



ORIGINAL ARTICLE

Strain improvement and statistical optimization as a combined strategy for improving fructosyltransferase production by *Aureobasidium pullulans* NAC8



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RSM

Abstract Strain improvement of a low fructosyltransferase-producing *Aureobasidium pullulans* NAC8 (Accession No. KX023301) was carried out using chemical mutagens such as ethidium bromide and ethyl methane sulfonate. The wild-type and mutant strain were distinguished using Random amplified polymorphic DNA PCR and DNA fingerprinting analysis. Plackett-Burman and Box Behnken design were statistical tools used to determine important media parameters and optimization, respectively. Phenotypically and genetically, the new improved strain was different from the wild-type. The most important media parameters from PDB influencing fructosyltransferase production were ammonium chloride, sucrose and yeast extract at $p < 0.05$. Some significant parameters obtained with the BBD exhibited quadratic effects on FTase. The F values (35.37 and 32.11), correlation coefficient (0.98 and 0.97) and the percent coefficient of variation (2.53% and 2.40%) were obtained for extracellular and intracellular FTase respectively. The validation of the model in the improved strain resulted in an overall 6.0 and 2.0-fold increase in extracellular and intracellular FTase respectively compared to the wild-type.

A relatively low FTase-producing strain of *Aureobasidium pullulans* NAC8 was enhanced for optimum production using a two-pronged approach involving mutagenesis and statistical optimization. The improved mutant strain also had remarkable biotechnological properties that make it a suitable alternative than the wild-type.

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1. Introduction

Fructosyltransferases (FTase) are enzymes involved in the transfer to a molecule of sucrose a fructosyl group. This may also transfer the fructosyl group to a fructooligosaccharide

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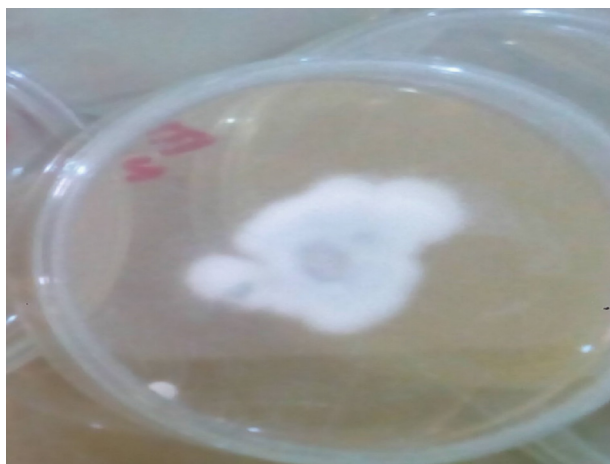
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Mutant strain of *A. pullulans* NAC8

Fig. 1a Mutant strain of *A. pullulans* NAC8.

elongating the chain by a unit of fructose [3]. FTase is known to be involved in several reactions such as polymerization reactions, in which the transfer of fructosyl units decreases the degree of polymerization maximally from fructans having a high molecular mass [24]. The mechanism by which FTase elucidate their action has been established to vary depending on the enzyme source. Mostly in some plants, the action of FTase occurs via the synergistic actions of several enzymes meanwhile in microorganisms, just a single enzyme elucidates this action. Also, it is noteworthy that the properties of FTase vary also in microorganisms depending on media parameters mostly the carbon source. Despite the fact that quite a number of microorganisms produce FTase, just a few have the potential for biotechnological or industrial production such as in the synthesis of fructooligosaccharides [15].

Fructooligosaccharides (FOS) are oligosaccharides derived from the addition of a fructose moiety to a sucrose molecule which can be carried out by the actions fructosyltransferase and/or β -fructofuranosidase [24,15]. Both enzymes are classified as FOS-producing enzymes. These FOS are obtained from several sources that include microorganisms and plants. FOS are known to play several important roles beneficial to human health. The production of these FOS is generally too low in plants and hence their significant health benefit is not so significant. *Aureobasidium* spp., and some other fungi

Table 2 Selected variables and their assigned levels by PDB.

Levels (g/L)			
Factors	Code name	Low (-1)	High (+1)
Sucrose	A	100	400
Yeast extract	B	0.2	1.0
NaNO ₃	C	0.2	2.0
NH ₄ Cl	D	2.0	5.0
KH ₂ PO ₄	E	0.2	1.0
NaCl	F	0.2	0.5

belonging to the *Aspergillus*, *Penicillium* and *Fusarium* genus have been reported to be producers of these fructooligosaccharides producing enzymes [7,23,25,6]. FOS are non-conventional sugars and they include kestoses (GF2), nystoses (GF3) and fructofuranosylnystoses (GF4). They are composed of β (2 \rightarrow 1)-linked fructose (F) units attached to a terminal glucose (G) moiety by a (2 \rightarrow 1) linkage [4]. These oligosaccharides have numerous health capabilities chief among which is their use as prebiotics because their intake aids the growth of bacterium beneficial to human health, decreases the level of cholesterol, prevents or reduces intestinal infections, allows for improved mineral absorption and also prevents incidences of colon cancer [8].

Due to FOS high demand, searching for new microbial strains with potential for FTase production thus increasing the rate of the enzymatic synthesis of FOS is of enormous importance. The most important factor that limits the industrial usage is the operational cost of enzyme production and its concomitant purification which can be reduced using a combinatorial approach that includes obtaining novel strains from established producers, optimizing the fermentation strategies, growth conditions and composition of the medium and using statistical approach to media screening and optimization [17].

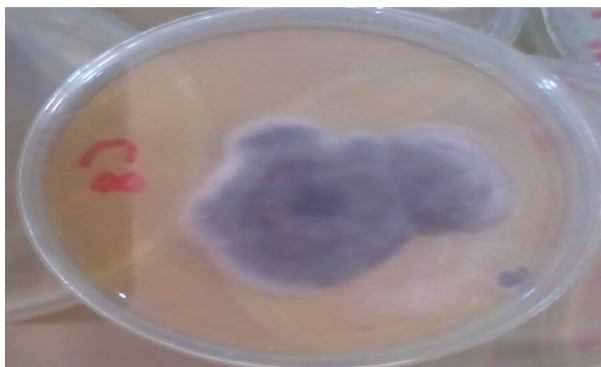
Strain improvement is defined as the science and technology of genetically modifying microbial strains to improve their potentials for numerous biotechnological applications and it majorly involves in iteration the genetic alterations, fermentation techniques and assay. Mutagenesis involves the use of a physical method (UV radiation) or chemical methods (mutagens) or both to obtain a unique strain with the desired improved biotechnological characteristics. From an economic point of view most industrial fermentation process evolve around lower fermentation process, manufacturing and capital cost. Strain improvement offers an advantage of decreased

Table 1 Properties of RAPD primers used for polymorphic analysis of the wild and mutant strain of *A. pullulans* NAC8.

S/N	Primer	Primer sequence	%GC content	Melting temperature (T_m)	Molecular weight
1	OPB05	5'-TGCGCCCTTC-3'	70	34	3035.26
2	OPB08	5'-CCACAGCAGT-3'	60	32	3077.33
3	OPH01	5'-GGTCGGACAA -3'	60	32	3157.39
4	OPH02	5'-TCGGAGGTGA-3'	60	32	3188.39
5	OPH06	5'-ACGCATCGCA-3'	60	32	3077.33
6	OPT02	5'-GGAGAGACTA-3'	60	32	3157.39
7	OPT04	5'-CACAGAGGGA -3'	60	32	3206.43
8	OPT05	5'-GGGTTTGGCA-3'	60	32	3179.35
9	OPT09	5'-CACCCCTGAG -3'	70	34	3053.30
10	OPT19	5'-GTCCGTATGG-3'	60	32	3139.35

Table 3 Selected variables and their assigned levels by Box-Behnken Design (BBD).

