Investigation of Static-Culture Exo-Inulinase Synthesis Unleashed by *Candida tropicalis* NRRL-Y-1552 Using Blackstrap Sugarcane Molasses

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Inulinases (β-fructanohydrolases) are hydrolyzing enzymes with an extensive range of industrial applications such as synthesis of fructose syrup, bioethanol, and certain chemicals, such as citric acid, lactic acid. In this work, extracellular inulinase was produced from *Candida tropicalis* NRRL-Y-1552 using a stationary culture technique (ScT) with molasses as the basal fermentation medium. The highest enzyme production (15.08 U/mL) was obtained at 30 °C, pH 4.5, 100 mL molasses, and 48 h incubation. Initially the enzyme activity was not encouraging (6.3 U/mL) but after optimizations, the enzyme production significantly increased (20.4 U/mL, $P \leq 0.05$). The enzyme yield was 69.1%. The size and age of inoculum (1.5 mL, 12 h old) was optimized for maximum production of enzyme. The exo-inulinase production data was subjected to artificial neutral network (ANN) to create a reliable association between the predicted and experimental outcomes. Decision tree techniques were used to forecast the validation of the model. The model's performance was significantly improved by the ANN's linear coefficient correlation value. The significance of the study lies in investigating ANN model of static-culture exo-inulinase synthesis enabled by *C tropicalis* NRRL-Y-1552 using blackstrap sugarcane molasses, making the process ecofriendly and economically feasible for scale up studies.

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INTRODUCTION

Enzymes are biological catalysts. They catalyze the chemical reactions without themselves suffering any overall change. As members of the glycoside hydrolases family 32 (GH32) inulinases, also known as β-fructanohydrolase (E.C. 3.2.1.80), are a significant class of enzymes that catalyze the hydrolysis of inulin, resulting in the primary products of fructose, glucose, and inulo-oligosaccharides. Bacteria, fungi, and yeasts are significant microbiological suppliers of inulinases (Saha 2006; Singh *et al.* 2017). Endoinulinases and exoinulinases are classed as two groups of inulinases based on how they function on inulin.

Endoinulinases (*E.C.3.2.1.7*) work arbitrarily on internal β-2,1 glycosidic links of inulin to form fructo-oligosaccharides, while exoinulinases (*E.C.3.8.1.80*) break down the terminal connections in inulin in order to produce fructose (Ricca *et al.* 2009; Singh and Singh 2017).

It is possible to use the endoinulinase to produce inulo-oligosaccharides and the exoinulinase to produce high fructose syrup using a natural inulin substrate (Ertan *et al.* 2003; Chi *et al.* 2009, 2011; Neagu and Bahrim 2011). Among the hydrolytic enzymes, inulinases has been receiving much attention because of their beneficial effects in human nutrition. Wassink and Flemming (1980) described a procedure that involves the extraction of inulin from *Jerusalem artichokes* and its subsequent hydrolysis with microbial inulinases (Kaur and Gupta 2002; Kim *et al*. 1989; Kim *et al*. 1997; Liu *et al*. 2010). For commercial production of inulinases, submerged fermentation (SmF) and solid-state fermentation (SSF) have been efficiently used (Mazutti *et al.* 2010). In SSF, microorganisms grow without free water; the moisture required by the organism present in the absorbed form in the solid matrix. But in case of SmF, a liquid medium in which fermentation substrate is diluted with water (Fonseca Amaral *et al.* 2007; Das *et al*. 2019). In the stationary culture technique (ScT), the organism is allowed to grow on the surface of a liquid medium without agitation. Molasses has been also used for the production of inulinase since molasses is known as a prime fermentation medium (Vallander and Eriksion 1985). Blackstrap molasses, which is cheap, dark, and viscous, is readily available substrate used for inulinase production (Bender *et al.* 2006; Lim *et al.* 2011; Mazutti *et al.* 2007).

Another critical element for yeast growth and metabolite formation in stationary culture is the medium's pH (Pandey 1992; Silva-Santisteban and Filho 2005). Depending on the kind of substrate, different microbial species have been found to have different pH values (Singh and Bhermi 2008). The incubation period significantly affects the synthesis of inulinase. Most yeast has been found to produce inulinase in growth-related ways, with the peak production occurring close to their stationary phase (Al-Dagal and Bazaraa 1998; Yuan *et al.* 2012). After a given time of incubation, the synthesis of inulinase may decrease due to a catabolic repression mechanism or a decline in the medium's carbon source (Vandamme and Derycke 1983). This decrease may result from the release of proteolytic proteins, which are known to denaturize proteins (Gupta *et al.* 1994). The size of inoculum has a great influence on inulinase production. Maximum production was acquired at 4% inoculum level, while minimal yield was obtained at the 1% inoculum level (Selvakumar and Pandey 1999). According to Shafiq and Ali (2002), minimal enzyme yield at an inoculum level less than 4% may be caused by insufficient biomass to utilize enzyme yield, whereas low enzyme yield following a 4% inoculum level may be the consequence of high inoculum concentrations depleting the amounts of substrate nutrients required for optimal product formation. Temperature significantly influences the metabolic activity of microbial organisms. Each organism has a certain optimum temperature that facilitates maximal growth and product yield. Several investigations have indicated that inulinase synthesis peaks at 30 °C in submerged fermentation (Cazetta *et al*. 2005). In SSF, Selvakumar and Pandey (1999) found that the greatest inulinase synthesis occurred at 37 °C. An organism's shift in optimal temperature may depend on the kind of microbe, the substrate's depth, porosity, and particle diameter (Vandamme and Derycke 1983). Inulinase production was enhanced through the screening and optimization of carbon source concentration (Rouwenhorst *et al.* 1988; Singh *et al*. 2017). Growth typically follows a pattern that is influenced by various environmental factors and the nutrients present in the environment.

At first, a single yeast unit will develop quickly. Following the exponential phase, there will be a plateau, also known as the stationary period, during which the organic nutrients will decrease, and a decline phase will ensue. Numerous supplements, including weak organic acids, phosphate sources, different forms of magnesium, and additional carbon sources, are necessary for the nutritional requirements of yeast. For yeast to develop, carbon is one of the most important sources. It is also well recognized that phosphorus has a significant physiological and biochemical function in microorganisms. Consequently, it controls microbial development and yield more thoroughly (Nuggehalli and Montanhalli 1936).

Inulinases constitute a major class of hydrolyzing enzymes, as they have a wide range of industrial applications. Different techniques are being developed by using various substrates to enhance its production (Onilude *et al.* 2011). In the present study, *Candida tropicalis* NRRL-Y-1552 was used for inulinase production using stationary culture technique (ScT) with molasses as substrate. Although in ScT less labor is required, it is time consuming, due to the lower amounts of enzyme units. The microbial strain was chosen because little research work has been done on it for inulinase production. In addition, it is a faster growing organism, and handling of this yeast is secure and facile. Hence, there has been a need to design a procedure for inulinase production using this particular strain.

