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Kinetics of an Extracellular β-D-Fructofuranosidase Fructohydrolase Production from a Derepressed Mutant of *Saccharomyces carlsbergensis* and Parameter Significance Analysis by 2-Factorial Design

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Abstract.- In the present study, over hundred strains of *Saccharomyces carlsbergensis* were isolated from various fruits including jaman, plum, peach, date, banana and mango. These isolates were screened for extracellular β-D-fructofuranosidase fructohydrolase (FFH) production under submerged fermentation (SmF). The strain GCBt-99 isolated from plum gave relatively better enzyme activity and it was exposed to ultraviolet radiations (UV dose ~1.2×10^2 J/m^2/s) to improve its FFH potential. Among 16 UV irradiated mutant strains, UV-dg4 was selected for batch culture optimizations as it exhibited ~17.86 fold higher enzyme activity compared to wild culture. The potential mutant was cultured overnight and plated on YPS-agar medium containing various levels of 2-deoxy-D-glucose (2dg). The cultural conditions and nutritional requirements were optimized in a stirred fermentor by the selected mutant culture. Over 75% enhancement in enzyme production (38.72 U/ml/min) on the basis of substrate consumption was achieved when the process parameters including sucrose concentration (10 g/l), incubation period (72 h), initial pH (6), inoculum size (12%, v/v) and soybean meal as a nitrogen source (5 mg/ml nitrogen) were further identified using Plackett-Burman design (PBD) and response surface methodology (RSM). On the basis of kinetic variables, notably Q_p (0.826 U/ml/h), $Y_{p/s}$ (2.845 U/ml/g) and q_p (0.102 U/g yeast cells/h), the mutant UV-dg4 was found to be a hyper FFH producer ($p\leq0.05$) indicating a viable utility (*HS*, LSD~0.031).

Keywords: *Saccharomyces carlsbergensis* / β-D-fructofuranosidase fructohydrolase / microbial fermentation / two-factorial design / enzyme kinetics / induced mutagenesis.

INTRODUCTION

The enzyme β-D-fructofuranosidase fructohydrolase (FFH, *EC 3.2.1.26*) catalyzes α-1,4 glycosidic linkages in sucrose to release glucose and fructose monomers (Li *et al.,* 1998). It is an intra- as well as extracellular enzyme. The extracellular form is a glycoprotein and exists as a dimer that associates to form tetramers, hexamers or octamers. The enzyme has wide range of commercial applications (Aranda *et al.,* 2006). It has the ability to produce inverted syrup *i.e.*, an equimolar mixture of fructose and glucose, which is used in several industrial processes such as the production of lactic acid and ethanol. The inverted syrup is preferred over chemically produced golden syrup because of its higher purity, stability, better taste and absence of by-products (Roitsch *et al*., 2003). The size and age of inoculum need in-depth investigation prior to scale up of a high-yielding batch process. The ever increasing demand has stimulated its production from microbial sources (Marianna *et al.,* 2005; Doaa and Mahmoud, 2007). Among the microorganisms, yeasts such as *Saccharomyces, Kluyveromyces, Candida, Debaryomyces, Sporotrichum, Pichia* and fungi, *Penicillium* and *Aspergillus* species are common FFH producers (Skowronek and Fiedurek, 2004). However, *Kluyveromyces* species viz. *K. fragilis* and *K. marxianus* have relatively higher potential for enzyme yield under batch and continuous cultures but industrially not acceptable (Singh *et al.,* 2007). Therefore, the increasing potential of FFH applications prompts isolation and screening for some novel enzyme producing isolates particularly among *Saccharomyces* spp. that can meet the conditions favourable for industrial applications. This yeast or its hybrids are preferable due to the GRAS status, high sucrose fermenting ability and relatively less fermentation time compared to other yeasts (Barlikova *et al.,* 1991). A

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potent yeast strain could secrete a large amount of FFH into the medium without additional nutrient supplementation by submerged fermentation (SmF). However, the industrial application of FFH will only be viable when it is available in large quantities at competitive market prices.