Variables	Component	Range	Levels of Variables Studied		
			-1	0	+1
A	Sucrose	100–400	10	200	400
B	Yeast extract	1.0–2.5	1.0	2.5	5.0
C	NH ₄ Cl	0.10–10	1.0	5.0	10.0

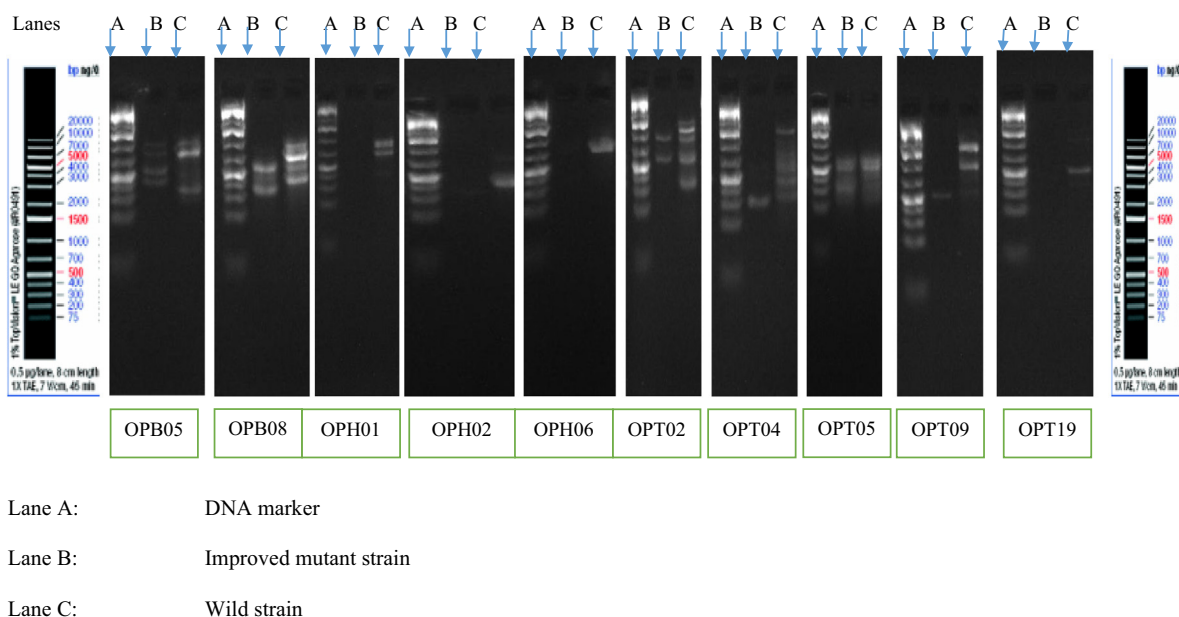
Wild strain of *A. pullulans* NAC8**Fig. 1b** Wild strain of *A. pullulans* NAC8.

cost of production simultaneously with no increased capital outlay [20]. The random amplified polymorphic DNA (RAPD) technique described by Williams et al. [30] offers a quick method to distinguish between closely related species. RAPD provides an approach to analyze polymorphisms between a newly obtained improved strain of the same specie [11].

The use of a statistical approach to optimization (response surface methodology) and screening of important media parameters (Plackett-Burman Design) provides a more reliable

and strong technique to investigating numerous process variables. This is because there are comparatively fewer experimental trials compared to the conventional varying a factor at a time technique which does not provide the information needed when the effect of the interactions among the variables is to be established. Also, one factor at a time approach is time consuming. According to Nascimento et al. [17], the conventional studying of one factor at a time is very time consuming and very tasking while also not providing the desired true optimum facilitated by the interactions among the variables.

Therefore, this work obtained a novel and relatively high fructosyltransferase secreting mutant strain from the wild-type of *Aureobasidium pullulans* NAC8 [2] using random sequential chemical mutagenesis. The wild-type was relatively a low FTase secreting strain isolated from soil containing decayed plant litters. After chemical mutagenesis, the wild-type was distinguished from the improved mutant strain using random amplified polymorphic DNA (RAPD) PCR and subsequent DNA fingerprinting analysis using GelQuest Sequen-tix, Germany version 3.2.1. Further enhancement of the production of fructosyltransferase (submerged fermentation) by the improved mutant strain was investigated using a statistical approach that involved the screening of media parameters using Plackett-Burman design and optimization using Box-Behnken based response surface methodology. This would be the first report on the use of this combinatorial approach to source for high secreting fructosyltransferase strains. This

**Fig. 2** RAPD profile generated by all primers.

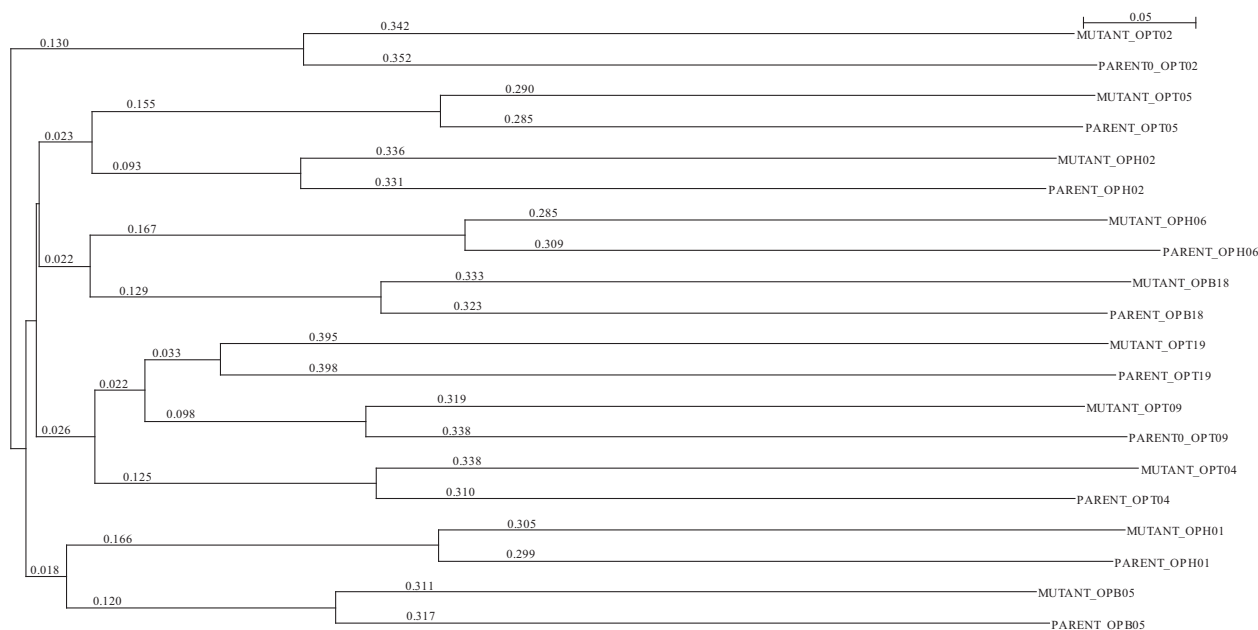


Fig. 3 Rectangular cladogram showing the Neighbor Joining (NJ) tree cluster analysis of agarose RAPD gel bands analyzed using GelQuest software (DNA fingerprint analysis software) to distinguish between parent and mutant strain of *Aureobasidium pullulans* NAC8.

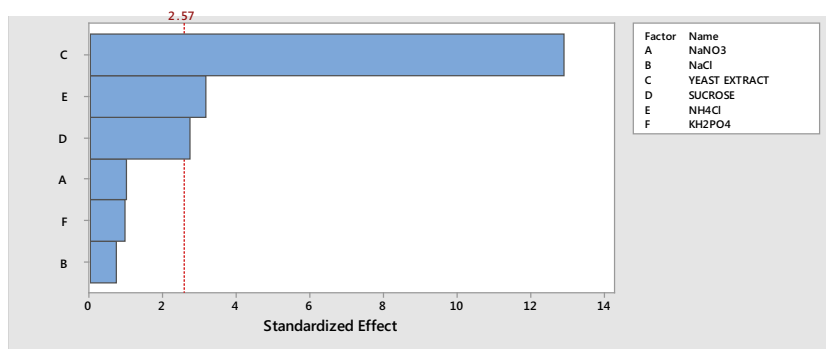


Fig. 4 Plackett-Burman design based Pareto chart for the effect of several media components on extracellular fructosyl transferase production by the improved mutant strain of *A. pullulans*.