The artificial neural network (ANN) approach was used to simulate non-linear statistical data linkages between input and output data. ANNs can be adaptive systems that change their structure based on the information that flows through the network while learning. An ANN model might be used to target the statistical analysis validation exo-inulinase released by *C. tropicalis* NRRL-Y-1552 because it is regarded as a significant mathematical modeling tool. Lee (2003) demonstrated the effectiveness of ANN in predicting the strength of concrete and suggested using ANN algorithms to accurately predict the concrete's compressive strength. With a high degree of accuracy, the model may be useful in predicting the coefficient of determination (R^2) value. Additionally, the method can forecast the values of the circular steel constrained concrete's compressive strength. It is also crucial to look into supervised statistical methods for predicting the strength property beforehand so that it can be compared to the actual outcome. This could allow researchers to examine the results of the input parameters without having to spend time on practical work.

EXPERIMENTAL

Materials and Methods

The chemicals including sodium acetate, peptone, dextrose, dinitrosalicylic acid (DNS), phenol, sodium metabisulphate, sodium potassium tartarate, and phosphoric acid were of the highest possible purity. Also, the commercial grade Coomassie Brilliant Blue without further purification and standard bovine serum albumin (BSA) were used.

Organism and Culture Maintenance

The culture *Candida tropicalis* NRRLY-1552 obtained from the stock culture of *Institute of Industrial Biotechnology (IIB), GC University Lahore* was utilized in this study. It was kept in yeast extract employing peptone dextrose agar (YPD medium, which was made by dissolving all of the ingredients (except from the agar) in about 900

mL of distilled water. The mixture was agitated until it turned clear. The pH was adjusted to 4.5 with 0.1N NaOH or 0.1N H_2SO_4. Agar was then added. The final volume was raised to 1 L) slopes containing 10 g/L yeast extract, 20 g/L dextrose, 20 g/L peptone, 20 g/L agar at pH 4.5. For sub-culturing, yeast cells were inoculated on solidified YPD slopes from the master slant culture. For best growth, the slant cultures were incubated for 24 to 48 hours at 30 °C. To prevent contamination, subculturing was done every two weeks and continually examined under a light microscope. The slant cultures were then kept in a cooled incubator at 4 °C (11-679-25C, Gallenkamp, England).

Inoculum Preparation and Cell Count

One hundred milliliters of YPD medium (without agar) were prepared at pH 4.5 in a 250 mL Erlenmeyer flask and autoclaved. The medium was set aside to cool at room temperature (25 °C). A loopful of yeast culture (2 to 3 day old) was inoculated aseptically. The flask was incubated at 30 °C, (200 rpm) for 24 h using a rotary shaker (Iremeco GmbH, Germany). A haemocytometer (Marienfield, Germany) was used to enumerate the number of cells and found to be 1.99×10^8 CFU/ mL.

Pretreatment of Molasses

Molasses pretreatment was done with conc. H_2SO_4. Initially 200 mL molasses (pH 5) was diluted with 200 mL distilled water and shake well. Then 14 mL diluted H_2SO_4 was added and allowed to stand at room temperature (25 °C) for 30 min. After that, it was placed on boiling water bath at 90 °C for 1 h, with constant stirring. The molasses was set aside at room temperature (25 °C) for overnight. Next day the clear shiny black layers decant off and sterilized. The clarified molasses was stored at room temperature (25 °C).

Fermentation Technique

The production of exo-inulinase was carried out using stationary fermentation in 500 mL Pyrex flasks. One hundred milliliter of molasses with 150 g/L sugar was used as a substrate. The flasks were cotton plugged and sterilized in an autoclave at 15 lbs/in^2 pressure (121 °C) for 15 min. The medium was allowed to cool at room temperature, and 1 mL of inoculum was added under aseptic conditions. The flasks were incubated at 30 °C for 72 h under static-culture conditions.

Analytical Techniques

Enzyme extraction

Following the specific period, the flasks were taken out of the incubator and their contents were filtered using Whatman filter paper No. 44, which had been previously weighed. For additional testing, the filtrate was centrifuged for 15 min at 4500 rpm, and the clear supernatant was utilized.

Enzyme assay

Nelson's method (1944) was used to measure the concentration of reducing sugars produced from inulin in order to determine the inulinase activity. Inulinase activity was measured by incubating a reaction mixture with 0.5 mL of acetate buffer (0.1M, pH 5.5) and 1% inulin at 50 °C. Following 20 min of incubation, 1.5 mL of DNS reagent (1.06 g of DNS was added in the 60 mL of hot distilled water and was shaken well to dissolve it. Then 30 mL of hot distilled water was added in it along with the 1.95

g of NaOH and was shaken to dissolve it. Phenol (0.75 mL) and sodium metabisulphate (0.83 g) was added in it and final volume was elevated up to 121 mL) was added and the sample was further examined correspond to DNS method (Miller 1959). Under controlled test conditions, one unit of inulinase activity is the quantity of enzyme that generates 1 µmol of reducing sugars per minute. Control samples were analyzed using the same procedure with the replacement of crude enzyme by distilled water. The absorbance A_{595nm} of reaction mixture was evaluated using a spectrophotometer (SP-300, Optima, Tokyo, Japan).

Sugar Consumption

Sugar residues were determined by using glucose standard curve. After incubation, the molasses was diluted according to our requirement. Test tubes were filled with (1 mL) of molasses, and then 1.5 mL of DNS was added to each tube. After a thorough shake, the tubes were submerged in a boiling water bath at (50 °C) for 15 minutes. About 1 mL of distilled water was used as a control in place of diluted molasses. Then absorbance A_{575} was quantified at 575 nm.

Protein Estimation

Bradford (1976) was used to estimate the protein content. In 50 mL of 95% ethanol, 5 mL of Bradford reagent (Hundred milligrams of Coomassie Brilliant Blue, or G-250) was added. After adding this solution to 100 mL of 85% (w/v) phosphoric acid, the final volume was increased to 1000 mL. To get rid of any remaining particles, it was thoroughly swirled and then filtered using Whatman filter paper No. 44. To prevent photo-oxidation, the reagent was kept in amber. It was then transferred to a test tube and 0.1 mL of enzyme extract was added. After swirling, the tubes were incubated for 15 mins at room temperature (25 °C). In parallel, 0.1 mL of distilled water was used in place of the enzyme extract to run a blank. The absorbance was determined at 595_{nm}. The curve's slope was employed for protein quantification, adhering to the established relationship, Protein contents (ug/mL) = slope × 5 × Dilution factor.

Determination of Dry Cell Mass (DCM)

A pre-weighed Whatman filter paper No. 44 was used to filter the fermented mash and it was then rinsed twice with distilled water. Subsequently, it was subjected to oven-drying at 105 °C for 30 mins. The total weight of the sample along with the Falcon tube was measured. After drying, DCM (g/L) was calculated by subtracting the weight of dry cell mass of Falcon tube from the total weight.

