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Enhanced Production of an Extracellular β-D-Fructofuranosidase Fructohydrolase from a 2-Deoxy-D-glucose Stabilized Mutant of *Candida utilis*

S. Ali · M. Ashiq

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Abstract The enzyme β-D-fructofuranosidase fructohydrolase (FFH) cleaves the α-1,4 glycosidic linkage between α-D-glucose and β-D-fructose molecules of sucrose, releasing monosaccharides by hydrolysis. In the present study, FFH production in *Candida utilis* GC-46, a lipolytic wild yeast strain was improved by exposure to *N*-methyl *N*-nitro *N*-nitroso guanidine (NG) and 2-deoxy-D-glucose (2dg) at various levels. The mutant strain NG-5 was obtained after exposure to 0.06 mg/ml of NG for 20 min. NG-5 offers improved extracellular FFH production (34 ± 2.6 U/ml/min) when compared to the wild strain (1.15 ± 0.01 U/ml/min). A 40-fold increase of FFH (45.65 ± 2.0 U/ml/min) was achieved when the process parameters, including incubation period (48 h), sucrose concentration (5.0 g/l), initial pH (6.0), inoculum size (2.0% *v*/*v*, 16 h old), and urea concentration (0.2%, *w*/*v*) were identified using Plackett–Burman design. The kinetic parameters viz. Q_p (0.723 U/g/h), $Y_{p/s}$ (2.036 U/g), and q_p (0.091 U/g yeast cells/h) indicate that NG-5 is a hyperproducer of extracellular FFH with a concomitant increase in growth rate. The volumetric productivity of NG-5 was over sixfold improved over the parental strain. The enzyme production improvement is highly significant (HS, LSD 0.042, $p \leq 0.05$), indicating commercial utility.

Keywords Plackett–Burman design · *Candida utilis* · β-D-Fructofuranosidase fructohydrolase · Microbial fermentation · Kinetic study · Two factorial experimental design

Introduction

Fermentation design and formulation is based on the microorganism, substrate, and the production process [1]. β-D-Fructofuranosidase fructohydrolase (FFH, *EC 3.2.1.26*) is a

S. Ali · M. Ashiq (✉)
Institute of Industrial Biotechnology (IIB), Government College University, Lahore, Pakistan
e-mail: zebshumail@yahoo.com

Present address:
M. Ashiq
School of Forensic Science, University of Central Lancashire, Preston, Lancashire PR1 2HE, UK

glycoenzyme with mannose the major component of the carbohydrate moiety. The enzyme attacks β-D-fructofuranosides such as raffinose, stachyose, or sucrose. FFH is useful in the production of confectionery with liquid or soft centers and in the fermentation of cane molasses into ethanol. Strain selection is critical in the development of a biotechnological process, and it is based on factors such as physiological stability, yield consistency, incubation time required for the maximum enzyme production as well as tolerance to temperature, aeration, and shear stress [2, 3]. The use of FFH is somewhat limited due to its high price; thus, optimization of the production process and enzyme characterization becomes important for feasibility [4]. Sucrose is considered to be the best sole carbon source for FFH production as the availability of glucose for yeast is dependent on sucrose hydrolysis by FFH. Therefore, sucrose concentration markedly influences FFH biosynthesis. Appropriate incubation period is of critical import for FFH synthesis as longer incubation can cause feedback repression of the enzyme [3, 5].

Saccharomyces cerevisiae has been identified for extracellular FFH production due to higher sucrose fermenting activity [6]. A faster growth rate and concomitantly higher enzyme secretion rate make alternative yeast strains such as *Candida utilis* prime targets for FFH production experimentation. *C. utilis* belongs to class *Saccharomycetes* and order *Saccharomycetales* and is also known as yeast fungus. It is dimorphic yeast whose morphology varies from ellipsoidal yeast cells to branched filaments in response to changes in culture conditions. *C. utilis* has a delicate cell wall, which can easily rupture [7]. In the present study, *C. utilis* GC-46, a lipolytic wild yeast strain, was treated with NG and tested for enzyme production. The effect of sucrose concentration, incubation period, initial pH, and inoculum size was investigated, for which two-factorial Plackett–Burman experimental design was used for identifying the significant variables influencing FFH hyperproduction. Kinetic parameters were also studied in order to study the improved performance of the batch fermentation process.