The design and formulation of the medium is largely dependent on the microorganism, substrate and type of cultivation. A well-defined statistical experimental design is considered to be necessary for optimization of a fermentation process (Aranda *et al*., 2006; Skowronek and Fiedurek, 2004). The Plackett-Burman design (PBD) is a two-level multifactorial design based on the rationale known as balanced incomplete blocks. It has frequently been used for screening process variables that make the greatest impact on a process (Roitsch *et al*., 2003). Hence, it is pressing to specify the effect of incubation period, sucrose concentration, initial pH, inoculum size and nitrogen sources along with kinetic parameters of a novel 2-deoxy-D-glucose (2dg) tolerant mutant culture. The two-factorial PBD could further identify the significant variables influencing FFH hyper production. Various combinations of the components were formed. Since the number of significant variables used in this study was 5, therefore a design consisting of 8 combinations was used. After optimizing the critical parameters, response surface methodology (RSM) using a central composite design was used and it was proved to be crucial in achieving a high performance batch process for FFH production.

MATERIALS AND METHODS

Isolation, identification and screening of microorganism

One hundred and five strains of *S. carlsbergensis* were isolated from different fruits such as jaman (*Eugenia jambolana* Lamk.), plum (*Prunus bokhariensis* Royle), peach (*Prunus persica* Stokes.), date (*Pheonex dactylifera* L.), banana (*Musa paradisiaca* L.) and mango (*Mangifera indica* L.) by serial dilution method (Clark *et al*., 1958). The samples were collected from local markets of Lahore in sterile polythene bags. One gram of fruit sample was aseptically added in 100 ml of sterilized distilled water and swirled in a rotary shaking incubator (160 rpm) for 15 min. The fruit suspension was diluted up to 10^6 times. Approximately 0.1 ml of the dilution was transferred to a Petri plate containing yeast-extract peptone sucrose (YPS) agar medium containing (g/l): yeast extract 3, peptone 5, sucrose 20 and agar 20 at pH 6. The addition of sucrose helped in the selection of colonies having higher enzyme activity. The Petri plates were incubated at 30ºC for 1-2 days. The independent initial yeast colonies were picked up and transferred to YPS-agar slopes. Incubation was carried out at 30°C (2-3 days) for optimal growth in a cooled incubator (MIR-153, Sanyo, Japan). The isolates were identified by observing the morphological characteristics (Bold *et al*., 1980; Onion *et al*., 1986) and screened for enzyme activity by shaking culture. The culture was stored at 4°C in a cold cabinet (MPR-1410, Sanyo, Japan).

Preparation of cell suspension

Cell suspension was prepared from a 48 h old slant culture of yeast. Fifteen millilitre of sterile 0.05% (w/v) diacetyle ester of sodium sulpho succinic acid (Monoxal OT) was transferred to the slant. Yeast cells were scratched with sterilized wire-loop and the tube was swirled gently to form a homogenous cell suspension.

Inoculum preparation

Fifty millilitre of YPS-medium, pH 6 was transferred to a 250 ml Erlenmeyer flask. The flask was cotton-plugged, sterilized in an autoclave at 15 lbs/in^2 pressure (121°C) for 15 min and allowed to cool at room temperature. Afterwards, 1 ml of the cell suspension was aseptically transferred and incubated in a rotary shaker (10×400 XX2.C Sanyo Gallenkamp PLC, UK) at 30°C for 24 h. The agitation rate was maintained at 200 rpm. The cell count (A_{546}~1) was made by a haemocytometer slide bridge (K-110, Neuberger, Germany).

Fermentation procedure, scale up and critical phases

The laboratory scale production of extracellular FFH was carried out by SmF using 500 ml Erlenmeyer flasks. Hundred millilitre of YPS-medium having sucrose 4 g/l was transferred to

separate flasks. The flasks were cotton plugged, sterilized in an autoclave and cooled at room temperature. One millilitre of inoculum (1.25×10^7 CFU/ml) was aseptically transferred to each of the flasks and incubated in a rotary shaker at 30°C for 72 h. The agitation rate was maintained at 240 rpm throughout the fermentation period. Batch culture experiments were run parallel in a set of triplicates.