Optimization of Cultural Conditions

Commercially available molasses gathered from a local market at Lahore was utilized as substrate for inulinase production. The sugar concentration in molasses was varied from 30, 60, 90, 120, 150, and 180 g/L, and their effects on the enzyme production were studied. The effect of different levels (25, 50, 75, 100, 125, and 150 mL) of medium was observed for maximum enzyme yield. The fermentation was carried out 60 g/L sugar concentration. Moreover, the effect of varying pH (4, 4.5, 5, 5.5, 6, and 6.5) was also studied, and maximum activity was observed at 100 mL of medium at pH 4.5. The fermentation was conducted for varying duration (12, 24, 36, 48, 60 and 72 h) to examine their effects on the production of enzyme, maintaining all other conditions at their optimal level. In addition, the fermentation was conducted at various temperatures such

as, 25, 30, 45, 40, 45, 50, 55, and 60 °C to observe maximum enzyme activity. All batch culture experiments were performed at optimal levels. Several inoculum levels (0.5, 1, 1.5, 2, 2.5, and 3 mL) were evaluated for inulinase synthesis. The fermentation was preceded for 48 h, with all other parameters maintained at optimal level. The age of inoculum was also varied (2, 4, 6, 8, 12, 16, 20, 24, 28, and 32 h) for maximum inulinase production (Duan *et al.* 2026).

Statistical Analysis *via* One-way ANOVA and ANN Model Validation

Following the methodology of Snedecor and Cochran (1980), statistical analysis was conducted using SPSS (version 27). All experiments were conducted in triplicate through independent biological replicates. One-way ANOVA was used to assess significant differences between treatments, and the findings were reported as probability (P) values. The data are presented as mean ± standard deviation (SD). This model was utilized to understand the multipart relationship among many important factors and their total mutual effect on exo-inulinase synthesis by artificial neural network (ANN) model (Lee 2003). As a result, several factors were chosen as input layers to aid in designing a variety of combinations for all hidden layers, and the end productivity was forecasted as the legitimate output layer. When presenting results, Y-error bars indicate standard deviations (±SD) among the three parallel replicates (LSD 2.415, Df 20). The ±SD for protein content and DCM was calculated at a level of 5%. The values in each set differ significantly at a level of $P \leq 0.05$. The ANN model's particle architectural parameters were developed using the experimental data for exo-inulinase synthesis unleashed by *C. tropicalis* NRRL-Y-1552 as given in Table 1.

Table 1. ANN Model's Particle Architectural Parameters were Used for Exo-Inulinase Production from *C. tropicalis* NRRL-Y-1552 under Static Culture*

Architectural Specifications	Static Culture	
	Pre-Optimization	Post-Optimization
Neurons in input layer	2	3
Neurons in hidden layer	3	5
Neurons in output layer	1	1
Training error	0.01452	0.00874
Test error	0.01216	0.00057
Hidden layer activation	Tanh	Tanh
Output activation	Identity	Identity

*Analysis of the hidden, output, and inline input layers was done following the ANN model.

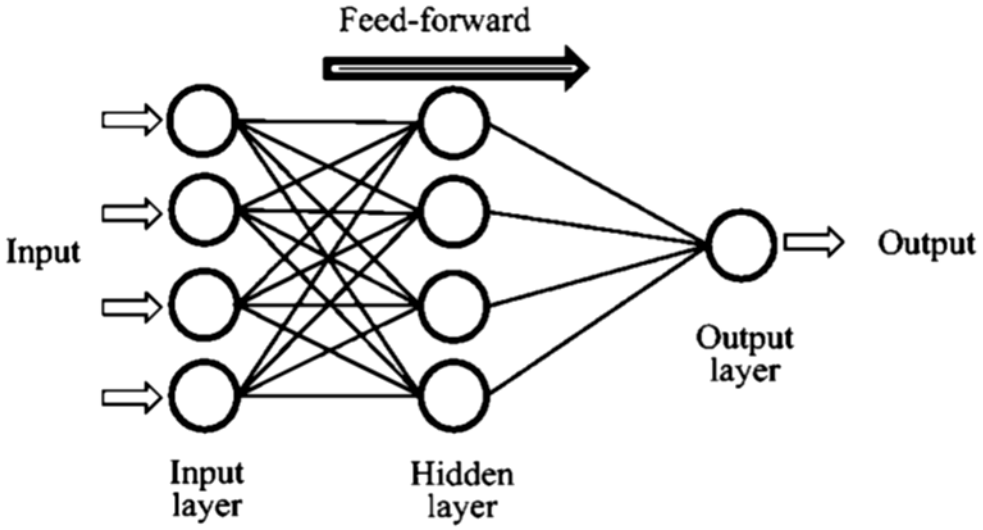

Fig. 1. ANN model showing that the location of hidden layer between input and output layers

Innovative advances in artificial intelligence (AI) have made it evident that ANN can solve multiplex problems (Lecun *et al*. 2015). During the static process, these tools consist of links between the input and output data and an adaptive system through the network. In feed-forward networks, the neurons were grouped in layers. The first layer is referred to as the input layer, and the output layer has an identical number of neurons as the beginning layer. A loop from the hidden layer's input to its output was chosen by the model. The activation function used in the present study for exo-inulinase synthesis unleashed by *C. tropicalis* NRRL-Y-1552 was adopted from a previous report (Jalal *et al.* 2021). The process of the ANN model has been given in Fig. 1.

RESULTS

Evaluation of Molasses Sugar

The effect of distinct molasses sugar on the production of exo-inulinase from *Candida tropicalis* NRRL-Y-1552 under stationary culture technique (ScT) was studied (Fig. 2). Molasses was used as a substrate, and to achieve optimal activity of inulinase, various concentration of sugar (30, 60, 90,120, and 150 g/L) were employed. An enzyme activity of 3.8 U/mL was observed when 30 g/L molasses sugar was used. Maximum enzyme production (6.3 U/mL) was achieved ($P \leq 0.05$) at 60 g/L. Enzyme activity declined (1.93 U/mL) at 120 g/L of sugars. At optimal level, protein content (81.5 mg/mL), sugar consumption (39.5 g/L) and DCM (11.5 g/L) were also observed. The enzyme yield was found to be 23.7%. Therefore, to maximize enzyme production 60 g/L sugar was chosen for further studies.

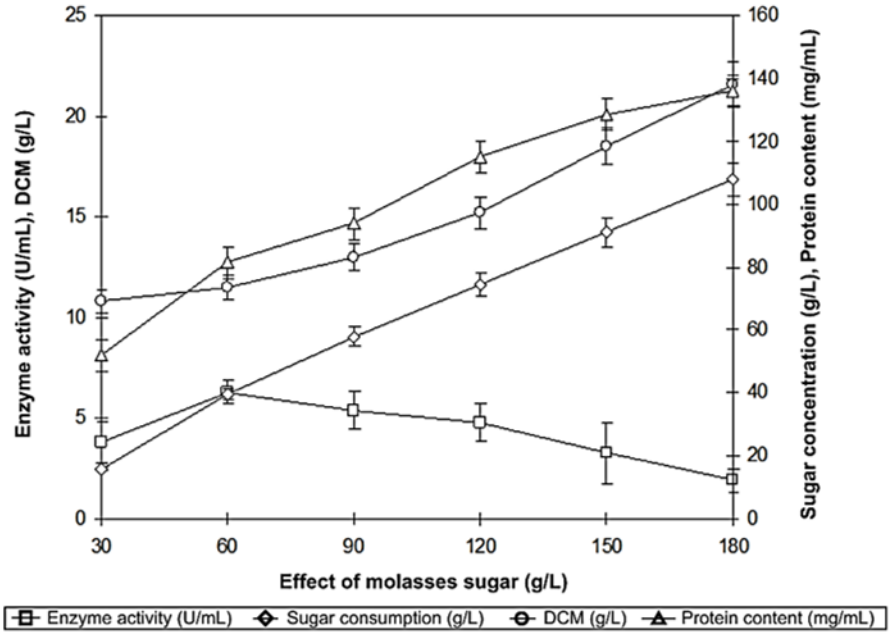

Fig. 2. The impact of varying concentration of molasses sugars on the synthesis of exo-inulinase from *C. tropicalis* NRRL-Y-1552. Volume of medium 100 mL, initial pH 5, Period of fermentation 72 h, and incubation temperature 30 °C