Materials and Methods

Organism, Media, and Chemicals A lipolytic strain of *C. utilis* GC-46 was obtained from the Biotechnology Culture Collection, GC University Lahore, Pakistan. Chemically defined SAPY medium containing 30 g/l sucrose, 15 g/l agar, 5.0 g/l peptone, and 3.0 g/l yeast extract at pH6.0 was used for culture growth and maintenance. All chemicals used in this study were acquired from Sigma Chemicals, Inc. (St. Louis, MO, USA).

Induced NG Mutagenesis and 2dg Resistance Yeast cells were suspended in 5.0 ml of sterilized 0.5% (*w*/*v*) sucrose acetate buffer, pH4.5 and washed twice, followed by suspension in 50 ml of buffer. A total of 10 ml of NG (0.02–0.10 mg/ml) was transferred to centrifuge tubes containing 5.0 ml of yeast cell suspension. The tubes were kept at room temperature for different time intervals (5–30 min), then centrifuged at 6,532×*g* for 15 min. The supernatant was discarded to remove the mutagen from the cells, and 10 ml of sterilized saline water (NaCl 0.85%, yeast extract 0.05%) was added to each of the tubes [8]. Approximately 0.5 ml of the treated suspension was transferred to petriplates containing SAPY medium with bromocresol green (40 ml/l of 1.0% dye), pH6.0 and 12 g/l sucrose as an additional carbon source, then incubated at 30°C. After 24–36 h, yeast colonies bearing large pinkish zones of sucrose hydrolysis (in comparison to control) were picked and transferred to agar slopes.

The mutant strain was harvested during the exponential phase of growth, washed with sterile distilled water, and plated on SAPY medium containing 2dg (0.02–0.10 mg/ml),

replacing the sucrose by raffinose [9]. Colonies appearing in 24–36 h were sub-cultured, selected for vigorous growth, and tested for stability during FFH production via shaking flask fermentation. Samples were drawn periodically, washed, and plated on the medium to select strains resistant to various 2dg levels. The master mutant strain has been preserved in liquid paraffin and stored at 4°C in a cold-cabinet (CE-110, Sanyo, Japan).

Inoculation and Fermentation Procedure A total of 1.0 ml of the suspension (1.25×10^6 yeast cells) was aseptically transferred to a cotton-plugged, sterilized, 250-ml Erlenmeyer flask containing 25 ml SAPY broth. A hemocytometer slide bridge (B124, Knobbier, Germany) was used for cell counting. The flask was incubated in a rotary shaker (JB-4043, Gallenkamp, London, UK) at 30°C for 24 h (160 rpm). The production of FFH was carried out using the shaking culture technique in 250-ml Erlenmeyer flasks. Sterile, cotton-plugged, SAPY flasks at pH6.0 were prepared as before. One milliliter of inoculum was aseptically transferred to each flask, which were then incubated for 48 h at 30°C and 200 rpm (found optimal).

Assay Methods

Dry Cell Mass Yeast dry cell mass was determined by centrifugation of fermented broth at 8,330×*g* for 15 min using pre-weighed centrifuge tubes. After decanting off the supernatant, cell mass was washed twice with distilled water. Final weight was measured after the tubes containing cell mass were oven-dried at 105°C for 1 h. The weight of tubes was subtracted from the final weight, and the net weight was converted into grams per liter.

Sugar Consumption Sugar was estimated by the DNS method [10]. One milliliter of supernatant along with 1.0 ml of DNS reagent was taken in test tubes. A blank control containing 1.0 ml of distilled water along with 1.0 ml of DNS was run in parallel. The tubes were placed in a boiling water bath for 5 min, then allowed to cool to room temperature. The % transmittance was noted at 546 nm using a UV/Vis double beam scanning spectrophotometer (Model: Cecil CE-7000, UK). Sugar concentration was determined in comparison with the standard curve.

Extracellular FFH Activity Enzyme activity was determined following the procedure of Akgol et al. [11]. Tubes containing 2.5 ml acetate buffer (50 mmol, pH5.5) and 0.1 ml sucrose (300 mmol) were pre-incubated at 35°C for 5 min. After adding 0.1 ml of diluted extracellular enzyme broth, incubation was continued for another 5 min. The reaction mixture was placed in a boiling water bath for 5 min, then allowed to cool to room temperature. A control was also run, replacing the enzyme solution by distilled water. To 1.0 ml of each reaction mixture, 1.0 ml of DNS reagent was added, and then the tubes were placed in boiling water for 5 min. After cooling to an ambient temperature of 25°C, the volume was raised to 10 ml. Transmittance (%) was measured at 546 nm. FFH was calculated from the standard curve of glucose. One FFH unit is defined as the amount of enzyme, which releases 1.0 mg of inverted sugar in 5 min at 20°C, pH4.5.