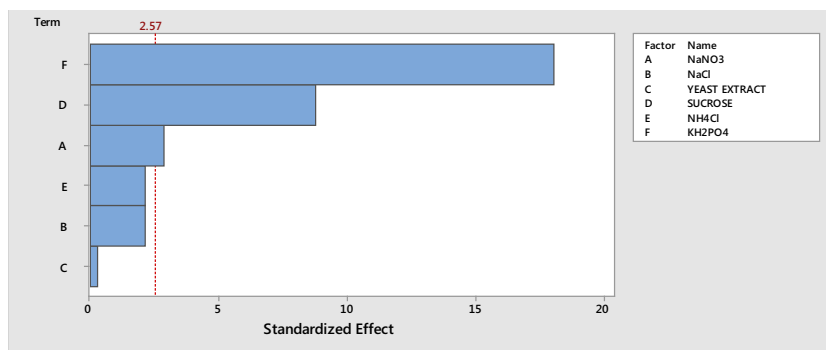


Fig. 5 Plackett-Burman design based Pareto chart for the effect of several media components on intracellular fructosyl transferase production by the improved mutant strain of *A. pullulans* NAC8.

study provides information on obtaining an improved mutant strain with potentials for increased production/synthesis of fructooligosaccharides while simultaneously decreasing the cost of production downstream.

2. Materials and methods

2.1. Reagents and chemicals

Glucose oxidase-peroxidase (GOD-POD) kit, ethidium bromide and ethyl methane sulfonate (EMS) were obtained from Sigma–Aldrich St. Louis, MO., USA. Primers for random amplified polymorphic deoxyribonucleic acid (DNA) (RAPD) analysis were purchased from Inqaba Biotec, Ibadan, Nigeria. Qiagen DNA Mini Kit was purchased from Qiagen, Valencia, CA. Deoxy nucleotide phosphate (dNTP) was purchased from Fermentas Inc. Maryland, USA. All other reagents and chemicals were of analytical grade. Random amplified polymorphic DNA analysis was carried out at the Bioscience Laboratory of the International Institute of Tropical Agriculture (IITA), Nigeria.

2.2. Fungi and conditions

The fungi strain, *Aureobasidium pullulans* NAC8, used in this study had been previously isolated from soil containing decayed plant litters and identified using molecular methods by sequencing of its internally transcribed spacer regions (ITS2 and ITS1). The sequences obtained were deposited on the NCBI database with an ascension number KX203301 [2]. *A. pullulans* was stored on malt extract agar at 4 °C. The fungus was subcultured every 7 days. *A. pullulans* strain was grown in medium as described by Kelly and Cateley [13]. The medium composition contained in 100 ml: glucose (11.25 g); KCl (0.04 g); MgCl₂ (0.38 g); Na₂SO₄ (0.006 g); Na₂HPO₄ (0.24 g); NaH₂PO₄ (0.28 g); NH₄Cl (0.11 g); FeCl₃·6H₂O (0.06 g); MnCl₂·4H₂O (0.04 g); CaCl₂ (0.60 g); CuSO₄·5H₂O (0.16 g) and yeast nitrogen base (0.65 g). An inoculum was prepared by suspending the organism, grown on malt extract agar, in sterile water. The inoculum was grown in 100 ml of the above medium for 48 h at 25 °C. Two milliliters of this culture was then used to inoculate a further 100 ml of medium

2.3. Genetic modification of *Aureobasidium pullulans* using sequential random chemical mutagenesis using ethidium bromide and ethyl methane sulfonate

Mutagenesis was carried out as described by Kelly and Cateley [13] with slight modification. A sample (10 ml) of an 18 h culture was transferred aseptically to 90 ml of the standard medium containing 40 µg/ml ethidium bromide (EtBr). The culture was incubated at 25 °C for 4 h, centrifuged (5000g, 10 min) and was washed in sterile growth medium without glucose. Samples were removed, spread on 2% (w/v) agar plates containing the standard growth medium containing deoxy glucose and incubated at 25 °C for 3 days. Selection of mutant strains was carried out using the method described by Kelly and Cateley [13]. Those colonies displaying an apparent increase in the proportion of yeast-like cells were re-plated. The above process

is repeated using ethyl methane sulfonate (EMS). Then again, the whole process is cycled again using EtBr and EMS. The obtained cells now serve as the improved mutant. The improved mutant strain and the wild-type type were used for the production of fructosyltransferase in the medium described by Ganaie et al. [9]. The increase in FTase activity was monitored in every cycle of the random mutagenesis step.

2.4. Isolation of fungal genomic DNA in the wild-type and mutant strain

The isolation of fungal DNA was carried out using methods and instructions described by ZR Fungal Bacteria DNA Mini-prep kit. The fungi (50–100 mg wet weight) that have been re-suspended in up to 200 µl of water or isotonic buffer are added to a ZR Bashing™ Lysis Tube. Thereafter 750 µl Lysis Solution is added to the tube, and the ZR Bashing Bead™ Lysis Tube is centrifuged in a micro-centrifuge at 10,000g for 1 min. Up to 400 µl supernatant is transferred to a Zymo-Spin™ IV Spin Filter in a Collection tube and centrifuged at 7000g for 1 min. Approximately 1200 µl of fungal/bacterial DNA Binding Buffer is added to the filtrate in the Collection tube, in which 800 µl of this mixture (1200 µl and 400 µl) is transferred to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuged at 10,000g for 1 min. The flow through from the Collection Tube is discarded and this step is repeated. DNA Pre-Wash Buffer (200 µl) is added to the Zymo-Spin™ IIC Column in new Collection Tube and centrifuged at 10,000g for 1 min. Fungal/Bacterial DNA Wash Buffer (500 µl) is added to the Zymo-Spin™ IIC Column and centrifuged at 10,000g for 1 min. Thereafter, there was the transfer of the Zymo-Spin™ IIC Column to a clean 1.5 ml micro centrifuge tube with the addition of 100 µl (35 µl minimum) DNA Elution Buffer directly to the column matrix. This was then centrifuged at 10,000g for 30 s to elute the DNA.

2.5. RAPD-PCR of wild-type and improved mutant strains of *A. Pullulans* DNA

Table 1 shows the properties and types of RAPD primers (decamers) used. Amplification conditions were optimized in a GeneAmp-2700 thermocycler. RAPD-PCR reaction profile includes 10 mM Tris–HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% (w/v) gelatin, 2 mM of each dNTP, Taq DNA polymerase, 40 pmol of each primer and 30 ng of DNA template. The PCR reaction profile programmed at 94 °C at 1 min denaturation period which was followed by primer annealing at 35 °C for 1 min and primer extension with a total of 40 cycles at 72 °C for 2 min. The denaturation (initial) of DNA was for 2 min at 94 °C. The final extension period was adjusted for 5 min at 72 °C, and then 3 µL of 6× loading buffer was added to each tube.

2.5.1. Agarose gel electrophoresis

Electrophoresis of the amplified PCR products was carried out using 1.5% agarose. The staining agent used was ethidium bromide. Gel documentation (Bio-Rad, Hercules, CA, USA) was used for documentation. To ensure reproducibility of the results, the experiment was repeated three times each for each primer. Reproducible fingerprints (DNA bands) produced by these primers were considered for the data analysis.

2.5.2. DNA fingerprinting analysis

The electropherogram for the PCR process for each RAPD primer (both the wild-type and mutant strain) was analyzed for the presence or absence of distinct band (fingerprints) using GelQuest version 3.2.2 (SequentiX, Germany) image analysis software. Each band was treated as an entity and the polymorphic information content was determined. Neighbor joining (NJ) cluster analysis was used for the determination of genetic variation/similarity of the wild-type from the mutant strain.