The effect of various volumes of molasses medium (25, 50, 75, 100, 125, and 150 mL) on inulinase production was undertaken (Fig. 3). The enzyme synthesis (2.86 U/mL) was less efficient at 25 mL. A marked enhancement in the enzyme yield (7.53 U/mL) was observed ($P \leq 0.05$) when 75 mL of molasses medium was used. Beyond the optimal level, enzyme yield gradually decreased. The enzyme activity was sharply decreased to 4.41 U/mL at 150 mL of the medium. The values for protein content (71 mg/mL), sugar consumption (45.5 g/L) and DCM (16.5 g/L) were also recorded. The enzyme yield reached 20.8% at the optimal level. Thus, 100 mL of molasses medium was optimized for enzyme production.

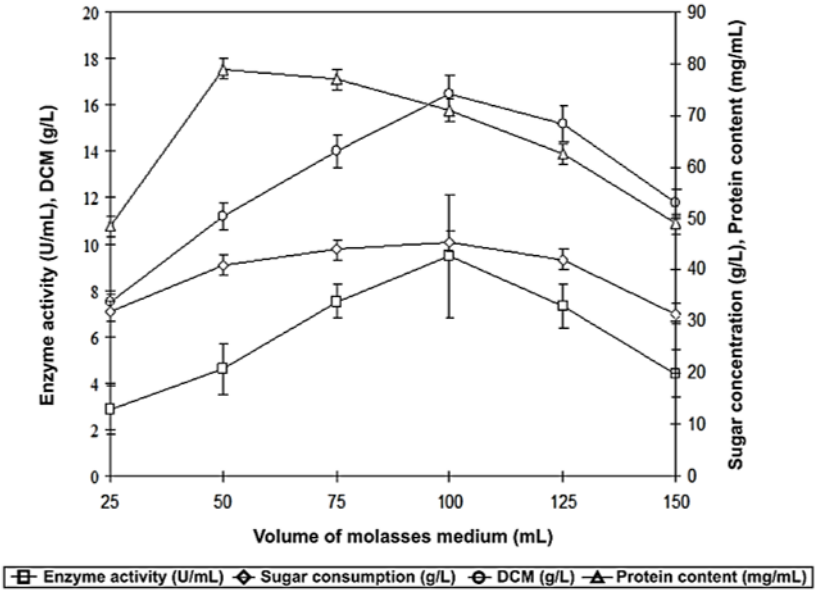

Fig. 3. The effect of distinct volume of molasses on the synthesis of exo-inulinase from *C. tropicalis* NRRL-Y-1552. Sugar concentration 60 g/L, initial pH 5, period of fermentation 72 h, and incubation temperature 30 °C.

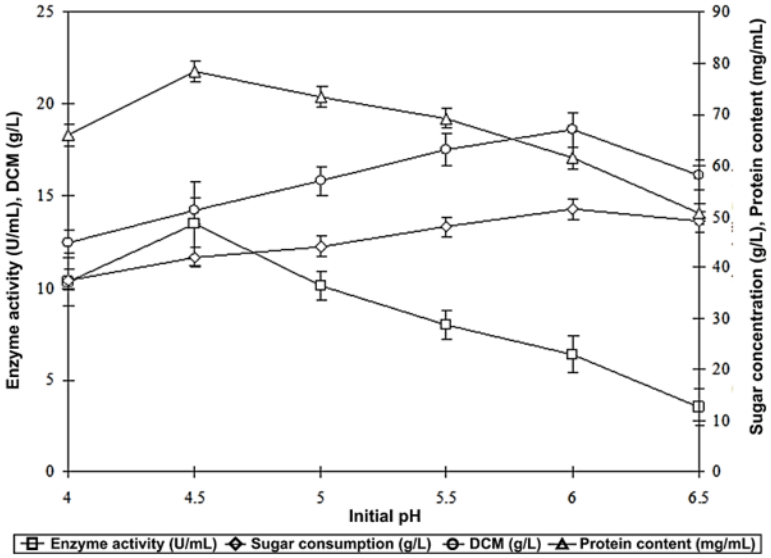

Fig. 4. The impact of initial pH on the production of exo-inulinase from *C. tropicalis* NRRL-Y-1552. Sugar concentration 60 g/L, volume of medium 100 mL, period of fermentation 72 h, and incubation temperature 30 °C

The effects of different pH (4, 4.5, 5, 5.5, 6, and 6.5) of medium on inulinase production from *C. tropicalis* NRRL-Y-1552 under ScT was noted (Fig. 4). Initially, less enzyme activity (10.34 U/mL) was observed at pH 4. Enzyme synthesis rose progressively with the rise in pH. The elevated enzyme production (13.5 U/mL) was noticed ($P \leq 0.05$) at pH 4.5. After that there was a significant decline in production (3.5 U/mL) at pH 6.5. The protein content (78.4 mg/mL), sugar consumption (42 g/L), and DCM (14.2 g/L) exhibited maximum values at the optimal pH range. The enzyme yield was also achieved (32.1%) at 4.5 pH. Hence, pH 4.5 of the medium was optimized.

Period of Fermentation

For optimal enzyme production, the rate at which *C. tropicalis* NRRL-Y-1552 produced inulinase during stationary culture was examined (Fig. 5). After the inoculation, the incubation period was adjusted between 12 and 96 h. After 24 h of fermentation, an enzyme activity of 6.12 U/mL was noted. A 48-h incubation period produced the highest levels of enzyme synthesis ($P \leq 0.05$). The enzyme yield was recorded to a maximal of 15.25 U/mL with protein content (91.5 mg/mL), sugar consumption (39.4 g/L), and DCM (12.95 g/L) were also noted. Subsequently, the synthesis of enzymes decreased steadily over the course of (60 to 84 h), reaching a very low level (4.4 U/mL) at 120 h of incubation. The production of the enzyme reached to 42.6%. Therefore, 48 h of incubation was ideal for the synthesis of enzymes.