Kinetic Study Kinetic parameters were studied according to the procedures of Pirt [12]. The growth yield coefficient ($Y_{x/s}$) was calculated as the dry cell mass of saccharide utilized from the test substrate following fermentation. The product yield coefficients $Y_{p/s}$ and $Y_{p/x}$ were determined by using the relationships $Y_{p/s}=dP/dS$ and $Y_{p/x}=dP/dX$, respectively. Volumetric rates for substrate utilization (Q_s) and product formation (Q_p) were determined

from the maximum slopes in plots of substrate utilized and extracellular FFH produced each vs. the time of fermentation. The volumetric rate for biomass formation (Q_x) was calculated from the maximum slope of cell mass formation vs. the incubation time period. The specific rate constants for product formation (q_p) and substrate utilization (q_s) were determined by the equations, i.e., $q_p = \mu \times Y_{p/x}$ and $q_s = \mu \times Y_{s/x}$, respectively. The specific rate for cell mass formation (q_x) was calculated by multiplying the specific growth rate (μ) by the growth yield coefficient ($Y_{x/s}$).

Statistical Analysis Treatment effects were compared according to Snedecor and Cochran [13]. Duncan's multiple range tests (Spss-14, version 5.6) were applied under one-way analysis of variance. Significance is presented in the form of probability ($p \leq 0.05$) values. The significant variables were identified using two factorial system, i.e., Plackett–Burman experimental design [14, 15]. The variables were denoted at two widely spaced intervals, and the effect of individual parameters on enhanced production of FFH was calculated by the following equations,

$$E_o = (\Sigma M_+ - \Sigma M_-)/N \tag{1}$$

$$E = \beta_1 + \Sigma\,\beta_2 + \Sigma\,\beta_3 + \beta_{123} \tag{2}$$

In Eq. 1, E_o is the effect of first parameter under study, while M_+ and M are responses of FFH production by yeast. N is the total number of optimizations. In Eq. 2, E is the significant parameter, β_1 is the linear coefficient, β_2 is the quadratic coefficient, and β_3 is the interaction coefficient for process parameters.

Results and Discussion

In the present study, over 200 mutant colonies of *C. utilis* were selected after NG treatment, and a total of six isolates were obtained from the plates having at least a death rate of 90%. Among the mutant strains, the best extracellular FFH mutant of *C. utilis* (34± 2.6 U/ml/min) was named NG-5. The strain NG-5 was cultivated on medium containing 2dg and its stability for extracellular FFH production was determined at various 2dg levels. Initially, high FFH-producing colonies were obtained at 0.02 mg/ml of 2dg; however, these strains lost stability after approximately 2 weeks. A potential cause for this loss of stability may be the development of resistance in yeast cells after a few generations, allowing unstable mutant strains to thrive. This instability of mutant strains is probably due to photo reactivation. To eradicate this problem, these strains were again grown on the medium containing different concentrations of 2dg. The concentration of 0.04 mg/ml was optimal as at this level NG-5 yielded consistent extracellular FFH production. It might be due to the fact that 2dg-resistant mutant strains lost reversion, and sustained it even after numerous generations.

In batch-wise FFH fermentation, enzyme production started after a lag phase of 8 h and reached maximal at the onset of late exponential or early stationery phase. Afterward, enzyme activity declined due to decreased nutrient availability in the medium or carbon catabolite repression as the expression of extracellular FFH in yeast is checked by the presence of monosaccharides like glucose and fructose [16]. This demonstrated that proper incubation time is critical for optimal enzyme production. The results for time course

profiles for extracellular FFH production by the wild *C. utilis* GC-46 and 2dg-stabilized mutant strain NG-5 are shown in Fig. 1. Enzyme activity was estimated after different time intervals (8–72 h). Maximum extracellular FFH production (34.72± 2.4 U/ml/min with 18.82± 2.0 g/l sugar consumption and 7.97± 2.5 g/l dry cell mass) was achieved 48 h after incubation by the mutant strain NG-5. The overall rate of volumetric production was many folds improved over the parental strain. Further increase in incubation period did not enhance extracellular FFH production, probably due to the decreased amount of available nitrogen in medium, age of organism, addition of inhibitors produced by yeast itself, and/or the protease production characteristic of decline phase [17]. Other research has reported extracellular FFH production by other yeasts incubated for 72–96 h [18, 19].

