2020; Grygier et al., 2020). Moreover, this microorganism can be utilized as the starter for the fermentation of some cheeses and beverages (Chebeňová-Turcovská et al., 2016). *G. geotrichum* was utilized in different studies like extracellular productions of lipid (Grygier et al., 2020), lipase enzyme (Fernández et al., 2006; Yan et al., 2007), ethanol from sugarcane bagasse hydrolysate (Lamounier et al., 2020), however, to our best of knowledge, β-fructofuranosidase production capability has not yet been tested.

Classical optimization methods are commonly preferred, but since the last decades use of statistical optimization methods have been increased. Classical methods involve fixing one factor at a time while the others kept constant (Nyanhongo et al., 2002). The whole factors are optimized step by step. This method is very useful, however, statistical optimization methods, like Taguchi DOE, present a randomized experimental designation. In this context, use of statistical optimization techniques can be applied in multi factors-optimization processes. Taguchi DOE is preferred to maximize the robustness of the products and the quality of the optimization process (Rao et al., 2008). This technique is also used for minimizing the variabilities of the target value, besides less experimental runs required than the full factorial design (Tan et al., 2005).

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma (USA) and Merck (Germany). The migratory locusts were bought from Mira Corp. (Antalya, Türkiye) and identified as Locusta migratoria by Gani Erhan Taşar. The migratory locusts were killed using ethyl acetate treatment. At the next step, the locusts were dried in an oven during 3 days at 70˚C. The dried material was then converted to powder using a fine powder mill. The powdered locusts were named as locust powder (LP).

Microorganism and medium

The enzyme producer-microorganism *Galactomyces geotrichum* TS61 (GenBank accession: MN749818) strain was used in this study was isolated and identified before (Altun et al., 2020). The cultures were grown on yeast exract agar slants at 4 ˚C and subcultured monthly. To prepare the yeast starter culture, 100 mL of potato dextrose broth was taken in 250 mL Erlenmeyer flask. One loopful of a 24-h-old culture of *G. geotrichum* TS-61 grown on potato dextrose agar (PDA) was employed as the inoculum material and then the flask was incubated at 30 ˚C and 200 rpm for 48 h on a shaking incubator. The cell density was adjusted to an absorbance at 1.5_{600nm}, and 1 mL of the starter was used for the inoculation. In the first experimental run, unoptimized condition, the medium contents were designated

following (g/L): 10 LP, 3 yeast extract, 5 $NaNO_3$, 0.5 $MgSO_4.7H_2O$, 1 KH_2PO_4, 0.3 $CaCl_2$. The sucrose was added in suitable value depending on the levels on the orthogonal array (Table 1). The initial pH of the medium was adjusted to the selected value with 1 N HCl and 1 N NaOH and autoclaved at 121 ˚C for 15 min. Then, the flasks were cooled to 24 ˚C and inoculated. The environmental conditions were fixed for all the experiments as 30˚C, 150 rpm and 48 h before optimization process (data not shown).

Taguchi DOE and ANOVA

Statistical optimization methods have many advantages and have been used in various fields. In this context, optimization of the production progress is a critical point. Large scaled productions are simulated in small scaled productions before mass production. In full factorial design method, all the factors at all levels are investigated. This method is very useful if there are few factors, however, more factors and levels need fairly high numbered experiments that cannot be available to run. For example, in a full factorial design, three factors with four levels require 4^3 experimental runs, however at the similar situation, Taguchi DOE requires an orthogonal array including 16 experiments, which is a part of the full factorial design. In brief, use of Taguchi DOE method supports less human power, energy consumption and time with high production yield (Canlı Taşar, 2017, 2020). This technique uses three characteristic categories as the larger-the better, the nominal-the better and the smaller-the better. In this study, it was aimed to gain higher enzyme production, hence, the larger-the better criterion was utilized and the equation is following:

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

where S/N value determines the statistical performance, n is determined as the number of repetitions, and *Yi* is determined as a performance value of the *i*th experiment. Designers use the S/N value for detection of the best results (Jean and Tzeng, 2003). In the present study, three optimization factors with four levels were studied (Table 1).

Table 1. Selected culture conditions and assigned levels.