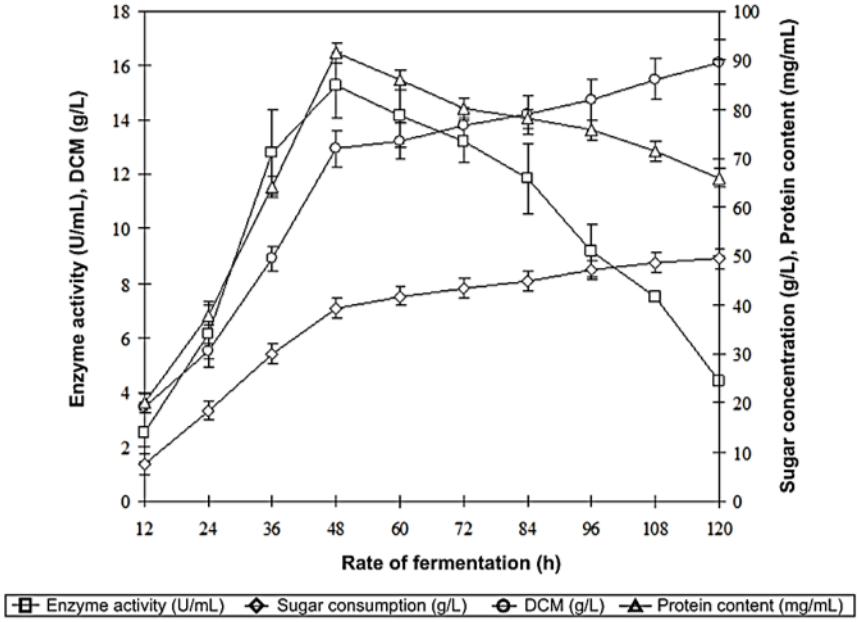

Fig. 5. The effect of different time period on the production of exo-inulinase from *C. tropicalis* NRRL-Y-1552. Sugar concentration 60 g/L, volume of medium 100 mL, initial pH of 4.5, and incubation temperature 30 °C

Incubation Temperature

The impact of incubation temperature (20, 25, 30, 35, 40, 50, 55, 60, 65, and 70 °C) on inulinase production from *C. tropicalis* NRRL-Y-1552 using stationary culture was investigated (Fig. 6). The enzyme production (8.5 U/mL) was not encouraging at 20 °C. However, when the temperature was changed to 30 °C for the duration of the 48 h fermentation, a substantial increase ($P \leq 0.05$) in enzyme yield was observed (15.08 U/mL). Enzyme synthesis was found to decrease when the temperature rose from 35 to 45 °C. Once the temperature rose to 55 °C, 11.5 U/mL of enzyme was produced. At optimal level, protein content (93 mg/mL), sugar consumption (41 g/L) and DCM (13.5 g/L) were also noted. The enzyme yield was found to be 36.7%. For the highest enzyme activity therefore, an incubation temperature of 30 °C was found.

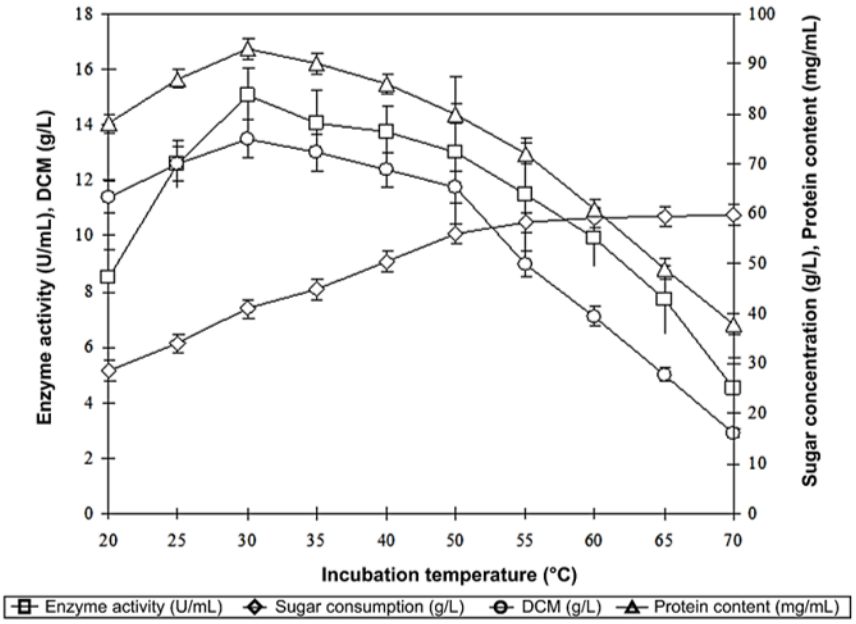

Fig. 6. The impact of varying incubation temperature on the production of exo-inulinase from *C. tropicalis* NRRL-Y-1552. Sugar concentration 60 g/L, volume of medium 100 mL, initial pH of 4.5, and period of fermentation 48 h

Age and Size of Inoculum

The impact of inoculum age (2, 4, 6, 8, 10, 12, 14, and 16 h) on inulinase production from *C. tropicalis* NRRL-Y-1552 under stationary culture was studied (Fig. 7). The enzyme production was not effective (11.8 to 15.18 U/mL) when 2 to 4 h old culture was chosen to inoculate the sterile culture media. A marked increase ($P \leq 0.05$) in enzyme production (18.56 U/mL) along with protein content (107.5 mg/mL), sugar consumption (39.5 g/L), and DCM (12.75 g/L) were achieved at 12 h old inoculum. Enzyme production significantly decreased at inoculum ages above optimum, with only 13.75 U/mL of enzyme activity detected at 16 h. The enzyme yield reached 46.9% at optimal level. So, 12 h old inoculum was optimized for maximum yield.

The effect of size of inoculum (0.5%, 1%, 1.5%, 2%, 2.5%, and 3%) on inulinase production from *C. tropicalis* NRRL-Y-1552 using stationary culture was also observed (Fig. 8). The enzyme production was not valuable (13.91 to 18.26 U/mL) when 0.5% to 1% of inoculum was used. An enzyme production of 20.42 U/mL ($P \leq 0.05$) along with protein content (121 mg/mL), sugar consumption (42 g/L), and DCM (15.95 g/L) were achieved when 1.5% inoculum was used. As an inoculum size increase than the optimal level, the enzyme production was decreased and enzyme activity 10.5 U/mL was obtained at 3% inoculum. The enzyme yield was also studied and was found to be 49.6%. Hence, 1.5% size of inoculum was used for maximum enzyme production.