The effect of sucrose concentration (1.0–10.0 g/l) on extracellular FFH production by the mutant strain *C. utilis* NG-5 (Fig. 2) indicates that maximum enzyme activity (35.56± 2.4 U/ml/min) was obtained at a sucrose concentration of 5.0 g/l. Sucrose concentration higher than 5.0 g/l caused an increase in sugar consumption and dry cell mass; however, there was no net increase in extracellular FFH production. Vitolo et al. [19] proposed that generation of higher concentrations of inverted sugar in the medium could result in glucose-

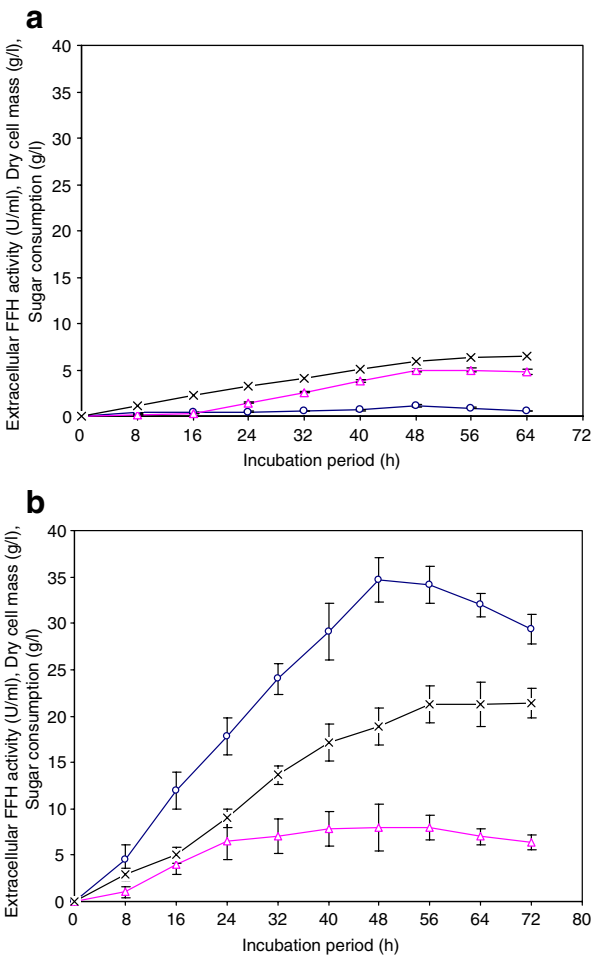

Fig. 1 Comparison of time course profile for extracellular FFH production by *C. utilis*, **a** GC-46, **b** NG-5 (*circles* extracellular FFH activity, *triangles* dry cell mass, *multiplication signs* sugar consumption). Sucrose concentration 30 g/l, temperature 30°C, initial pH6.0, agitation rate 200 rev/min. Y-error bars indicate standard deviation (±SD) among the three parallel replicates

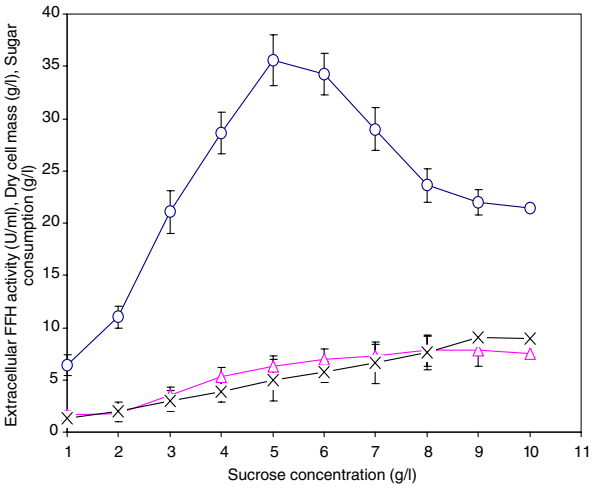

Fig. 2 Effect of sucrose concentration on the extracellular FFH production by the mutant strain *C. utilis* NG-5 (*circles* extracellular FFH activity, *triangles* dry cell mass, *multiplication signs* sugar consumption). Incubation period 48 h, temperature 30 °C, initial pH6.0, agitation rate 200 rev/min. Y-error bars indicate standard deviation (±SD) among the three parallel replicates

induced repression of extracellular FFH. The effect of initial pH on enzyme production by the mutant strain *C. utilis* NG-5 is shown in Fig. 3. Maximum production of extracellular FFH (38.42± 1.6 U/ml/min) was obtained when the initial pH was adjusted to 6.5. Similarly, dry cell mass and sugar consumption were optimal at pH6.5, i.e., 7.43± 1.0 and 4.99± 0.5 g/l, respectively. Less enzyme activity, accompanied by a decrease in dry cell mass and sugar consumption, was noticed at pH values other than the optimal, with similar results reported by Persike et al. [20]. It was noted that during submerged fermentation of *C. utilis*, the final pH of the fermentation mixture was less than the initial pH, with the extent of the decrease in pH proportional to extracellular FFH activity.