2.6. Submerged fermentation for the production of fructosyltransferase

2.6.1. Extracellular FTase

Erlenmeyer flasks (250 ml) containing 50 ml of medium were inoculated with one agar plug of the wild-type and improved mutant strain of *Aureobasidium pullulans*. The medium for enzyme production will contain the following (w/v): sucrose 20%, yeast extract (0.5%), NaNO_3 (1%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%), KH_2PO_4 (0.25%), NH_4Cl (0.5%) and NaCl (0.25%) with an initial pH of 5.5. Incubation period was for 72 h after which centrifugation of the culture broth was carried out at 6000g for 10 min at 4 °C. The supernatant was used as source of extracellular free enzyme and remaining pellet as source of mycelial FTase [9].

2.6.2. Intracellular FTase

The intracellular FTase in the mycelia of the improved mutant strains of *A. pullulans* was extracted by grinding with acid washed sand (pH 7.0) (1:1) in a clean and pre-chilled mortar using 0.1 M acetate buffer, pH 5.0 (5.0 w/v). The homogenization was carried out at 4 °C. The homogenate obtained was centrifuged at 4000g for 20 min. The supernatant obtained was used as the source of intracellular FTase. All operations were performed on ice.

2.7. Fructosyltransferase assay

The transfructosylating activity was determined by incubating 0.1 ml appropriately diluted enzyme with 0.9 ml of 1.75 M sucrose in 0.1 M acetate buffer (pH 5.5) at 45 °C for 60 min in a water bath. To stop the reaction, the reaction mixture was boiled at 100 °C for 20 min. Transfructosylating activity was estimated by taking 0.01 ml of appropriately diluted reaction mixture and mixing it with 1 ml test reagent (Glucose

oxidase-peroxidase, GOD-POD kit, Sigma). Glucose released was estimated using a spectrophotometer by measuring absorbance at 505 nm. One unit of fructosyltransferase was defined as the amount of enzyme activity required to produce 1 μmol of glucose per minute under the described conditions [9].

2.8. Statistical based screening and optimization for fructosyltransferase production

2.8.1. Plackett-Burman design using fractional factorial for screening for important nutritional parameters

Plackett-Burman design offers a very unique and powerful strategy to identify, screen and possibly evaluate very important variables that otherwise affect the response [21]. As a result, the number of experimental procedures needed is brought down significantly to identify the most important variable. In this study, various medium components such as sucrose, NaCl , KH_2PO_4 , yeast extract and ammonium chloride were investigated as variables using PB design to identify the components that significantly affected FTase production.

A typical PB design is such that each desired variable was studied at two levels. A high (+) level indicating a higher concentration of the studied variable and a low (−) level indicating a lower concentration of the variable were determined Table 2. Using the selected levels for each variable with 12 runs of experiment were generated using the MINITAB software. The Plackett-Burman experimental design is based on the first order model: $Y = \alpha_0 + \sum \alpha_1 X_i$, where Y is the response (enzyme activity), α_0 is the model intercepts, α_1 is the linear coefficient and X_i is the level of independent variable. Table 3 illustrates the Plackett-Burman design for screening of important nutritional parameters for fructosyl transferase production by the wild-type and improved mutant strains of *A. pullulans*.

2.9. Response Surface Methodology (RSM) based optimization for FTase production by the improved mutant strain of *A. Pullulans* NAC8

The significant variables obtained from PBD which may influence the fructosyltransferase production significantly were analyzed and optimized by Box-Behnken Design (BBD). One of the downsides of Response surface methodology (RSM) is its limits for the number of variables (up to five) but it becomes impractical when a large number of variables are used, expectedly due to the high number of experimental runs that will be

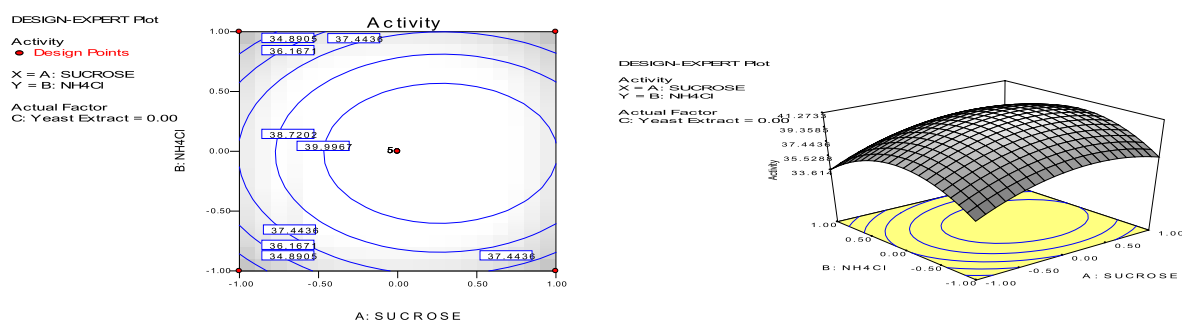


Fig. 6 The contour and three-dimensional surface plots for the combined effect of sucrose and NH_4Cl on extracellular fructosyltransferase production by the improved mutant strain of *A. pullulans* NAC8.

Table 4a Plackett-Burman Design for fructosyltransferase production (extracellular and intracellular) by the wild and improved mutant strains of *A. pullulans* after 72 h of submerged fermentation.

	A	B	C	D	E	F	Extracellular FTase (Units/ml)	Intracellular FTase (Units/ml)
1	+	–	+	–	–	–	30.39	6.66
2	+	+	–	+	–	–	14.31	8.82
3	–	+	+	–	+	–	30.00	6.27
4	+	–	+	+	–	+	32.75	15.88
5	+	+	–	+	+	–	22.16	10.00
6	+	+	+	–	+	+	30.98	12.35
7	–	+	+	+	–	+	31.37	14.31
8	–	–	+	+	+	–	34.32	9.80
9	–	–	–	+	+	+	20.39	14.61
10	+	–	–	–	+	+	18.82	14.02
11	–	+	–	–	–	+	14.90	11.17
12	–	–	–	–	–	–	11.76	6.08

Table 4b Statistical analysis of Plackett–Burman design showing coefficient values, *t*- and *p*-values for each variable on extracellular and intracellular activity of wild and improved mutant strains of *A. pullulans* NAC8.

Variable	Effect	Coefficient	<i>t</i> -Value	<i>p</i> -Value
Constant		^A 541.78 ^B 1217.60	^A 67.60 ^B 43.19	^A 0.000* ^B 0.000*
NaNO ₃	^A 45.76 ^B 55.50	^A 22.88 ^B 27.80	^A 2.86 ^B 0.99	^A 0.036* ^B 0.370
NaCl	^A –34.32 ^B –39.30	^A –17.16 ^B –19.60	^A –2.14 ^B –0.70	^A 0.085 ^B 0.517
Yeast extract	^A 4.90 ^B 729.00	^A 2.45 ^B 364.50	^A 0.31 ^B 12.93	^A 0.077* ^B 0.000*
Sucrose	^A 140.56 ^B 153.60	^A 70.28 ^B 76.80	^A 8.77 ^B 2.72	^A 0.000* ^B 0.042*
NH ₄ Cl	^A 34.33 ^B 176.60	^A 17.16 ^B 88.30	^A 2.14 ^B 3.13	^A 0.085 ^B 0.026*
KH ₂ PO ₄	^A 289.28 ^B 523.00	^A 144.64 ^B 26.20	^A 18.05 ^B 0.93	^A 0.000* ^B 0.396

Keys:

^A Intracellular fructosyltransferase by the improved mutant strain.^B Extracellular fructosyltransferase by the improved mutant strain.* Significant values at *p* < 0.05.