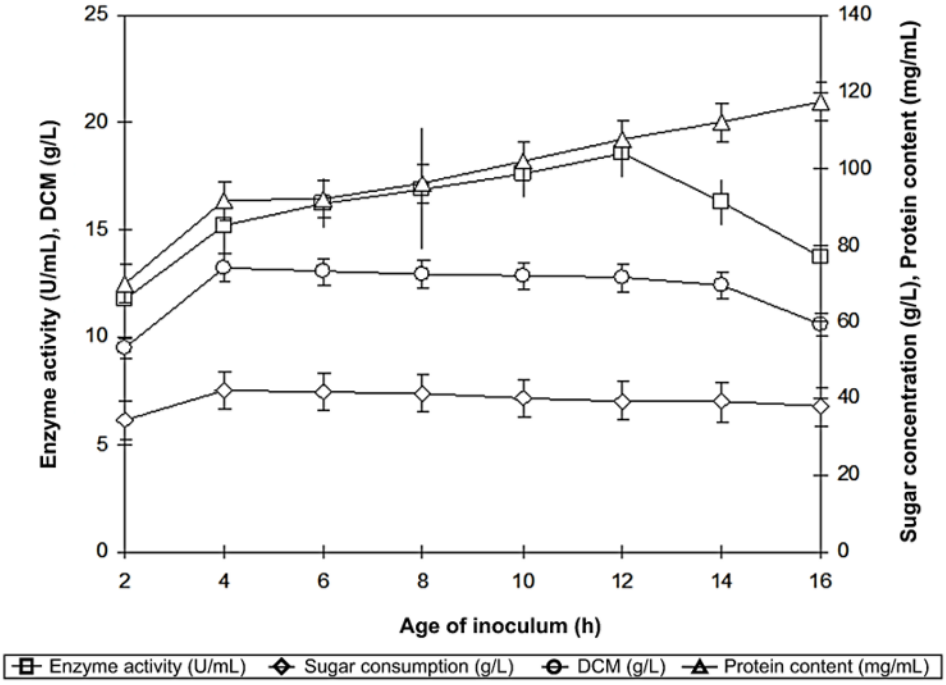

Fig. 7. The effect of age of inoculum on the production of exo-inulinase from *C. tropicalis* NRRL-Y-1552. Sugar concentration 60 g/L, volume of medium 100 mL, initial pH 4.5, period of fermentation 48 h, and incubation temperature 30 °C

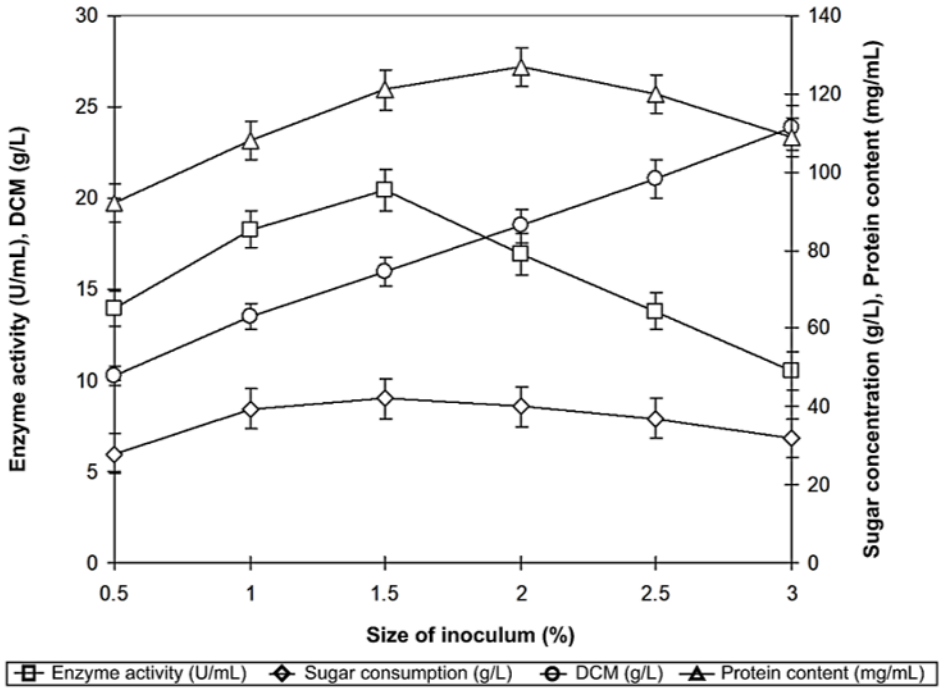

Fig. 8. The effect of size of inoculum on the production of exo-inulinase from *C. tropicalis* NRRL-Y-1552. Sugar concentration 60 g/L, volume of medium 100 mL, initial pH 4.5, rate of fermentation 48 h, and incubation temperature 30 °C

Mathematical Modeling for Prediction of Exo-inulinase Production *via* ANN

To create a reliable mathematical correlation between the exo-inulinase synthesis released by *C. tropicalis* NRRL-Y-1552 and other important parameters, ANN modeling was performed to the experimental data. This correlation could then be used to predict exo-inulinase activity. Regarding the exo-inulinase synthesis released by *C. tropicalis* NRRL-Y-1552, the data presented in Table 2 showed some of the important experimental factors, notable experimental outcomes, anticipated outcomes as calculated by the ANN model, and variance among specific parameters.

Table 2. ANN Model Validation for Exo-inulinase Production from *C. tropicalis* NRRL-Y-1552 under Static Culture*

Run	Sucros. conc. (g/L)	Molass. vol. (mL)	Initial pH	Time of incubation (h)	Temp (°C)	Size of inocul. (mL)	Exo-Inulinase production (U/mL)		Variance (%)
							Experi-mental	Predi-cted	
1	30	25	4	12	25	0.5	10.06	10.65	0.056
2	60	50	4.5	24	30	1	18.25	16.92	– 0.115
3	90	75	5	36	35	1.5	20.82	22.05	0.124
4	120	100	5.5	48	40	2	23.18	25.36	0.164
5	150	125	6	60	45	2.5	17.45	20.72	0.112
6	180	150	6.5	72	50	3	12.94	12.64	– 0.072

*A total of six variable runs were examined for the experimental and predicted enzyme production using blackstrap sugarcane molasses.

As can be seen from the ANN model findings, there was little difference between the significant experimental and projected values. The data also showed that ANN validation worked well on the optimization findings of this study. To illustrate this, Fig. 9 shows an ANN plot comparing the anticipated and experimental outcomes of the exo-inulinase production that *C. tropicalis* NRRL-Y-1552 released based on radial function and multilayer perception. In the present study, the data for model ANN summary and independent variable importance of ANN predicted results of exo-inulinase synthesis unleashed by *C. tropicalis* NRRL-Y-1552 is given in Tables 3 and 4.