The size and age of inoculum are of prime importance among the factors that determine morphology and the general course of yeast fermentations. Early research to standardize the inoculum for extracellular FFH production in shaking culture has previously been reported [21, 22]. In the present investigation, a 16-h-old vegetative inoculum was found optimal for maximum extracellular FFH production (45.65± 2.0 U/ml/min) when added at a level of

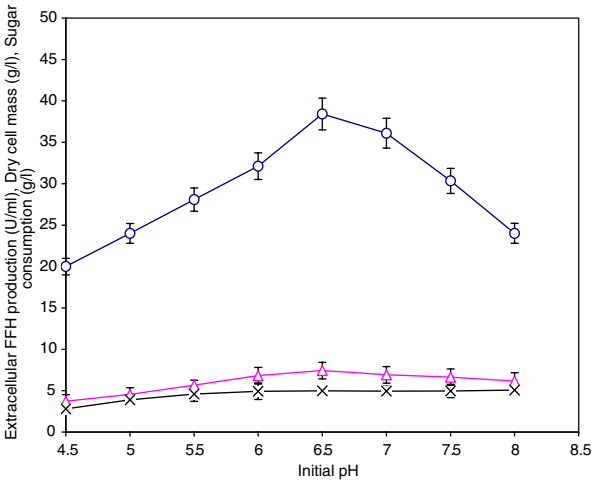

Fig. 3 Effect of initial pH on the extracellular FFH production by the mutant strain *C. utilis* NG-5 (*circles* extracellular FFH activity, *triangles* dry cell mass, *multiplication signs* sugar consumption). Incubation period 48 h, sucrose concentration 5.0 g/l, temperature 30 °C, agitation rate 200 rev/min. Y-error bars indicate standard deviation (±SD) among the three parallel replicates

2.0% (*v*/*v*). An inoculum greater or smaller than 2.0% (*v*/*v*) resulted in the reduction of extracellular FFH production (Fig. 4). This reduction may reflect that at a lower concentration, insufficient yeast cells were available in the medium to convert more substrate into enzyme. Bokosa [18] optimized 3.0% (*v*/*v*) vegetative inoculum for extracellular FFH production. In contrast to our studies, Roitsch et al. [5] found that 48-h-old cells are as good as those a 72- to 96-h-old slant culture for extracellular FFH production, which suggested that the age of yeast cells may not have a bearing on the enzyme production. The lag associated with inoculum from the stationary phase of a culture may be attributed to the cellular reorganization necessary to reverse changes caused by cessation of growth.

A ≅40-fold enhancement over the wild strain (1.15± 0.01 U/ml/min) was achieved with the mutant strain NG-5 after optimizing the nutritional conditions, leading to an enzyme production of 45.65± 2.0 U/ml/min. The drop in enzyme activity with the wild strain might be due to enzyme inactivation as well as cessation of synthesis. In the present study, the comparison of Q_s (g cells/l/h) for extracellular FFH production demonstrated that the mutant strain NG-5 has a higher value for volumetric rate of substrate consumption (Q_s = 0.355± 0.05 g/l/h) than the wild strain (GC-46). Several-fold improvement in terms of