The scale up studies for the production of extracellular FFH was carried out in a stirred fermentor (New Brunswick Scientific Bioflo 110, USA) of 7.5 L capacity with working volume of 5 L. The working vessel containing basal medium, was sterilized in an autoclave at 121°C for 20 min. The inoculum was transferred at different levels (1-4 %, v/v). Sterilized solutions of 0.1 N HCl or 0.1 N NaOH were used for pH adjustment. The temperature was kept at 30°C. Agitation speed of the stirrer was maintained at 200 rpm while aeration rate was set at 1.0 l/l/m (vvm). The dissolved oxygen (DO) was maintained by the proportional integral derivative (PID) cascade controller, which changed the speed of agitation. Pure oxygen was automatically supplied to the fermentor to keep the DO level at the set point after the agitation speed reached the maximum allowable set point.

Induced mutagenesis through UV radiations

The induced mutagenesis of the selected yeast isolate *S. carlsbergensis* GCBt-99 was carried out through UV radiations (Perlman *et al*., 1986). For this, 5 ml of an 8-h yeast culture was subjected to centrifugation in a refrigerated spinning machine (738k, Eppendorf, Hamberg, Germany) at 9500×*g*. The cells were suspended in 5 ml of sterilized 0.5% sucrose acetate buffer, pH 4.8, washed twice and resuspended in 10 ml of the buffer. Five milliliter of this suspension was transferred to individual sterile Petri plates and exposed to UV radiations for different time intervals (5 to 30 min). A fixed distance of 5 cm (dose 1.2×10^2 J/m^2/s) was maintained for UV treatment. Approximately 0.1 ml each of the treated cell suspensions were transferred to the plates containing YPS agar medium. Colonies that appeared within 36-48 h of incubation at 30°C were transferred to YPS agar slopes while those exhibiting the most growth were replica plated, and one set was exposed to a glucose measuring kit solution (Sigma, St. Louis, USA). The initial colonies that were surrounded by the largest reddish pink zones were selected for further study.

Induction of tolerance in mutant yeast cells against 2dg

The potential mutant strains were sub-cultured overnight in YPS medium, harvested during the exponential phase of growth (1.2×10^6 cells/ml), washed with sterilized distilled water and plated on 2dg-YPS agar medium containing (mg/ml): yeast extract 3, peptone 5, raffinose 20, agar 20 and 2-deoxy-D-glucose 0.01-0.08. Raffinose was used instead of sucrose because sucrose hydrolysis by yeast FFH results in glucose formation (Negoro and Kito, 1973). Colonies appearing between 2-3 days were cultured again and those exhibiting vigorous growth were tested for FFH production by shake flask fermentation. Samples were drawn periodically, washed and plated on YPS agar medium to select the strains resistant against 2dg. The master mutant culture was preserved in sterile paraffin oil at –20°C.

Significant cultural and nutritional parameters

Carbon sources

Carbon is not only the basic element of cells but is required by the living organisms for the maintenance of their metabolic processes (Costaglioli *et al*., 1997). In the present study, the effect of different sucrose concentrations (1-30 mg/ml) was investigated on FFH production by *S. carlsbergensis* UV-dg4.

Time of incubation

Time of incubation not only determines the length of exponential phase of microbial growth but also establishes the efficacy of metabolite production (Samia, 2008). Therefore, the time of incubation was varied from 12-96 h after inoculation.

Initial pH

The effect of initial pH on extracellular FFH production by the selected yeast was also carried out. It was varied from 3.5 to 8.5 under SmF. The culture was cultivated using 100 ml of the medium for 72 h.

Size of inoculum

The optimization of inoculum is of prime importance as it not only determines the rate of substrate utilization by yeast cells but also defines the limits of product formation under controlled conditions (Mona and Mohamed, 2009). An inoculum level of 2-16% (v/v) was used to seed the liquid cultivation media. Incubations were carried out at 30°C (pH 6) for a period of 72 h after inoculation.