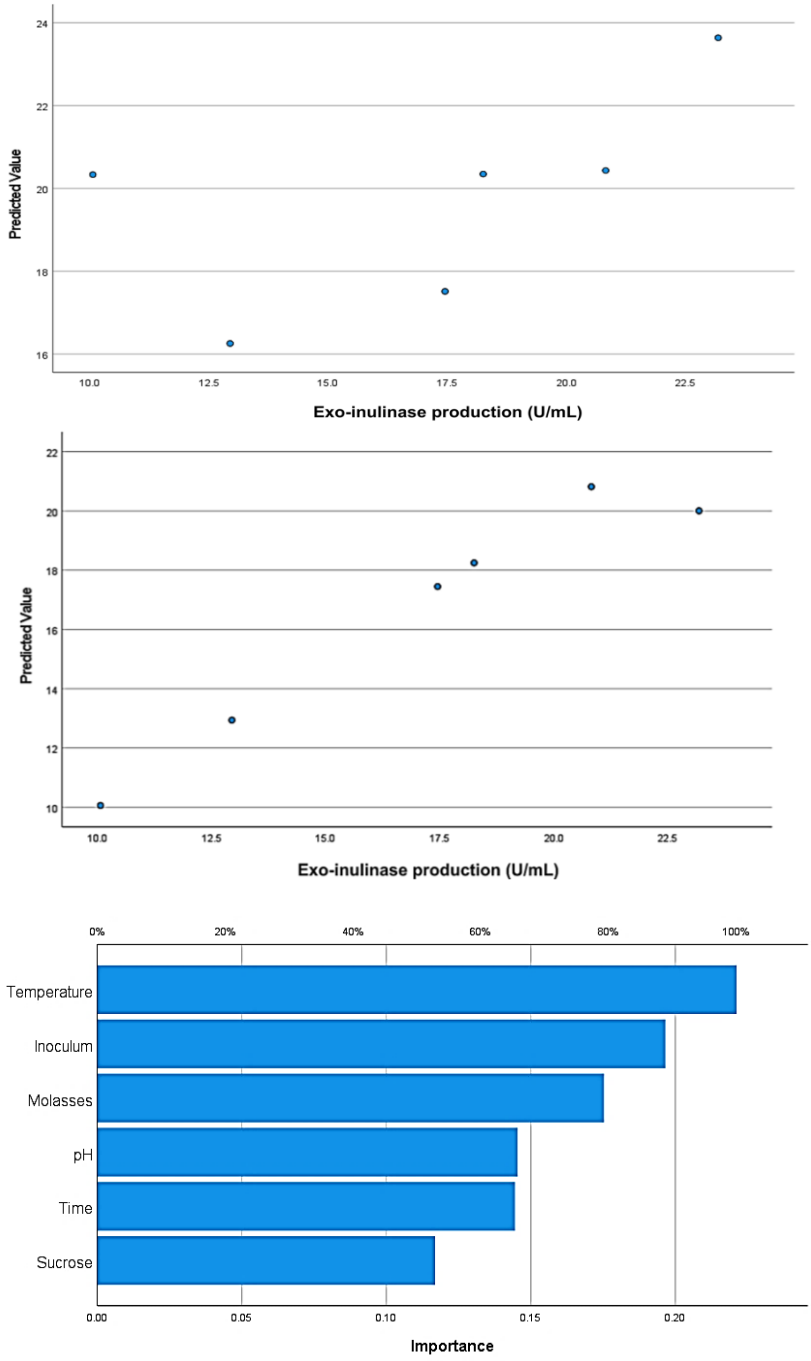

Fig. 9. Experimental *vs* ANN predicted results of exo-inulinase synthesis by *C. tropicalis* NRRL-Y-1552 on the basis of multilayer perception, radial function, and normalized importance

Table 3. Model Summary of ANN Predicted Results of Exo-inulinase Synthesis Unleashed by *C. tropicalis* NRRL-Y-1552*

Training	**Sum of Squares Error**	0.022
	Relative Error	0.022
	Stopping Rule Used	1 consecutive step(s) with no decrease in error[a]
	Training Time	0:00:00.00
Testing	**Sum of Squares Error**	7.291
	Relative Error	3.503

* Dependent variable: Exo-inulinase production (U/mL)
[a] Error computations are based on the testing sample.

Table 4. Independent Variable Importance of ANN Predicted Results of Exo-inulinase Synthesis Unleashed by *C. tropicalis* NRRL-Y-1552

Significant Parameters	Importance	Normalized Importance (%)
Sucrose conc. (%)	0.117	52.8
Molasses vol. (mL)	0.175	79.3
Initial pH	0.145	65.8
Time of incubation (h)	0.145	65.3
Temp. (°C)	0.221	100.0
Size of Inoculum (%, v/v)	0.197	88.8

DISCUSSION

In this study, inulinase production from *Candida tropicalis* NRRL-Y-1552 using molasses as a carbon and nitrogen source was carried out under stationary condition. Cultural conditions such as different concentration of molasses sugars, volume of molasses medium, initial pH of medium, incubation time, age and size of inoculum, and temperature were optimized for maximum enzyme production using ScT (Wei *et al.* 1999). Inulinases have been produced using different substrates including, plant material containing raw inulin to agro-industrial residues. Inulinases is particularly used in producing fructooligosaccharides, high fructose syrup, and maltotriose syrup (Paul *et al.* 2020). Enzymes that hydrolyze inulin work on its glycosidic bonds to generate different compounds (Singh *et al.* 2019; Mathur and Sadana 2021). Agro-industrial residues have recently drawn the interest of researchers due to their low cost and eas *Chrysosporium pannorum* availability; however, naturally occurring inulin-rich materials are preferable for the manufacture of inulinase (Chi *et al*. 2011; Xiao *et al.* 1988). Choosing the right substrate is a crucial step in the development of an enzyme production process because raw material availability and cost are significant factors (Bender *et al*. 2006; Mazutti *et al*. 2007; Sguarezi *et al*. 2009; Mazutti *et al.* 2010; Chen *et al*. 2011). Since molasses has a higher concentration of total sugar, reducing sugars, and other nutrients, it is regarded as the ideal fermentation medium (Vallander and Eriksion 1985). Moreover, the molasses sugars provided a sufficient amount of carbon and nitrogen source, which is necessary for yeast proliferation and biosynthesis of enzyme (Basso *et al*. 2010). As noted by Kalil *et al*. (1999), in addition to the molasses used as a carbon source, other medium components such as yeast extract, peptone, dextrose, and K_2HPO_4 also affect cellular growth and product formation, which may might explicate the variation in the degree of product formation on various substrates. A variety of substrates, including rye, barley, banana,

garlic, pure inulin, wheat, chicory, dahlia, and orange, were employed by Sharma *et al*. (2006) to produce inulinase. Garlic, however, exhibited the highest inulinase activity. To promote microbial growth and the synthesis of enzymes, Abiodun *et al*. (2011) also employed a range of substrates, including bagasse, molasses, banana, orange, and wheat bran. Guttikonda *et al.* (2017) described an effective bacterial isolate, *Bacillus sp*, capable of producing inulinase enzymes via enrichment culture technique at 37 ºC and pH 6 utilizing dahlia as raw inulin.

For inulinase production, medium has profound effect on the physiology of yeast and the production of inulinases (Ward 1989). In the present study, molasses medium was used as a sole fermentation medium. Initially production was less effective at 100 mL of medium. After that, enzyme production declined when 150 mL of medium was used. Enzyme synthesis decreased to such a high degree because the increased medium volume did not fulfill the particular needs for yeast growth. Kumar *et al*. (2005) have published a similar study. Further, optimization of vitamin, mineral, yeast extract, and peptone medium concentrations boosted *Aspergillus niger* inulinase activity from sugar beet molasses. It was shown that fungal growth, enzyme synthesis, and sugar depletion may all be successfully defined by the suggested kinetic models (Germec and Turhan 2020; Yu *et al.* 2009). Guerrero-Urrutia *et al*. (2021) examined *Aspergillus brasiliensis*' extracellular inulinase production from minimal culture media with high starting sucrose concentrations in SSF and SmF conditions. One band of inulinase implicated in sucrose hydrolysis was visible on the zymographic analysis.