needed. As a definition, RSM comprises of well-defined empirical procedures/processes that are very important to examine the relatedness or differences between clusters of measured responses and its controlled variables or experimental factors. RSM approach requires a prior knowledge as well as bioprocess understanding which is very important for a more realistic model. Once the critical factors were identified through the PBD screening, the Box-Behnken design (BBD) was used to obtain a quadratic model, consisting central points to estimate the pure process variability with FTase production as response. In this study, concentrations of the three major medium components (identified using Plackett–Burman design) were optimized, keeping temperature, pH and moisture and inoculum size constant. Each factor in the design was studied at three different levels (–1, 0, +1) (Table 3). All variables were set at a central coded value of zero. Minimum and maximum ranges of variables were obtained based on previously conducted experiments. For each experimental run, the FTase

activity was measured in triplicate. Data (FTase activity) were analyzed using a second-order polynomial equation. Multiple regression procedure was used to fit the data into the equation. With BBD, those determined factors having the highest confidence levels were thus assigned into three levels termed 1, 0 and –1 which indicated high concentration, central (middle) and low concentrations (or values), respectively. For the correlation of the relationship that exists between the response (Fructosyltransferase activity) and the independent variables, the second-order polynomial function was fitted. This was used to predict the optimal point. For three factors the equation is $Q = \alpha_0 + \alpha_1 R_1 + \alpha_2 R_2 + \alpha_3 R_3 + \alpha_{12} R_1 R_2 + \alpha_{13} R_1 R_3 + \alpha_{23} R_2 R_3 + \alpha_{11} R_{12} + \alpha_{22} R_{22} + \alpha_{33} R_{32}$ where *Q* is the predicted response, α_0 is the model constant, *R*₁, *R*₂ and *R*₃ are independent variables, α_1 , α_2 and α_3 are linear coefficients, α_{12} , α_{13} and α_{23} are cross product coefficients and α_{11} , α_{22} and α_{33} are the quadratic coefficients. Experiments were performed in triplicates and mean values are given. The model

Table 5a Full experimental Box–Behnken design with coded and actual level of variables and the response function.

Variables	Sucrose %(w/v)		NH ₄ Cl %(w/v)		Yeast extract %(w/v)		Fructosyltransferase activity (Units/ml)			
	A		B		C		Extracellular		Intracellular	
	Coded	Real	Coded	Real	Coded	Real	Observed	Predicted	Observed	Predicted
1	-1	10	-1	0.10	0	0.50	34.54	33.93	9.84	9.60
2	-1	10	+1	0.50	0	0.50	35.65	36.40	10.24	10.40
3	+1	40	-1	0.10	0	0.50	34.36	33.61	9.72	9.56
4	+1	40	+1	0.50	0	0.50	35.61	36.22	10.16	10.39
5	-1	10	0	0.25	-1	0.10	34.91	35.69	9.92	10.11
6	-1	10	0	0.25	+1	1.00	34.77	34.19	10.04	9.83
7	+1	40	0	0.25	-1	0.10	30.19	30.77	8.61	8.81
8	+1	40	0	0.25	+1	1.00	38.11	37.33	10.91	10.72
9	0	20	-1	0.10	-1	0.10	33.53	33.36	9.52	9.56
10	0	20	-1	0.08	+1	1.00	33.80	33.77	9.64	9.61
11	0	20	+1	0.50	-1	0.10	33.11	33.14	9.40	9.43
12	0	20	+1	0.50	+1	1.00	32.04	32.21	9.36	9.32
13	0	20	0	0.10	0	0.50	40.38	40.93	11.98	11.78
14	0	20	0	0.10	0	0.50	41.03	40.93	11.74	11.78
15	0	20	0	0.10	0	0.50	40.66	40.93	10.78	11.78
16	0	20	0	0.10	0	0.50	41.72	40.93	11.66	11.78

Table 5b Analysis of Variance (ANOVA) for the Box Behnken model quadratic model for both extracellular and intracellular fructosyltransferase by the improved mutant strain.

Source	Sum of squares	DF	Mean square	F-value	Prob > F	
Model	^A 1.98 × 10 ⁶	9	2.2 × 10 ⁵	32.110	< 0.0001	Significant
	^B 1.75 × 10 ⁵	9	19279.56	35.370	< 0.0001	Significant
A: Sucrose	^A 1.29 × 10 ⁵	1	11.29 × 10 ⁵	18.740	0.0034	Significant
	^B 13240.37	1	13240.37	24.290	0.0017	Significant
B: NH ₄ Cl	^A 1297.49	1	1297.49	0.190	0.6770	Not significant
	^B 17.72	1	17.72	0.033	0.8620	Not significant
C: Yeast extract	^A 15894.29	1	15894.29	2.320	0.1780	Not significant
	^B 868.38	1	868.38	1.59	0.2473	Not significant
Sucrose * sucrose	^A 2.146 × 10 ⁵	1	2.146 × 10 ⁵	31.28	0.0008	Significant
	^B 50166.80	1	50166.80	38.34	0.0004	Significant
NH ₄ Cl * NH ₄ Cl	^A 5.564 × 10 ⁵	1	5.564 × 10 ⁵	81.11	< 0.0001	Significant
	^B 50166.80	1	50166.80	92.03	< 0.0001	Significant
Yeast extract * yeast extract	^A 7.335 × 10 ⁵	1	^A 7.335 × 10 ⁵	107.21	< 0.0001	Significant
	^B 61708.60	1	61708.60	113.21	< 0.0001	Significant
Sucrose * NH ₄ Cl	^A 48.25	1	48.25	7.03 × 10 ⁻³	0.9355	Not significant
	^B 3.94	1	3.94	7.23 × 10 ⁻³	0.9346	Not significant
Sucrose * yeast extract	^A 1.623 × 10 ⁵	1	1.623 × 10 ⁵	23.66	0.0018	Significant
	^B 11913.19	1	11913.19	21.86	0.0023	Significant
NH ₄ Cl * yeast extract	^A 4509.06	1	4509.06	0.66	0.4442	Not significant
	^B 63.01	1	63.01	0.12	0.7438	Not significant
Residual	^A 48023.53	7	6860.50	–	–	–
	^B 3815.64	7	545.09	–	–	–
Lack of fit	^A 37857.84	3	12619.28	4.97	0.0778	Not significant
	^B 3249.05	3	1083.02	7.65	0.0392	Not significant
Pure error	^A 10165.69	4	2541.42			
	^B 566.59	4	141.65			

Table 5b (continued)

Source	Sum of squares	DF	Mean square	F-value	Prob > F
Correlation total	^A 2.031 × 10 ⁶ ^B 1.773 × 10 ⁵	16 16			

R^2 for A is **0.98** while R^2 for B is **0.97**; **CV for A is 2.53% while CV for B is 2.40.**

Keys:

^A Extracellular FTase.

^B Intracellular FTase.

equation for analysis is given as representing the constant process effect in total, the linear (R_i), quadratic effect (R_j) and the interaction effect between R_i and R_j for the production of FTase.

2.9.1. Model validation

The predicted and experimental values for fructosyltransferase (FTase) activity were confirmed by an experiment using the optimum values for the variables obtained by the BBD response optimization

2.9.2. Statistical analysis

Design Expert 7.1.5, a statistical package software was used for analyzing the experimental data for BBD while MINITAB version 17 was used for the PBD. Positive effects were considered significant for p -values lower than 0.05 [10].

3. Results and discussion

3.1. Mutagenesis, isolation of improved mutant strain and RAPD-PCR

In terms of colony appearance and morphology, not too distinct several colonies were obtained after the first round of mutagenesis using ethidium bromide. Just one relatively hyper fructosyltransferase producing strain was isolated after the several rounds of random mutagenesis. Kelly and Cateley [13] used just ethidium bromide as a mutagen for mutagenesis of *Aureobasidium pullulans* whereas in this study, the combined effect of ethidium bromide and ethyl methane sulfonate was used to select a mutant strain of *Aureobasidium pullulans* that carried beneficial mutation such as hyper production of fructosyltransferase and perhaps the absence of dark pigment. The wild-type and newly obtained improved mutant strain (after the random mutagenic process) are shown in Figs. 1a and 1b. It was observed that the dark pigment (melanin) was lost in the mutant strain as evidenced by the whitish color it now had. *Aureobasidium pullulans* are black yeast-like fungi and the removal of this pigment had no adverse effect on the growth of fungi. The lack of melanin-like pigment is of another important biotechnological use such as in the production and purification of pullulan. The melanin pigment is of adverse effects making it necessary for further purification. This increases the cost of production downstream [27,22]. With the lack of melanin pigment by the newly obtained the effect downstream is a possible significant cut in the cost of production.

