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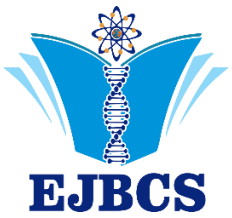

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Use of onion peels as an economical substrate for microbial inulinase production under solid state fermentation

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Abstract: Onion (*Allium cepa*) is a valuable vegetable and a candidate for sustainable waste management in agri-food industry. The purpose of the current paper was to research the utilization of onion peels to an economical substrate for inulinase production by *Yarrowia lipolytica* ISF7 strain under solid state fermentation (SSF). SSF is preferred to obtain an effective and low-cost inulinase production. The medium designation was optimized using Taguchi design of experiment. For this purpose, Taguchi L_9 orthogonal array layout was applied using the moisture content, initial pH and incubation time as the selected factors at three levels. The results showed that the minimum inulinase activity 22.7 U g^{-1} of dry substrate (ds) was determined using the 6th experimental setup while the highest inulinase activity 292.2 U gds^{-1} was measured from 5th experimental setup. The predicted value was determined as 311.6 U gds^{-1} which was closer to the obtained result (305.1 U gds^{-1}). Consequently, an effective inulinase production can be achieved by *Y. lipolytica* ISF7 using onion peels as an economic substrate under SSF.

Keywords: Solid state fermentation, Taguchi design, enzyme, optimization

1. Introduction

Waste management is going to be more important due to increase at the world population. One of the major challenges of the current age is the conversion of waste materials to valuable products, especially in agri-food industry. The waste materials like egg shells, vegetable peels, coffee grounds have attraction as being candidates for biotechnological applications (Budžaki et al. 2022). The governments investigate policy for food management and safety, decreasing the food loss and evaluation of the non-edible food parts for value-added products. The greatest part of the waste management includes food wastes (Sharma et al. 2022). The onion (*Allium cepa*) is generally cultivated and consumed globally and in terms of economic importance. It is accepted as the second valuable vegetable after tomatoes among all the vegetables. At household kitchen or food industry level, huge amounts of non-edible parts of onions, like peels, top and bottom parts, and corrupted layers are thrown away as garbage (Pathak et al. 2016; Abd-Elsalam et al. 2021; Zhivkova, 2021; Kumar et al. 2022). Furthermore, waste onion parts present environmental problems and cannot be used within animal fodder or fertilizer production depending on its strong aroma (Benítez et al. 2011). An alternative disposal of onion-derived wastes may be used in food ingredients that have positive effects on human health, due to their rich ingredients like dietary fibre, polysaccharides, polyphenols, antioxidants, fructooligosaccharides, alkyl cysteine sulphoxides and flavonoids (Griffiths et al. 2002). Moreover, the onion peels were determined as “suitable” candidates as raw materials for immobilized enzyme carriers; however, a sustainable conversion-production process using these waste materials is not available yet (Budžaki et al. 2022).

Inulin exists the β-2,1-linked D-fructofuranose linear chains residue through a sucrose-type bonding at the reducing end that placed in the roots and tubers. Inulin is a main carbohydrate reserve material and stores energy in many plants like garlic, leek, Jerusalem artichoke, dahlia, chicory, burdock, onion, asparagus, agave, etc. (Van Loo et al. 1995). Onion is an indispensable economic food source and it has been easily available relatively all the time. As a result of regular consumption of this plant for households or food industry, waste onion peels exist in significant amounts every day. It’s about 450.000 tonnes of onion wastes exist annually in Europe (‘Conversion of

environmentally-unfriendly onion waste into food ingredients', 1999). Bioconversion of the waste materials are considered as economic, environmentally friendly (Bhatnagar et al. 2015). Onion has many valuable contents in its natural structure like, free fat (0.31%), total sugars (4.29%), reducing sugars (3.10%), total dietary fibre (16.02%), digestible carbohydrates (4.7%), crude protein (2.61%), magnesium (1285 mg kg-1), sodium (1021 mg kg-1), phosphorus (881 mg kg-1) and copper (4.58 mg kg-1) (Zhivkova 2021). Besides, the onion contains inulin 2-6% in its natural structure (Van Loo et al. 1995; Rawat et al. 2021). The fructooligosaccharides (FOS) production is made using different enzymes by the transfructosylation of sucrose catalysed by inulinase or β-fructosyltransferase. FOS expands the shelf-life of many products due to their ability (Sangeetha et al. 2005). The disadvantage of microbial inulinase production is its availability in only large quantities at competitive market prices. Thus, the inulinase production by a microorganism should be well optimized using the environmental conditions like pH, incubation temperature and incubation time, content of the medium, etc. (Sguarezi et al. 2009; Tasar, 2020).