Serial No	Factors	Level 1	Level 2	Level 3	Level 4
1	LP	10	20	30	40
2	Sucrose	0	10	20	30
3	pH	4	5	6	7

The signal-to-noise (S/N) ratio are preferred to interpret the obtained results instead of the average value (Tan et al., 2005). S/N ratios enables the highest results that was selected by the designer (Jean and Tzeng, 2003). The analysis of variance (ANOVA) test was used to determine the effects of the factors. Minitab® 19.1.1. Software (USA) was utilized for statistical design and analysis. The whole experiments were done in triplicate.

Enzyme Assay

The extracellular activity of β-fructofuranosidase was assayed according to Pessoni et al., (Pessoni et al., 1999) with some modification. Briefly, 100 µL of enzyme solution (culture filtrate) and 900 µL of 0.1 M sodium acetate buffer (pH 5.5) containing 2% sucrose (w/v), were taken in glass test tubes and incubated at 50˚C for 30 min. The reaction mixture was then assayed for the reducing sugar using the 3.5-dinitrosalicylic acid (DNS) method (Miller, 1959). Then, 1 mL of DNS reagent was placed into each tube and the glass tubes were placed into boiling water for 5 min to stop the reaction and allowed to cooled to 24˚C. The total volume of each tube was raised to 15 mL with distilled water. The percentage of transmittance at 550 nm was determined using a spectrophotometer. Distilled water was used as the blank instead of the enzyme source. Preparation of DNS reagent was reported in detail before (Canli et

al., 2011). One unit of β fructofuranosidase activity is defined as the amount of enzyme in 1 mL that catalyses the hydrolysis of 1 μmol sucrose per min.

RESULTS AND DISCUSSION

Medium ingredients and environmental conditions are the fundamental factors for the production progress of valuable products. Optimization process is inevitable for efficient use of resources and obtaining high quality products. In this context, optimization tools are employed depending on the factors and their levels investigated. Statistical optimization tools are going to be popular. The obtained results showed that, LP and additional sucrose had strongly affected β-fructofuranosidase production (Table 2). The S/N ratios minimized the undesired effects that causes higher enzyme activity. The highest enzyme activity (36.21 U/mL) was gained from the 15th experimental run, while the lowest activity (9.91 U/mL) was determined at the first experimental run, sucrose-free medium. Sucrose is a type of carbohydrate that contains glucose and fructose at equimolar concentrations, additionally, it is an important substrate for β-fructofuranosidase enzyme. Hence, sucrose-free medium showed fairly low enzyme production. It was reported in a paper that, use of glycerol for extracellular β-fructofuranosidase production by *Y. lipolytica* caused higher enzyme activity than sucrose, glucose and glucose + fructose mixture, respectively. Glycerol had the highest enzyme results, but sucrose caused closer enzyme activity to the glycerol (Lazar et al., 2011). This result confirmed that β-fructofuranosidase enzyme is strongly affected by sucrose.

Table 2. Taguchi L16 orthogonal array and β-fructofuranosidase activity and S/N ratios

Exp. No.	LP	Sucrose	pH	β-fructofuranosidase (U/mL)	S/N ratios
1	10	0	4	9.91	19.9215
2	10	10	5	13.50	22.6067
3	10	20	6	18.77	25.4693
4	10	30	7	16.11	24.1419
5	20	0	5	14.32	23.1189
6	20	10	4	15.21	23.6426
7	20	20	7	17.68	24.9496
8	20	30	6	31.33	29.9192
9	30	0	6	25.63	28.1750
10	30	10	7	27.55	28.8024
11	30	20	4	24.74	27.8680
12	30	30	5	35.39	30.9776
13	40	0	7	33.61	30.5294
14	40	10	6	34.14	30.6653
15	40	20	5	36.21	31.1766
16	40	30	4	31.25	29.8970

In a previous paper about use of powdered insects for food industry, a mixture of LP (15%, w/w) with wheat flour caused enrichment of the protein and fat content with less carbohydrate, and higher metabolizable energy when compared pure wheat flour for the production of protein rich-muffins. On the other hand, use of LP caused unpleasant smell in baked muffins that had negative sensorial impact (Çabuk 2021). A similar situation was reported in another study, using *Schistocerca gregaria* (grasshopper) powder to enrich the wheat bread's nutritional and sensorial properties, the results showed that protein content was increased, however, a distinctive odour existed and this caused lower score with 200 g /kg. Consequently, it was suggested that, powdered insects can serve as a supplement for the production of bread, up to 100 g /kg without alterations in sensorial aspects (Haber et al., 2019).