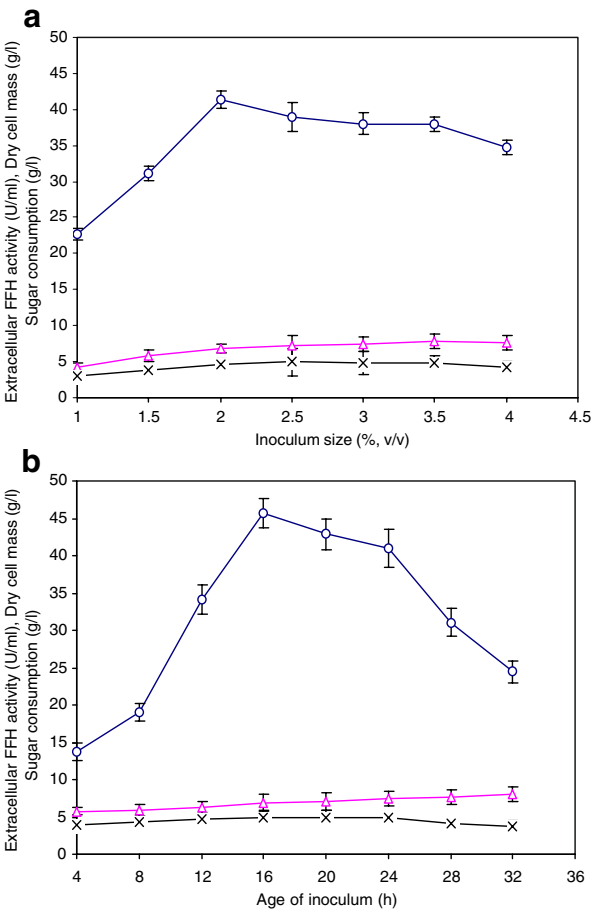

Fig. 4 Effect of inoculum on the extracellular FFH production by the mutant strain *C. utilis* NG-5, **a** inoculum size, **b** inoculum age (*circles* extracellular FFH activity, *triangles* dry cell mass, *multiplications signs* sugar consumption). Incubation period 48 h, sucrose concentration 5.0 g/l, temperature 30 °C, agitation rate 200 rev/min. Y-error bars indicate standard deviation (±SD) among the three parallel replicates

Table 1 Comparison of production and kinetic variables for extracellular FFH activity by *C. utilis* in shake flask (200 rpm, 30°C) 48 h after the incubation.

Production and kinetic variables	Extracellular FFH activity	
	GC-46 (wild strain)	NG-5 (mutant strain)
Total enzyme produced (U/ml/min)	1.15 ± 0.01	45.65 ± 2.0
Product formation parameters		
Q_p (U/g/h)	0.14 ± 0.03	0.846 ± 0.27
$Y_{p/s}$ (U/g)	0.190 ± 0.02	2.036 ± 0.63
$Y_{p/x}$ (U/g)	0.239 ± 0.11	4.356 ± 1.12
q_p (U/g yeast cells/h)	0.005 ± 0.001	0.091 ± 0.03
Substrate consumption parameters		
$Y_{x/s}$ (g yeast cells/g)	0.793 ± 0.13	0.467 ± 0.02
Q_s (g/l/h)	0.121 ± 0.02	0.355 ± 0.05
q_s (g/g yeast cells/h)	0.026 ± 0.01	0.045 ± 0.01
Q_x (g yeast cells/l/h)	0.096 ± 0.02	0.174 ± 0.02
Least significant difference (LSD)	0.016	0.042
Significance level <*p*>	S	HS

HS highly significant, *S* significant values, ±standard deviation (±SD) among the three parallel replicates

volumetric extracellular FFH production was noticed with the mutant strain NG-5 on all examined rates (Table 1). Although the wild strain GC-46 achieved a higher value ($Y_{x/s}$ = 0.793 ± 0.13 g yeast cells/g) than the mutant strain NG-5, the latter demonstrated improvement in terms of volumetric rate of product formation. In addition, when both strains were monitored for comparison of specific rate constant, the mutant strain NG-5 gave higher values for q_p (>18-fold improvement). Based on kinetic variables, it was found that the mutant strain showed four- to sixfold improved values for Q_p, $Y_{p/x}$, $Y_{p/s}$, and q_p over the parental strain (LSD 0.042). Similar findings were reported by Pirt [12]. However, Neto et al. [6] found that aeration rate and substrate moisture content influenced the substrate consumption rate, specific growth rate, and subsequent enzyme production.