Minimum free water content is the main structural property of the SSF. This method has been used since the ancient times for bread and cheese fermentation (Libardi et al. 2017; Soccol et al. 2017). SSF has been used commonly in biotechnological applications like microbial enzyme productions as amylase (Selvam et al. 2016), protease (Kandasamy et al. 2016), laccase (Srinivasan et al. 2019). Although, microbial inulinase production were obtained both of submerged (SmF) and solid-state fermentation (SSF) techniques with different kinds of microorganisms and substrates (Mughal et al. 2009; Erdal et al. 2011; Canli et al. 2013; Tasar, 2020), however, to the best of our knowledge, there have been limited studies using onion peels as substrate for inulinase production under SSF (Ayyachamy et al. 2007; Yazici et al. 2020). SSF method benefits from the low moisture content near or at the surfaces of solid materials for microbial growth and product formation (Selvakumar and Pandey 1999). SmF method needs more energy consumption and labour, besides, SSF presents similar growing condition to the microorganisms with their original habitat with less production cost than SmF (Singhania et al. 2010). Use of statistical optimization designs contributes more efficient fermentation progress, hence, SSF effects were investigated in this study. The researchers applied the optimization methods due to their advantages to enhance the production capacity.

Y. lipolytica is a dimorphic and strict aerobic yeast that has an oleaginous nature. This yeast has ability on the bioconversion of hydrophobic substrates. *Y. lipolytica* has also a GRAS (generally recognized as safe) status, that means this yeast is approved for many applications in food industry by the United States of America Food and Drug Administration (FDA) (Groenewald et al. 2014; Desnos-Ollivier et al. 2020; Fraga et al. 2021; Madzak, 2021). *Y. lipolytica* has a great potential to collect the lipids in large amounts and its whole genome sequencing caused valuable tools for genetic manipulation (Beopoulos et al. 2009; Wang et al. 2013; Hughes et al. 2017; Shi et al. 2018). *Y. lipolytica* is one of the most studied unconventional yeast, however, according to our best knowledge there is not any report for inulinase production using onion peels. This study aimed to research the inulinase production capability of *Y. lipolytica* ISF7 using waste onion peels as substrate under SSF cultivation.

2. Materials and Method

The whole chemicals were purchased and pure inulin (chicory) from Sigma Chemical Co. (USA) and Merck (Germany). Onions were bought from markets in Erzurum, Turkey. Considering the fact that, waste onion peels were needed to be totally free from apparent damage or microbial infections. The washed and dried onion peels were sliced in a blender to 0,5 mm particle-sized fine powder and the main substrate was called as onion peel powder (OPP).

2.1. Microorganism and medium

The microorganism was isolated from 1% inulin enriched-potato dextrose agar containing plates using 0.1 mL cheese whey directly as the microorganism source which was provided from Food Engineering Department Dairy Products, Ataturk University, Turkey. Dairy products are generally contaminated with inulin-hydrolysing microorganisms; thus, cheese whey was used for microorganism isolation target. The best grown isolate named as IN-3 was identified as *Y. lipolytica* ISF7 using 16S rRNA sequence analysis (Fig. 1). The ITS region was amplified under in vitro conditions using universal ITS1 and ITS4 primers. The pGEM-T Easy Vector Systems (Promega UK) was employed for cloning of PCR products and amplification of ITS gene region for sequencing at Macrogen (The Nederlands). The results were determined from the database and were analysed with BioEdit. Y. lipolytica ISF7 was maintained in potato dextrose agar at 4˚C and subcultured. Potato dextrose broth was prepared in 250 mL flasks for the yeast starter culture and one loopful of a 1-d-old *Y. lipolytica* ISF7 was used for the inoculation. The inoculum material was cultivated at 30˚C and 200 rpm for 48 h on an orbital shaking incubator (Zhicheng ZHWY-200B, China). The cell density was adjusted to an absorbance at $1.5_{600\ nm}$ and 1 mL of the suspension was used for the inoculum material. The growth medium contains (g L-1); 3 $(NH_4)_2SO_4$; 5 $NaNO_3$; 0,5 $MgSO_4.7H_2O$; 0,3 $CaCl_2$. 250 mL Erlenmeyer flasks with cotton stoppers were used for cultivation containing five grams of OPP and growth medium. The flasks were autoclaved at 121℃ for 15 min, cooled to room temperature and inoculated. The incubation temperature was adjusted to 35˚C for all experiments.