Use of various substrates have been investigated for enzyme production to gain economical production or management of waste/low cost materials. Fruit peels were utilized as substrate in a previous study for the production of β-fructofuranosidase by *Aspergillus flavus*, and the results showed

that the optimal pH was obtained as 6.0 similar to the current study (Uma et al., 2010). If the characteristic is affected differently, a main effect exists. LP showed the greatest effect on the 4 th level (40 g/L), while the sucrose and the pH had the same effects at the 4 th (30 g/L) and 3 rd (6.0) levels, respectively (Figure 1).

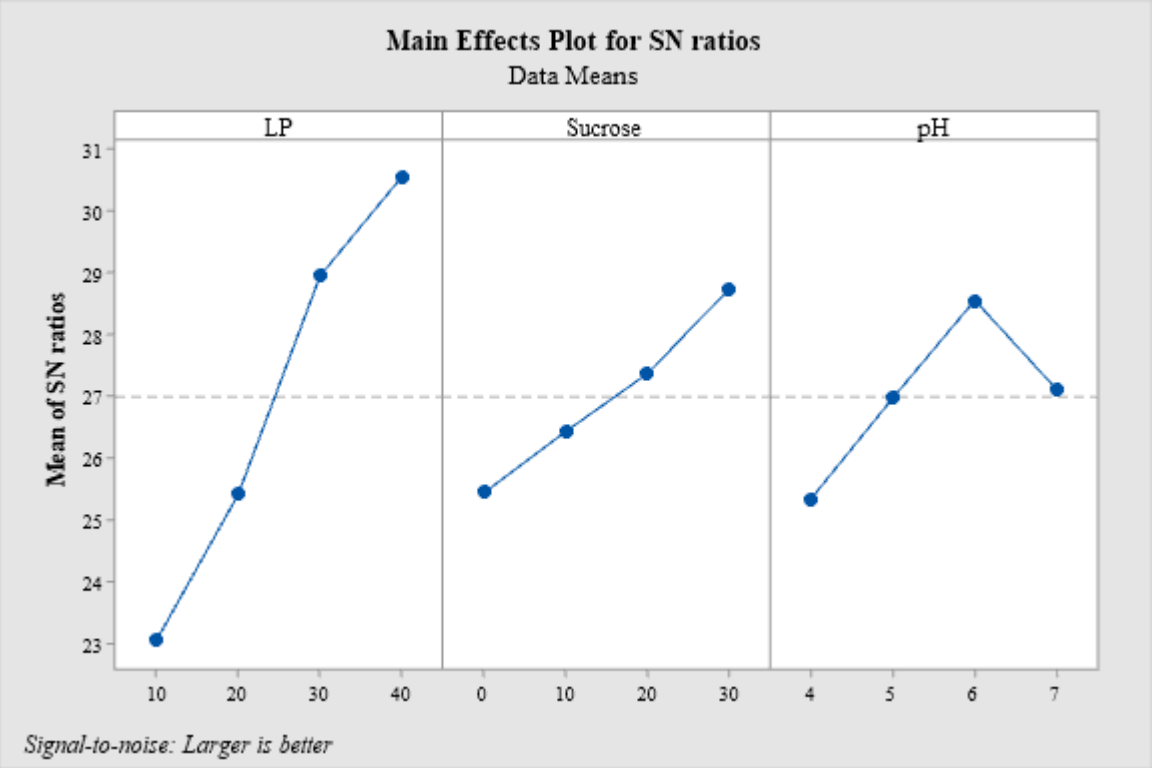

Figure 1. Main effects plot for S/N ratios

Response results of the experiments showed in Table 3. The response table for the means showed that, LP was the most effective factor on the enzyme production. LP was followed by sucrose and pH, respectively.