There was a relatively rapid growth in the mutant compared to the wild-type; this might be attributed to the mutation of some genes necessary for control of cell proliferation. Similar result was obtained for the ethidium bromide-based mutagenesis of *Aureobasidium pullulans* where there was an increase in cell mass for the treated cells compared with the untreated cells [13]. This suggests that a link exists that might possibly explain the pigment loss and mycelial biomass increase. In this study, molecular markers were used to determine the polymorphism i.e. genetic variation and similarity between the wild-type and mutant strain. The RAPD primers distinguished the wild-type and the mutant strain from each other as evidenced by the lack of some bands (fingerprints) in the mutant strain in the PCR electropherogram but present in the wild-type (Fig. 2). These markers are very good instruments to understand and analyze genetic relatedness and dissimilarity among related taxa. Several authors have reported the use of RAPD markers to distinguish genetic similarities and differences between mutants [26] although no reports have been found on the use of this technique to distinguish mutants of *A. pullulans*. The advantage of RAPD provides over other methods is its non-requirement of the need for a prior genetic sequence of the species to be determined [19]. The profiles indicate that the amplification products provided needed information to establish polymorphism through estimation of genetic distances between the wild-type and improved mutant strain. There was a remarkable silencing/inactivation of all the fingerprints in the mutant strain when the OPH01 and OPH02, OPH06 and OPT19 primers were used meanwhile the wild-type had more than three or four prominent bands at least higher than 200 base pair (bp). The absence of some bands in the electropherogram in the mutant strains is indicative of a successful mutagenic procedure where the genetic manipulation process resulted in the deletion of some genes and or mutations of these gene fragment. These differences might have resulted in the mutant strain outperforming the wild-type during FTase production. Fig. 3 shows the Neighbor-Joining (NJ) tree constructed from binary matrix data obtained using the ClustaVix package embedded in GelQuest version 3.2.2 to examine the degree of polymorphism between the wild-type and mutant strain. This compared the number of shared RAPD fingerprints from all primers between the wild-type and mutant strains. The use of RAPD technique to distinguish between wild-type and improved mutant strain of *Aureobasidium spp.* is the first of its kind, more so is the use of DNA GelQuest, an imaging software, provides a novel means to study polymorphisms between closely related species. Our study utilized the electropherograms from the RAPD-PCR

process to establish polymorphisms utilizing this software (see Fig. 4).

3.2. Fructosyltransferase activity in the wild-type and mutant strain

The improved mutant and wild-type were further assessed for their potentials to produce FTase using submerged fermentation and there was over twofold increase in the extracellular FTase production compared to the wild-type i.e. the extracellular FTase activity obtained for the improved mutant and wild-type was 7.64 ± 0.51 Units/ml and 16.8 ± 1.00 Units/ml respectively. The improved mutant intracellular FTase production was not much significantly different from the intracellular wild-type FTase i.e. 5.9 ± 0.12 Units/ml and 7.84 Units/ml respectively. Hence strain improvement allowed for enhanced secretion of FTase by the mutant strain more than twofold. These genetic alterations have significant benefits as it potentially drives down the cost of production while increasing yield in several industrial fermentation processes. Several authors have used chemical induced mutation to enhance the production of several industrial enzymes such as cellulase [26], where a combination of both chemical and physical mutagens was used to induce mutations. Also, there was the use of both ethidium bromide and hydroxyl amine to induce mutations in *Aspergillus niger* NCIM 563 for the improved production of phytase [5]. In this study about 5.98-fold increase was observed after both mutagenesis and statistical optimization.

3.3. Statistical based screening and optimization of media parameters

Several factors such as the cultivation time, medium composition for cultivation and storage of microorganism affect the activity and production of fructosyltransferase by *Aureobasidium pullulans*. In fact, differences were noted in the optimal conditions for the same strain of *Aureobasidium* due to the conditions stated above [3]. The media components varied were NaNO_3 , NaCl, yeast extract, sucrose, ammonium chloride and KH_2PO_4 . Yeast extract provides nitrogen needed for growth and it is known to be a reputable source of growth factors and necessary vitamins. Yeast extract plays a role in the

growth morphology of *Aureobasidium pullulans* where there is a concomitant increase in cell biomass as well as FTase production in medium supplemented with yeast extract compared to those with only inorganic nitrogen. Also, NaNO_3 has been regarded as the most common source of inorganic nitrogen for production of FTase by *Aureobasidium pullulans*. It is known to exert its influence on the morphology of the fungi, *Aureobasidium* spp. K_2HPO_4 serves its role as a necessary buffer component as well as a very important source of microelement necessary for fungal cell growth. An omission of this salt had a negative effect on FTase production although there was increased biomass.

Initial statistical media component screening carried out using PBD analyzed the most important media parameter influencing FTase production. The Pareto chart was used for statistical analysis of variable responses and each bar shows the decreasing order of magnitude. The length of each bar is directly proportional to the standardized effect. The red line indicates that all observed results are statistically significant at 95% confidence levels [17]. The influence of media parameters (NaNO_3 , NaCl, yeast extract, sucrose, ammonium chloride and KH_2PO_4) on FTase activity by the improved mutant using PBD indicated that KH_2PO_4 , sucrose and NaNO_3 had a significant effect ($p < 0.05$) on intracellular FTase production as indicated in the Pareto chart in Fig. 5. Meanwhile the medium parameters eliciting significant effects ($p < 0.05$) for extracellular FTase were yeast extract, sucrose and ammonium chloride (Fig. 6).

It can be concluded that sucrose plays significant role in the production of FTase both intracellularly and extracellularly at 20% (w/v). Lateef et al. [14] obtained a 20% sucrose concentration was optimal for FTase production by *Rhizopus stolonifer* LAU 07. A higher value, 25% (w/v), was optimal for *Aureobasidium pullulans* [12]. The variation in the concentration of sucrose as a requirement for FTase production might be largely dependent on the source of microorganism and storage. It is largely acknowledged that sucrose concentration higher than 20% (w/v) causes glucose-induced repression of fructosyltransferase. This results in decreased FTase activity and perhaps growth rate of the fungi. Thus, most reports find 10% (w/v) a safe choice for transfructosylating activity as well as synthesis of fructooligosaccharides. The results obtained in our study indicated that the optimum production of FTase

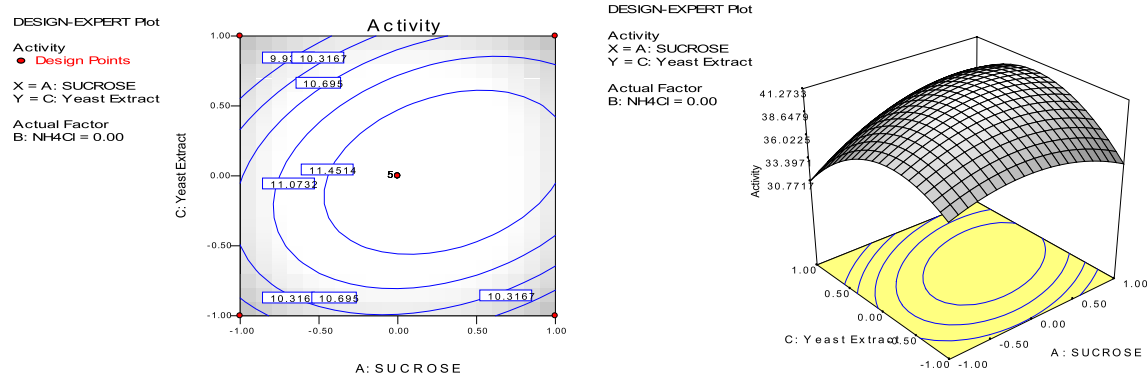


Fig. 7 The contour and three-dimensional surface plots for the combined effect of sucrose and yeast extract on extracellular fructosyltransferase production by the improved mutant strain of *A. pullulans* NAC8.