In ScT, the medium's pH has a significant impact on the development of yeast and the generation of metabolites because it influences both the organisms' metabolic processes and the synthesis of products (Pandey 1992; Sheng *et al.* 2007; Silva-Santisteban and Filho 2005). *Kluyveromyces marxianus var. bulgaricus*'s ability to produce enzymes was mostly influenced by its pH, which was found to be optimal at 3.5 in sucrose medium (Rosa *et al.* 1986; Rouwenhorst *et al.* 1990; Kalil *et al*. 1999) and to be optimal at 6 in inulin medium (Selvakumar and Pandey 1999). It has been noted that the pH varies among the different microbial species based on the kind of substrate. Salts and other chemicals found in complex media have the ability to hinder cellular growth and even certain microbial enzymes (Singh and Bhermi, 2008). Aeration was found to be critical in raising the amount of inulinase produced and the growth of the fungus ($P < 0.05$). A similar study has been carried out by Mazutti *et al*. (2010) and observed that inulinase production by SmF showed the highest activity at pH 4.5. Elsoud *et al.* (2023) chose a bacterial isolate *i.e.*, *Bacillus subtilis Inu* for inulinase production. For enzyme purification, aqueous two-phase system was used for partitioning the inulinase enzyme. Compared to prior investigations, the isolated *Bacillus subtilis* strain was found to produce inulinase efficiently.

Incubation period has a marked effect on inulinase production. Most yeasts have been found to produce inulinase in growth-associated ways, with the peak production occurring close to their stationary phase (Al-Dagal and Bazaraa 1998). After a certain period of incubation, the production of inulinase may decline due to a catabolic repression mechanism or a decrease in the medium's carbon source (Vandamme and Derycke 1983). According to Gupta *et al*. (1994), it was brought on by the release of proteolytic proteins, which are known to denaturize proteins. The two primary features that are typically impacted by incubation duration are microbial growth and enzyme synthesis (Ellaiah *et al*. 2002). The least efficient enzyme synthesis occurred after 12 h of incubation. The reason for this was that the yeast was in its lag phase. According to Dercyke *et al*. (1984), yeast growth did not occur during the lag period. The longer the incubation period following

inoculation, the more production there was in the fermented mash (Wang *et al.* 2024). After 48 hours after inoculation, the highest amount of enzyme (15.25 U/mL) was produced. This was because the yeast was in a phase of growth that was advantageous for microbial growth, either the late exponential or the early stationary stage. The synthesis of enzymes declined following the ideal incubation period. It happened when the yeast entered its decline phase, which led to a buildup of toxins and inhibitors and a reduction in the concentration of nutrient supplement in the fermented broth. Vandamme and Derycke (1983) studied inulinase production increase with time of fermentation. However, they have showed maximum enzyme production at 72 h of incubation. Rawat *et al.* (2021) presented work on the ongoing production of fructose from various underutilized plant sources, using thermostable exo-inulinase generated by *A. fumigatus*. An economical approach for producing inulinase from *Rhizopus oryzae* may be useful in fructose syrup manufacture and other industries (Yazici *et al.* 2021).

Temperature has great effect on the metabolic activity of microbial cells. Every organism has its own optimal temperature at which it grows its maximum and produces the desired product maximally. Hence maintenance of the optimal temperature is a must. Various researchers have used 30 ºC as optimal temperature for the synthesis of inulinase from *K. marxianus* (Selvakumar and Pandey 1999; Kalil *et al.* 2005; Silva-Santisteban and Filho 2005). Enzyme production is significantly affected by incubation temperature. The development of microorganisms and the synthesis of enzymes were hampered by the fact that high temperatures caused the medium's moisture levels to decrease and the enzyme to become denatured (Moussa and Jacques 1987). Abiodun *et al*. (2011) demonstrated that 35 ºC was the optimal temperature for inulinase synthesis. Nonetheless, numerous investigations have documented that SmF produces the most enzymes at 30 °C (Cazetta *et al*. 2005). Different microorganisms may have different optimal temperatures depending on the substrate's depth, porosity, particle diameter, and kind (Pandey *et al*. 1999).

The amount of the pre-grown inoculum is crucial for the generation of inulinase since it is widely known that the inoculum concentration has a significant impact on the creation of pellets (Mutanda *et al*. 2009). A smaller inoculum size resulted in less enzyme synthesis since there were fewer yeast cells present. The maximum inulinase production was obtained when maximum inoculum was used. This was because there were adequate yeast cells for the maximum amount of enzyme activity. At 3% of the inoculum, the synthesis of inulinase significantly decreased. Due to the yeast's rapid development and the early phases of fermentation's consumption of growth-related nutrients, secondary or tertiary metabolites and other byproducts accumulated. However, at high inoculum concentrations, the exceptional growth of yeast cells may cause the fermentation medium's viscosity to increase, leading to a nutritional imbalance (Wei *et al*. 1998). Selvakumar and Pandey (1999) utilized a 5% inoculum for inulinase synthesis from *Kluyveromyces* Y-85, achieving optimal inulinase activity with *K. marxianus* at the same inoculum concentration. The age of inoculum also have great effect on inulinase production. The enzyme production was not effective when 2 to 4 h old inoculum was utilized. The optimal outcome in terms of enzyme synthesis were attained when 12 h old inoculum was used.

Additionally, in order to investigate ANN model validation, the model's performance was confirmed using the k-fold cross method and statistical check. Sevim *et al*. (2021) proposed a method for predicting different composites with waste material using pertinent methodologies, such as an ANN network-based strategy. ANN outperformed using the provided data in terms of accuracy and variance. In a related study, Basyigit *et al*. (2010) reported their research on the use of ANN to forecast the compressive strength

of heavyweight concrete. The models were run using various experimental findings. Nonetheless, Gupta *et al*. (2019) assessed the influence of input parameters used to forecast the ANN model's performance and reported on the effectiveness of the ANN technique in predicting the mechanical characteristics of precast concrete exposed to high temperatures. The results of this investigation into the exo-inulinase synthesis released by *C. tropicalis* NRRL-Y-1552 were expected to advance future understanding and recommendations about the use of waste material in concrete to lessen its adverse environmental effects. Therefore, it contributed to the creation of the eco-friendly concrete. Moreover, there was no association between the neurons in the same layer, it is clear that all of the accessible neurons in the various ANN layers stayed connected to one another. However, exo-inulinase production can be further enhanced by mutagenesis and biochemical characterization prior to scale up studies in a fermentor.

CONCLUSIONS

The goal of this study is to produce inulinase, a multipurpose enzyme with high activity in a briefer period of time, in an economical manner. Molasses is a carbon source with a wide range of industrial uses since it is readily available and inexpensive in comparison to other agro-industrial leftovers.

1. The maximum activity was obtained by fermentation of sugarcane molasses at 30 °C after 48 h incubation. The results show the feasibility of using stationary culture for inulinase production by *Candida tropicalis* NRRL-Y-1552 and suggest new experimental runs for optimization of enzyme production.

2. The crude inulinase produced by this yeast showed the highest activity at temperature 30 °C, pH 4.5, and 12 h old inoculum at flask level. By effectively applying the mathematical model ANN to the exo-inulinase findings, the isolate *Candida tropicalis* NRRL-Y-1552 was able to establish a reliable correlation between the experimental and anticipated values.

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Competing Interests

The authors declare that there are no conflicts of interest.

Availability of Data and Material

All the data generated in this research work have been included in this manuscript.

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