Table 3. Response table for means

Level	LP	Sucrose	pH
1	14.57	20.87	20.28
2	19.63	22.60	24.86
3	28.33	24.35	27.47
4	33.80	28.52	23.74
Delta	19.23	7.65	7.19
Rank	1	2	3

The ANOVA results illustrated the ranking made on the basis of the amplitude of S/N ratio variation (Table 4). The most effective factor was determined as LP in this study. This may be resulted by the rich structural content of the LP. Sucrose free medium showed less enzyme production naturally. There are many prior studies in the literature that were made by a mixture of edible insect extract with a usual medium component or in different food forms to present them as an alternative source (Belluco et al., 2013; Hamerman, 2016; Haber et al., 2019; Çabuk 2021; Fombong et al., 2021;), however, utilization of *L. migratory* as an effective substrate for microbial β-fructofuranosidase enzyme production has not been investigated yet.

Minitab programme uses the F value for the calculation of the P value, to determine the statistical significance of the terms and model. The LP had the greatest effect on the enzyme production. It also had the largest F value; hence, the lowest P value was obtained by the LP (Table 4).

Table 4. Analysis of variance for means.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
LP	3	890.88	890.88	296.96	22.67	0.001
Sucrose	3	129.19	129.19	43.06	3.29	0.100
pH	3	106.61	106.61	35.54	2.71	0.138
Residual Error	6	78.59	78.59	13.10		
Total	15	1205.26	1205.26			

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.

LP has a P-value that is less than 0.05, thus, LP is statistically significant at a significance level of 0.05. Sucrose had less impact than the LP, on the other hand sucrose-free medium composition showed lower enzyme production. In a previous study, *L. migratoria* was utilized as the substrate for the inulinase production by *G. candidum* and similar results were obtained to this study. LP was obtained as the most effective factor for inulinase production (Canli et al., 2013). These results showed that LP is an effective substrate for microbial enzyme production.

The percentage contribution supported the effects of each factors. The individual effects of the factors on the mean response were calculated using the sequential sum of square of a factor to the total sequential sum of square (Figure 2).

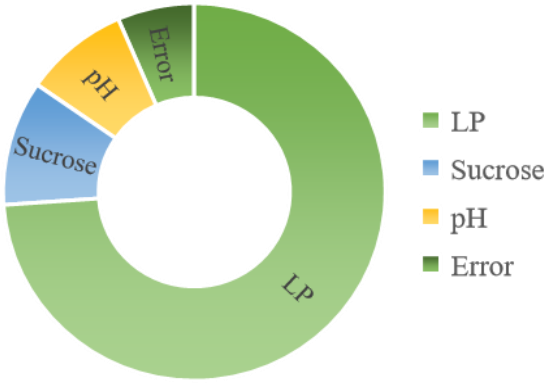

Figure 2. Contribution (%) of each factor on enzyme production

In the last step of the optimization, Taguchi DOE suggests a predicted value using the main effects plot results (Figure 1). The optimized conditions were existed as following: 40 g/L LP, 30 g/L sucrose and pH 6.0. The predicted value was calculated by Minitab software as 41.62 U/mL, and the obtained experimental result was found as 40.91 U/mL. The experimental result was closer to the predicted value; hence, the statistical evaluation was validated. In addition, the optimization of the β-fructofuranosidase production was resulted as approximately 4-fold (40.91 U/mL) higher than the unoptimized condition (9.91 U/mL).

CONCLUSION

Commercially valuable enzymes such as β-fructofuranosidase, require economical production progress. Selection of the fermentation components and optimization of the environmental conditions are the fundamental factors for commercially enzyme production. In the current study, β-fructofuranosidase was produced by *G. geotrichum* using an edible insect (*L. migratoria*) as a substrate. There are many reports about use of edible insects as substrates for different enzymes and valuable products. *L. migratoria* is an edible insect that has an increasing role in the food industry as supplement due to its rich protein and lipid with less carbohydrate content. This study was planned to investigate the effects of powdered *L. migratoria* as a substrate for β-fructofuranosidase production by *G. geotrichum*. The results showed that this edible insect may serve as an effective substrate for commercially valuable

enzyme production. Taguchi DOE proved that, it's a powerful statistical tool for robust production and optimization.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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