2.2. Extraction of the enzyme

The crude enzyme was extracted from the medium at the end of the incubation period. For this purpose, 100 mL of sodium acetate buffer (0.1 M pH 5.5) were placed to each flask and incubated in a rotary shaker for 30 min at 150 g at room temperature. The culture filtrates were identified as crude enzyme at the end of centrifugation at 5000 g for 15 min (Hettich Universal).

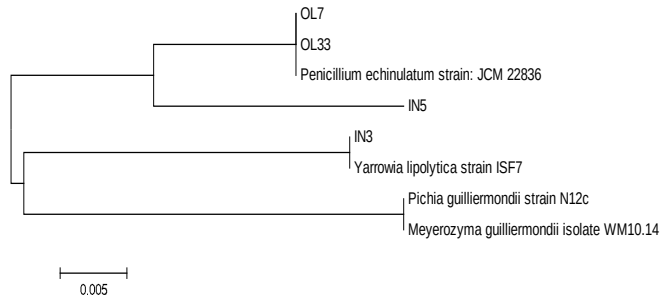

Fig. 1. The phylogenetic tree on the basis of 16S rRNA gene sequence data of *Y. lipolytica* ISF7 strain using neighbour joining method.

2.3. Analytical methods

Extracellular inulinase activity was determined using released reducing sugar from the inulin as described by Pessoni et al. (Pessoni et al. 1999) with some modifications (Ge and Zhang 2005; Mughal et al. 2009). One inulinase unit was determined as the enzyme amount that liberates 1 µmol of fructose from inulin per minute in 1 mL. For this purpose, 100 µL of enzyme source was added to 900 µL sodium acetate buffer (0.1 M pH 5.5) containing 2% (w/v) pure inulin and the glass test tubes were taken to the incubator at 50°C for 15 min. 1 mL of DNS was added to glass test tubes and left for boiling in a water bath for 10 min. Boiling water stopped the enzyme activity. The total volume of the tubes was raised to 8 ml using distilled water and released reducing sugar was measured at 592 nm (Thermo MultiSkan Go, Finland) (Lin and Huang 2000). Distilled water was used as the blank.

2.4. Taguchi DOE methodology and ANOVA analysis

In the current study, there were three efficient factors with three levels, that affect the inulinase production, following; initial pH, the moisture content (%) and incubation time (Table 1). In full factorial design, 27 (3^3) runs of experimental setups would be required. However, Taguchi DOE L_9 design suggests only 9 experimental setups, which is a part of full factorial design. This means less human power, less energy and time consumption that are the main advantages of a production process. This method applies the quadratic loss function to measure the loss for departure of the target Taguchi DOE is based on three different characteristic categories as the bigger-the better, the nominal-the better and the smaller-the better (Hsieh et al. 2005; Tasar, 2022). The bigger-the better criterion was selected to increase the enzyme activity using the equation shown below:

$$S/N = -10 \log10 \left(1/n \sum_{i=1}^{n} \mathbf{1}/\boldsymbol{Yi}^{2}\right)$$

where S/N are performance statistics. The n determines the repetition of the numbers and the Yi is a performance value of the i^{th} experiment in the equation. The calculation of S/N ratio was used for the detection of the maximum yield (Jean and Tzeng, 2003).

Table 1. Optimization parameters and their levels

Levels	Factors		
	pH	Moisture content (%)	Time (d)
1	4.0	55	1
2	5.0	65	2
3	6.0	75	3

Analysis of variance (ANOVA) of the obtained results was used to find out the characteristics variation using the selected factors. Minitab® 19.1.1 Statistical Software (United States) was utilized for all the statistical and experimental analysis. The results were determined as the average value of three runs for each setup.