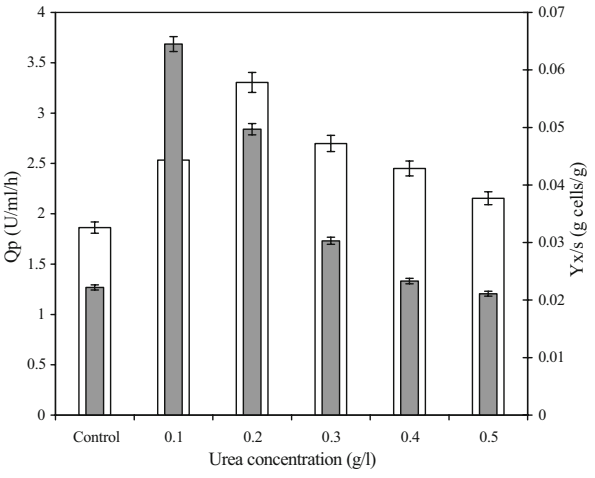

Fig. 5 Effect of urea concentration on the Q_p (*filled squares* U of FFH produced/ml/h) and $Y_{x/s}$ (*empty squares* g cells/g sugar consumed). Sucrose concentration 20 g/l, incubation period 48 h, temperature 30°C, initial pH6.0, agitation rate 200 rev/min. Y-error bars indicate standard deviation (±SD) among the three parallel replicates

Table 2 Application of Plackett–Burman design at various process parameters for extracellular FFH production by *C. utilis*.

Process parameters at two factorial design					Extracellular FFH activity (U/ml)	
Incubation period (h)^A	Sucrose conc. (g/l)^B	Inoculum$^{C}{}_{1+2}$		Urea conc. (g/l)^D	Observed	Predicted
		Size (%, *v*/*v*)	Age (h)			
32	4	1.5	12	0.1	26.46	32.90
40	4	2.0	12	0.2	35.81	45.58
48	5	2.0	16	0.2	45.92	51.25
48	5	2.0	16	0.3	39.80	50.64
56	6	2.5	20	0.4	33.48	47.06

The different letters represent significant process parameters for FFH fermentation. Statistical analysis of the model was based on two factorial design

The effect of urea as an additional nitrogen source in the fermentation medium on the production of FFH by *C. utilis* NG-5 was studied. Maximum enzyme activity was observed at urea concentration of 0.2% (*w*/*v*). Sugar consumption and dry cell mass were 24.72 and 1.02 g/l, respectively. A less amount of urea concentration was probably not enough to induce the enzyme urease in amount sufficient to promote FFH production, and also, it did not fulfill nitrogen requirements of the yeast cells, thus yielding a decreased level of the enzyme. Concentration of urea higher than optimal also produced less amount of FFH as it induces denaturation of yeast cells [6, 7], and this is also supported by Q_p and $Y_{x/s}$ (Fig. 5), indicating reduction in cell mass with an increase in urea concentration while increasing enzyme yield at optimal concentration of urea. In the present study, the cost of optimal enzyme produced by *C. utilis* NG-5 was reduced to USD ~2.85 per 10 U/ml fraction compared to the current cost (USD ~4.15 per 10 U/ml), which is more than 30% improvement.

The process parameters were determined under Plackett–Burman design for extracellular FFH production by *C. utilis* (Eqs. 1 and 2). The data are given in Table 2. The validation of the model was investigated under the conditions predicted against the responses obtained for enhanced enzyme productivity. A slightly differential correlation was observed between the observed and predicted values. The optimal levels of the significant process parameters for improved FFH production in shaking culture were incubation period (48 h), sucrose

Table 3 Statistical analysis of two factorial experimental design at various significant process parameters for extracellular FFH production by *C. utilis*.

Significant process parameters	Sum mean values	*F* value	Degree of freedom	Probability <*p*>
A	743.42	7.55	1	0.062
B	832.95	28.42	1	0.00258
C_{1+2}	1.634*E*+0025	14.08	3	0.091
D	955.02	42.94	1	0.00724
Correlation	2.003*E*+0025			

CM—19.24; R^2—0.238. The letters represent significant process parameters for enzyme production

concentration (5.0 g/l), initial pH (6.0), inoculum size (2.0% *v*/*v*, 16 h old), and urea concentration (0.2%, *w*/*v*). The statistical analyses of the responses for enzyme production were also performed and are represented in Table 3. The correlation, *B* and *D* for *F* values, depicts that the model is highly significant ($p < 0.05$). Correspondingly, the lower probability values also indicate that the model terms are significant. The analysis of linear, quadratic, and interaction coefficients were performed on the fermentative results, which highlight that FFH production is a function of the independent parameters [14, 15]. The addition of urea as a nitrogen source (degree of freedom 3) was necessary for maintaining the spatial conformation of the enzyme and thus has an important physiological role in the enzyme stability [7, 22].