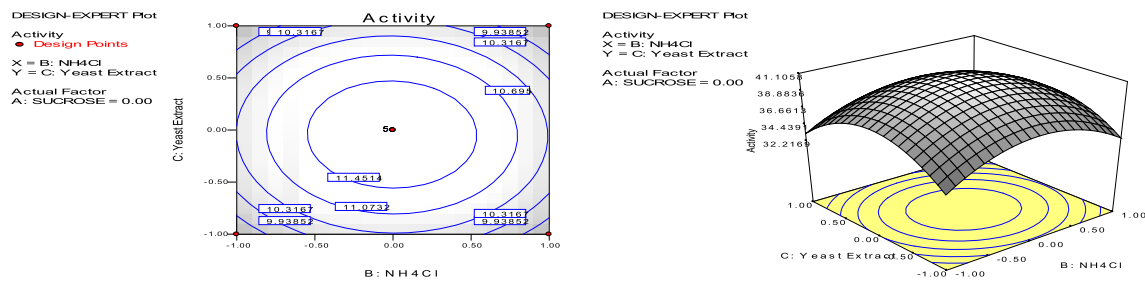


Fig. 8 The contour and three-dimensional surface plots for the combined effect of yeast extract and NH_4Cl on extracellular fructosyltransferase production by the improved mutant strain of *A. pullulans* NAC8.

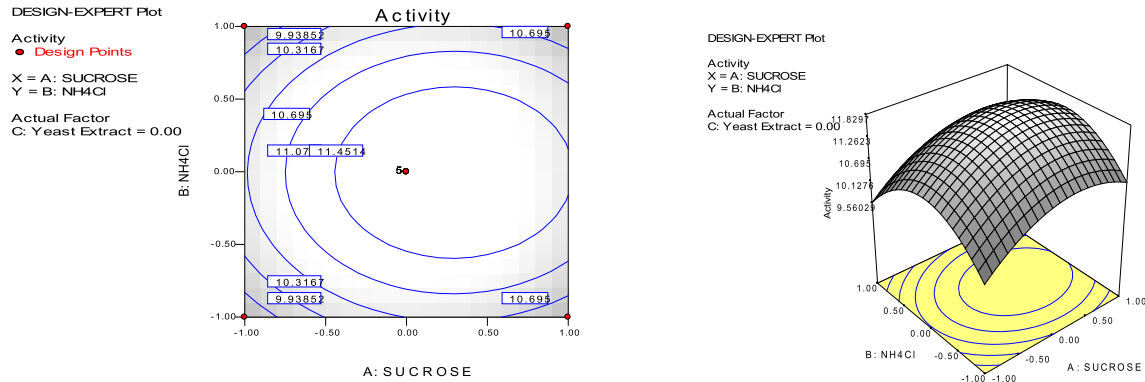


Fig. 9 The contour and three-dimensional surface plots for the combined effect of sucrose and NH_4Cl on intracellular fructosyltransferase production by the improved mutant strain of *A. pullulans* NAC8.

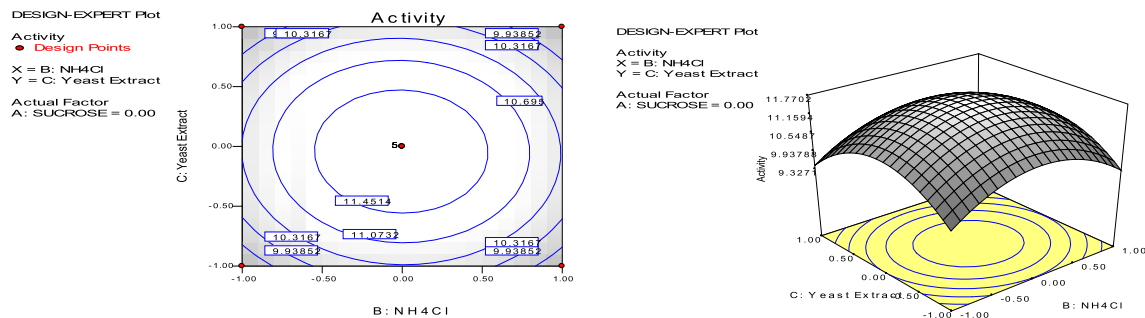


Fig. 10 The contour and three-dimensional surface plots for the combined effect of yeast and NH_4Cl on intracellular fructosyltransferase production by the improved mutant strain of *A. pullulans* NAC8.

occurred at 100 g/L i.e. 10% (w/v) as the sucrose concentration was varied at a low and high level of 100 g/L and 400 g/L respectively (using Plackett-Burman design).

The apparent decrease in fructosyltransferase activity at a high concentration of sucrose is thus explained by the glucose-induced repression of the enzyme. Interestingly, sucrose isn't the only reported inducer for FTase production. In our study, we never utilized any other carbon source as most literature agree that sucrose is the best carbon source for FTase production. There have been reports of induction with maltose as carbon source. There was a double yield of FTase by *Aspergillus foetidus* NRRL 337 using maltose as the source of carbon [29]. These results indicate that the requirements for production of FTase differ as the intracellular

enzyme required some inorganic salts for higher activity while the extracellular form of FTase required a mixture of both organic and inorganic salts for optimal activity. Of particular interest is the effect of yeast extract concentration on intracellular FTase production by the improved mutant strain. Numerous report exists that indicated that increasing yeast concentration inhibits FOS production by *Aureobasidium pullulans* [18] but this seems not to apply to the improved mutant strain's potential in the production of intracellular FTase. Yeast extract serves as a source of vitamin and growth factors for cell growth and production of fructosyltransferase. The non-requirement of yeast extract for intracellular production of fructosyltransferase might be explained in terms of the genetic alterations might have tinkered with its use of nitrogen

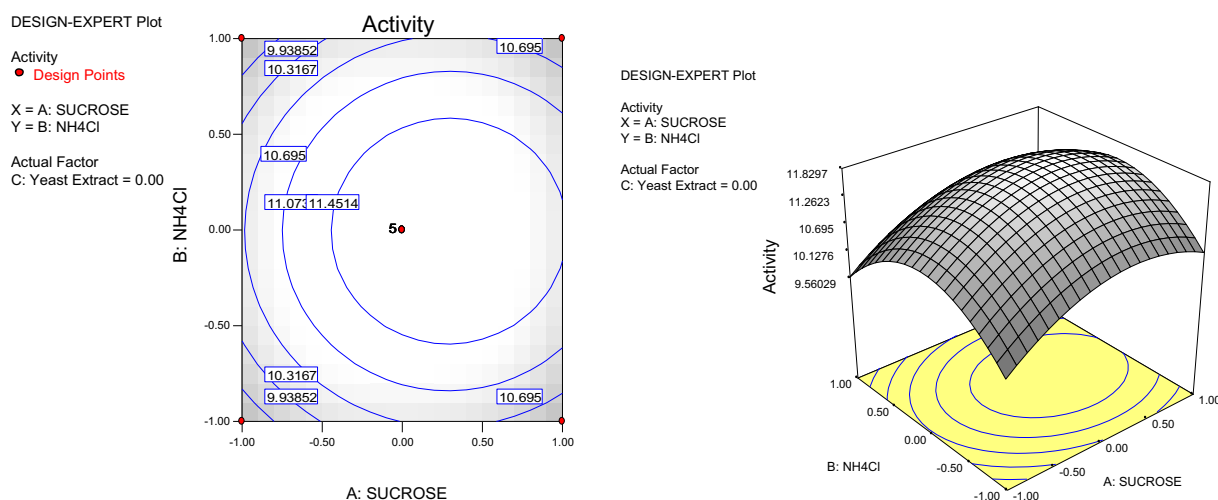


Fig. 11 The contour and three dimensional surface plots for the combined effect of sucrose and NH_4Cl on intracellular fructosyltransferase production by the improved mutant strain of *A. pullulans* NAC8.

source although both required sucrose as carbon source. Hence it is concluded that nitrogen source seems to vary but the requirement of sucrose is very important for the expression of FTase both intracellularly and extracellularly. This conclusion is drawn from the fact that production of FTases depends largely on the media composition for cultivation, organism type and storage conditions [28]. Tables 4a and 4b indicates the results obtained using the Plackett-Burman design and the summary of the statistical analysis showing the coefficient values, t and p -values for each variable on extracellular and intracellular activity of the improved mutant strains of *A. pullulans* NAC8 respectively. Hence it is noteworthy to mention that PBD allowed for the determination of important nutritional parameters for further optimization.