3. Results and Discussion

The exo-inulinase enzyme catalyses the removing the terminal fructose ending molecules from the non-reducing end of the inulin in one step and the final products exist as fructose and glucose at major and minor ratios, respectively (Zhao et al. 2010). *R. glutinis, A. fumigatus, P. brevicompactum, G. candidum* were studied for inulinase production before (Silva et al. 2011; Canli et al. 2013; Tasar et al. 2015; Tasar, 2020; Rawat et al. 2021).

3.1. Taguchi design results

In the current study, Taguchi DOE was employed for the optimization process of inulinase production by *Y. lipolytica* ISF7. The results showed that the cultivation conditions had great effect on enzyme activity. In a recent paper, it was reported that, use of onion peels, wheat bran and maltose had positive effects on inulinase production by *Rhizopus oryzae* under SSF. The optimization of the temperature, initial pH and incubation time were done using Placket-Burman design, and the obtained results showed that optimal values found as 35˚C, pH of 5.5 and 5 days, respectively (Yazici et al. 2020). However, in the current study, optimal values were found for pH as 6 and 3 days for incubation time. This difference may be resulted from inulinase-producer microorganisms. Onion has been reported for its antioxidant activity as dry onion scales which are thrown away as garbage. In a previous study, extracted onion scales were investigated for their quercetin quantity (Abd-Elsalam et al. 2021). Quercetin is an antioxidant that naturally exists as a free aglycone or glycosidic form as conjugated to one or more sugar molecules (Li and Row 2019).

Table 2. Taguchi L9 orthogonal array and inulinase activity and S/N ratios

Exp. No.	pH	Moisture	Time	Inulinase (U gds^{-1})*	S/N ratios
1	1	1	1	30.6±0.7	29.71
2	1	2	2	43.6±0.51	32.78
3	1	3	3	269.4±5.6	48.60
4	2	1	2	126.6±9.3	42.04
5	2	2	3	292.2±11.4	49.31
6	2	3	1	22.7±0.8	27.12
7	3	1	3	216.2±10.3	46.69
8	3	2	1	23.4±0.5	27.38
9	3	3	2	231.4±12.7	47.28

*Values mean ± standard deviation.

The maximum inulinase activity (292.2 U gds^{-1}) was gained from the 5th experimental setup while the minimum activity 22.7 U gds^{-1} was obtained from the 6th experimental setup (Table 2). It is clear from the Table 2, different environmental conditions combinations caused variation on the results. S/N ratios also approved that the higher inulinase activity had the higher S/N ratio. In a previous study, the INU1 gene encoding exo-inulinase cloned from *Kluyveromyces marxianus* CBS 6556 that was ligated into the surface display plasmid and expressed in *Y. lipolytica*, and the optimal pH was found as 4.5 for the expressed inulinase that was immobilized on the yeast cells. In addition, pH stability was obtained in the pH range of 3-8 (Liu et al. 2010). In another study with the same microorganisms using Jerusalem artichoke extract, the highest inulinase activity was obtained as 41.7 U ml^{-1} at 72^{th} hour of the fermentation, which was similar with the current study (Zhao et al. 2010).

Response data for S/N ratios and their comparison were given. The ranking in Table 2 demonstrates that incubation time had relatively strong impact, while the moisture content and initial pH of the medium had relatively weak impacts on the inulinase production by *Y. lipolytica* ISF7. Taguchi DOE uses the S/N ratio for the deviation of the quality characteristics of the results (Sharma et al. 2005). *Y. lipolytica* is commonly known as an oleaginous yeast and used for biofuel and single cell oil production (Shi et al. 2018; Zhao et al. 2010), however, it's inulinase production capability was investigated before, either naturally or recombinantly (Cui et al. 2011; Liu et al. 2010; Zhao et al. 2010). In a previous study, INU1 gene encoding exoinulinase cloned from *K. marxianus* CBS 6556 into the *Y. lipolytica* ACA-DC 50109 resulted as 41.7 U ml-1 inulinase activity after cell growth for 78 h in a 2-L fermenter with 50.6% (w/w) oil extract from Jerusalem artichoke tubers in its cells within 78 h (Zhao et al. 2010), which was similar to the current study for incubation time (3 days). On the other hand, in a previous study, the optimal pH and temperature were obtained as 4,5 and 50˚C, respectively for *Y. lipolytica* that had the INU1 exo-inulinase gene encoding cloned from *K. marxianus* CBS 6556 (Liu et al. 2010).