Conclusion

In the present study, we identified a 2dg-stabilized mutant strain *C. utilis* NG-5 with a 40-fold increase (45.65 ± 2.0 U/ml/min) in extracellular FFH production under optimal conditions (5.0 g/l sucrose, 2.0 inoculum, 48-h incubation period, and pH5.0) compared to wild strain GC-46. The enhancement in enzyme production was highly significant and was of commercial level (HS, LSD 0.042). It is noted that addition of 0.2% (*w*/*v*) of urea in the culture medium resulted in a highly significant increase in FFH production. This enhancement was attributed to sucrose in the medium which, after hydrolysis, produced glucose and fructose. In addition, if not fully utilized, this sucrose could lead to carbon catabolite repression of enzyme. The process parameters were determined using Plackett–Burman design for extracellular enzyme production by yeast. The correlation, *B* and *D* for *F* values, depicts that the model is highly significant ($p \leq 0.05$). Further work on enzyme characterization prior to scale up is in progress.

References

1. Barlikova, A., Svorc, J., & Miertus, S. (1991). *Analytical Chemistry, 247*, 83–87.
2. Dynesen, J., Smits, H. P., Olsson, L., & Nielsen, J. (1998). *Applied Microbiology and Biotechnology, 50*, 579–582. doi:10.1007/s002530051338.
3. Rincon, A. M., Codon, A. C., & Benitez, T. (2001). *Applied and Environmental Microbiology, 67*, 4279–4285. doi:10.1128/AEM.67.9.4279-4285.2001.
4. Sanchez, M. P., Huidobro, J. F., Mato, I., & Sancho, M. T. (2001). *Journal of Agricultural and Food Chemistry, 49*, 416–422. doi:10.1021/jf0003350.
5. Roitsch, T., Balibrea, M. E., Hofmann, M., & Sinha, A. K. (2003). *Journal of Experimental Botany, 54*, 513–524. doi:10.1093/jxb/erg050.
6. Neto, J., Infanti, P., & Vitolo, M. (1996). *Applied Biochemistry and Biotechnology, 58*, 407–412. doi:10.1007/BF02941720.
7. Murray, R., Walsh, K., & Foley, G. (2007). *Biotechnology Progress, 23*, 79–87.
8. Haq, I., Ali, S., & Baig, M. A. (2004). *Biologia, 50*, 25–33.
9. Ginka, I. F., Emilina, D. S., & Dora, M. B. (2004). *Zurich Naturforsch, 59*, 99–103.
10. Miller, G. L. (1959). *Analytical Chemistry, 31*, 426–428. doi:10.1021/ac60147a030.
11. Akgol, S., Kacarb, Y., Denizlia, A., & Aricab, M. Y. (2001). *Food Chemistry, 74*, 281–288. doi:10.1016/S0308-8146(01) 00150-9.
12. Pirt, S. J. (1975). *In principles of microbe and cell cultivation* (2nd ed.), pp. 116–124. London: Blackwell Scientific.
13. Snedecor, G., & Cochran, W. G. (1980). *In statistical methods* (7th ed.), pp. 80–86. Iowa: Iowa State University.
14. Plackett, R. L., & Burman, J. P. (1946). *Biometrika, 33*, 305–325. doi:10.1093/biomet/33.4.305.

15. Ahuja, S. K., Ferreira, G. M., & Morreira, A. R. (2004). *Biotechnology and Bioengineering, 85*, 666–675. doi:10.1002/bit.10880.
16. Herwig, C., Doerries, C., Marison, I., & Stockar, U. V. (2001). *Biotechnology and Bioengineering, 82*, 247–258. doi:10.1002/bit.10004.
17. Haq, I., Baig, M. A., & Ali, S. (2005). *World Journal of Microbiology & Biotechnology, 21*, 287–292.
18. Bokosa, I., Krastanov, A., & Roshkova, Z. I. (1992). *Nauchni Tritical, 39*, 269–279.
19. Vitolo, M., Duranti, M. A., & Pellegrim, M. B. (1995). *Journal of Industrial Microbiology, 15*, 75–79. doi:10.1007/BF01569803.
20. Persike, D. S., Bonfim, T. M., Santos, M. H., Lyng, S. M., Chiarello, M. D., & Fontana, J. D. (2002). *Bioresource Technology, 82*, 79–85. doi:10.1016/S0960-8524(01) 00121-3.
21. Gancedo, J. M. (1998). *Microbiology and Molecular Biology Reviews, 62*, 334–361.
22. Hashimoto, S., Ogura, M., Aritomo, K., Hoshida, H., Nishizawa, Y., & Akada, R. (2005). *Applied and Environmental Microbiology, 71*, 312–319. doi:10.1128/AEM.71.1.312-319.2005.