Using Design Expert, the optimum levels were determined by analyzing the response surface contour, surface plots and regression equations [1]. The media composition eliciting the highest response for the intracellular and extracellular FTase is conditions NaNO_3 (2 g/L), NaCl (5 g/L), yeast extract (5 g/L), sucrose (200 g/L), ammonium chloride (1 g/L) and KH_2PO_4 (1 g/L). F test analysis of variance indicated that the regression is statistically significant for FTase production (Table 5a). The values thus obtained for maximal activity ranged from 40.38 U/ml to 41.72 U/ml for extracellular FTase while the optimal intracellular FTase activity ranged from 10.78 to 11.98 (Table 5b). This value corresponds to approximately 6.0-fold and 2.0-fold increase in FTase activity both intracellularly and extracellularly compared to the non-optimized wild-type. The values obtained in our study (in terms of fold increase) were quite higher than those obtained for *P. simplicissimum* [16]. They obtained an increase in FTase activity for both intracellular and extracellular FTase from 61 IU/ml to 72 IU/ml and 92 IU/ml to 118 IU/ml respectively.

The model F value obtained for intracellular and extracellular FTase is 35.37 and 32.11 respectively and this implies that the model is significant. Values of “Prob < F < 0.05” indicate that such model terms are significant. In this case, A, A^2 , B^2 , C^2 and AB are significant model terms for both intracellular and extracellular FTase (Table 5b). The “lack of fit values” of 2.76 and 3.38 for extracellular and intracellular FTase indicates that these lacks of fit values were not significant, relative

to pure error. The non-significant lack of fit is good for the model to fit. The multiple correlation coefficient (R^2 value) obtained was 0.98 and 0.97 for extracellular and intracellular FTase. There was a better correlation between the obtained observed and predicted values because they were closer to one.

The coefficient of variation (CV) obtained for intracellular and extracellular FTase is 2.53% and 2.40%. The CV signifies to what extent the degree of precision for experimental comparison. For an experiment to be considered reliable, the CV value must be low. In the present case, a low CV indicates that the experiments performed are reliable. The adequate precision measures or estimates the signal-to-noise ratio. A ratio greater than 4 is desirable. In our case, the ratio obtained for intracellular and extracellular adequate precision is 15.50 and 14.50 respectively, which indicates an adequate signal. The regression equations in uncoded units for intracellular FTase production (Units/ml) by the improved mutant are $+11.77022 + 0.40682 * \text{SUCROSE} - 0.014888 * \text{NH}_4\text{Cl} - 0.10421 * \text{Yeast Extract} - 0.69566 * \text{SUCROSE}^2 - 1.08264 * (\text{NH}_4\text{Cl})^2 - 1.20169 * (\text{Yeast Extract})^2 + 9.92500 \times 10^{-3} * \text{SUCROSE} * \text{NH}_4\text{Cl} + 0.54572 * \text{SUCROSE} * \text{Yeast Extract} - 0.039700 * \text{NH}_4\text{Cl} * \text{Yeast Extract}$ while the regression equation in uncoded unit for extracellular FTase activity (Units/ml) = $+41.10476 + 1.26774 * \text{SUCROSE} - 0.12861 * \text{NH}_4\text{Cl} - 0.44698 * \text{Yeast Extract} - 2.34223 * (\text{SUCROSE})^2 - 3.71743 * (\text{NH}_4\text{Cl})^2 - 4.26160 * (\text{Yeast Extract})^2 + 0.034750 * \text{SUCROSE} * \text{NH}_4\text{Cl} + 2.01448 * \text{SUCROSE} * \text{Yeast Extract} - 0.33327 * \text{NH}_4\text{Cl} * \text{Yeast Extract} * (\text{SUCROSE})^2 - 3.71743 * (\text{NH}_4\text{Cl})^2 - 4.26160 * (\text{Yeast Extract})^2 + 0.034750 * \text{SUCROSE} * \text{NH}_4\text{Cl} + 2.01448 * \text{SUCROSE} * \text{Yeast Extract} - 0.33327 * \text{NH}_4\text{Cl} * \text{Yeast Extract}$.

The use of three dimensional (3D) plots provides information on how the interaction between the optimum values of the concentration and studied media components required affects the maximum FTase production while contour plots are used to provide information on areas of operability as dictated by the experimental region. These help to characterize or analyze the responses. Figs. 6–10 indicate the response surface plots as well as the contour plots and they were obtained by a complete evaluation of the total combinations possible of the entire variables for which the test was determined [17].

From the response surface plots, it was largely observed that there were moderate interactions between the media components for extracellular FTase production but some responses as indicated by the 3D-plots showed relative steep curves which indicated sharp effects due to the increase or decrease in concentration of the media parameters mostly for intracellular FTase production. Fig. 7 shows the 3D surface plot of the effect of both yeast extract and sucrose on the extracellular FTase production with NH_4Cl held constant. It can be observed that increase in the concentration of sucrose from 10 to 40% (w/v) resulted in a minor overall negative effect on FTase activity but compared to the effect of yeast extract, the curve tends to be steep which indicates that increase in yeast extract concentrations had a more negative effect on FTase activity than increase in sucrose concentration. Fig. 11 shows the response surface plots for intracellular FTase production by the improved mutant strain in which sucrose and NH_4Cl concentrations were varied while the concentration of yeast extract was held constant. From the shape of the curve, a sharp decline in the production of FTase intracellularly occurred as the concentrations of both sucrose and yeast extract were increased. The regression equations obtained after analysis of variance (ANOVA) are a function of ammonium chloride, yeast extract and sucrose concentration (g/L).

3.4. Validation of the model

The experiments were validated under the conditions predicted by the statistical model, that is, NaNO_3 (2 g/L), NaCl (5 g/L), yeast extract (2.5 g/L), sucrose (200 g/L), ammonium chloride (5 g/L) and KH_2PO_4 (1 g/L). The value obtained was 43.2 ± 2.5 Units/ml and 11.9 ± 1.2 Units/ml and it corresponds to an overall 6.0 and 2.0-fold increase for both extracellular and intracellular FTase respectively. The values obtained for the improved mutant strain are much higher than those obtained for some known industrial strain such as *Aspergillus oryzae* CFR 202 (16.5 U/ml) [24]. The findings in this study established that, using a combinative approach, a very low FTase producing wild-type can be enhanced for increased FTase activity using both strain improvement and statistical optimization.

4. Conclusion

Presently, no reports exist on the use of this combinatorial approach to enhance the production of fructosyltransferase by *A. pullulans* in the literature. The combined approach that involved using both strain improvement and two-staged statistical screening and optimization is the first ever study conducted for the enhancement of fructosyltransferase production and it was very successful in enhancing the increased production of FTase. Hence this serves as a template for further studies. In the two-staged screening and media optimization, it was concluded statistically, that the model was deemed significant and the experimental data were satisfactorily adjusted to the quadratic model as evidenced by the F value, R^2 and CV obtained. There was approximately a 6.0 and 2.0-fold increase in both extracellular and intracellular FTase production respectively post optimization process of the improved mutant strain.

The improved mutant strain secreted copious amount of FTase relative to the wild-type (with the possibility of increased FOS synthesis) and it had remarkable biotechnological properties that make it a suitable alternative to the wild-type.

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