Table 3. Response table for means

Level	pH	Moisture	Time
1	114.53	124.47	25.57
2	147.17	119.73	133.87
3	157.00	174.50	259.27
Delta	42.47	54.77	233.70
Rank	3	2	1

Taguchi DOE uses the prediction analysis of the obtained results. Fig. 1 illustrated the main effects plot for S/N ratios. For validation analysis, the proposed experimental methodology, inulinase production was re-designated using the suggested optimal levels. The last step of the study was to predict and verify the optimal conditions using the suggested levels of each individual factors. Using the data showed in Figure 1, the optimal experimental setup was indicated using levels at 3, 3 and 3 that's pH 6, 75% of moisture content, and 3 days for incubation time. The predicted inulinase activity was obtained as 311.6 U gds^{-1} which was closer to the obtained result (305.1 U gds^{-1}).

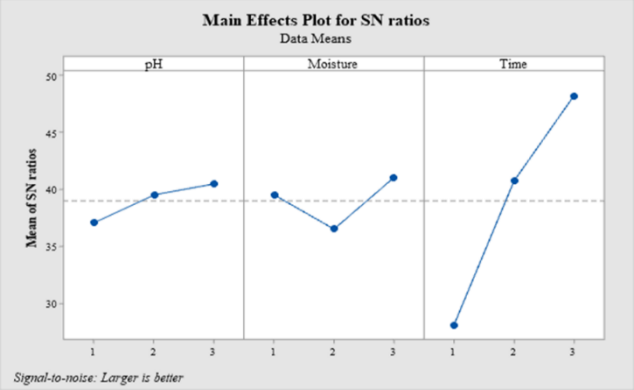

Fig. 2. Main effects plots for S/N ratios

3.2. ANOVA results

ANOVA table illustrates the influencing factors for the inulinase production by *Y. lipolytica* ISF7 (Table 4). The calculated F values indicated the significance of the factors for inulinase production. As a result of these values, the incubation time had the most significant effect, while the pH had less effects. In a previous study, incubation temperature had the significant effect on inulinase production while the incubation time had less effect (Tasar 2020).

Table 4. Analysis of variance for means.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	2	2965	2965	1483	0.24	0.806
Moisture	2	5525	5525	2763	0.45	0.690
Time	2	82070	82070	41035	6.67	0.130
Residual Error	2	12304	12304	6152		
Total	8	102864				

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.

ANOVA analysis showed the ranking made on the basis of the amplitude of S/N ratio variation. Figure 2 showed the individual contribution of each factors on inulinase production. It is clear that incubation time had the maximum effect and the initial pH of the medium had the minimum effect on inulinase production. In a prior study, incubation temperature had the greatest impact and the incubation time was the second most effective factor on inulinase production (Tasar 2020).

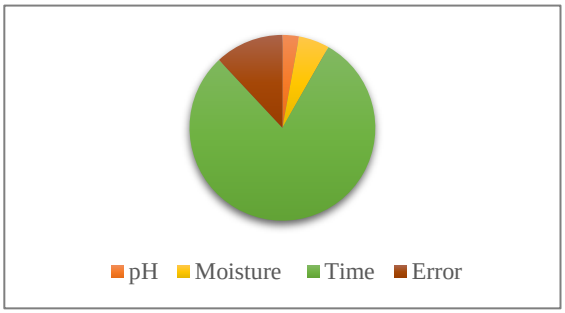

Fig. 3 Contribution of each factor (%)

4. Conclusion

Conclusion

In the current study, the waste onion peels, an important member of the domestic and agri-food industry derived wastes, were employed as an effective, economic and easily available substrate for inulinase production under SSF conditions. SSF commonly presents an economical fermentation for many valuable products like bread and cheese. Taguchi L_9 orthogonal array was utilized for optimization. The obtained results showed that, the environmental conditions have great impact on the fermentation progress. Onion peels can be indicated as a suitable substrate for enhanced inulinase production, besides, optimization of the fermentation is necessary and a powerful tool for effective enzyme production. The moisture content was found as the second effective factor after incubation time on inulinase production. This result is significant because the world stock of the clean water is decreasing day-by-day and there would be a water-crisis in the near future. The next studies can be performed using different agricultural wastes for bio-conversion process of different valuable products.

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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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