Nitrogen sources

Nitrogen requirement is also one of the basic necessities of living homeostasis. In addition, it is an integral part of protoplasm and regulates a number of life processes in a cell (Tammi *et al*., 1987). So, different sole nitrogen sources (urea, peptone, soybean meal, ammonium sulphate, ammonium nitrate, ammonium chloride) were tested. The concentration of nitrogen was adjusted at 4 mg/ml for extracellular FFH production by the mutant yeast culture under SmF. These were compared with the control. At optimal conditions, the concentration of nitrogen in soybean meal was varied from 1-8 mg/ml. Batch culture experiments were carried out under the optimized cultural conditions (pH 6, 30°C) for 72 h.

Analytical techniques and FFH assay

Yeast dry cell mass was determined after drying harvested cells at 70°C for 2 h. Total reducing sugars (as glucose) were estimated by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The enzyme activity was determined according to Burkert *et al*. (2006). 'One unit (U) is defined as the amount of enzyme that produces 1 μmole of glucose per minute under standard assay conditions.' A reaction mixture containing 0.1 ml of the enzyme extract and 0.9 ml of a sodium acetate buffer (0.1 M, pH 6) containing 2% inulin was incubated at 50°C for 15 min. Thereafter, the enzyme was inactivated by keeping the reaction mixture at 90°C for 10 min. The reaction mixture was then assayed for glucose as a reducing sugar using the DNS method. The mixture was placed in a boiling water bath for 5 min to stop the reaction and allowed to cool at room temperature. A blank with distilled water instead of the enzyme solution was run parallel. To 1 ml of each mixture, 1 ml of DNS reagent was added and placed in boiling water for 5 min. After cooling to an ambient temperature (20°C), the volume was raised to 8 ml with distilled water. A spectrophotometer (110, Shimadzu, Tokkyo, Japan) was used to determine $\%T_{592}$. Protein contents were determined after Bradford (1976).

Table I.- Comparison of kinetic variables for FFH productivity by *S. carlsbergensis* at 72 h of fermentation in shake flasks.

Kinetic variables	GCBt-99 (wild-type)	UV-dg4 (mutant)
Specific growth rate		
μ (h^{-1})	0.097 ± 0.03^{ab}	0.182 ± 0.04^{a}
Enzyme production variables		
Q_p (U/ml/h)	0.124 ± 0.06^{cde}	0.826 ± 0.13^{a}
$Y_{p/s}$ (U/ml/g)	0.328 ± 0.12^{def}	2.845 ± 1.03^{ab}
$Y_{p/x}$ (U/ml/g)	0.236 ± 0.03^{defg}	4.515 ± 1.53^{a}
q_p (U/g yeast cells/h)	0.011 ± 0.002^{de}	0.102 ± 0.07^{abc}
Substrate consumption variables		
$Y_{x/s}$ (g yeast cells/g)	1.116 ± 0.21^{ab}	0.762 ± 0.11^{bc}
Q_s (g/L/h)	0.102 ± 0.02^{bc}	0.286 ± 0.03^{a}
q_s (g/g yeast cells/h)	0.094 ± 0.03^{bcd}	0.181 ± 0.05^{ab}
Q_x (g yeast cells/L/h)	0.034 ± 0.02^{bc}	0.195 ± 0.02^{a}
Least significant difference (LSD)	0.056	0.031
Significance level <p>	*S*	*HS*

Kinetic variables: μ (h^{-1}) = specific growth rate, Q_p = FFH U/ml/h, $Y_{p/s}$ = FFH U/ml/g substrate consumed, $Y_{p/x}$ = FFH U/ml/g yeast cells formed, q_p = FFH U/g yeast cells/h, $Y_{x/s}$ = g yeast cells/g substrate utilized, Q_s = g substrate consumed/l/h, q_s = g substrate consumed/g yeast cells/h, Q_x = g yeast cells formed/L/h. HS is for the 'highly significant' while S for 'significant' values. LSD denotes least significant difference, p is for probability. ± Indicates standard deviation among three parallel replicates. The values designated by different letters in each row differ significantly at $p\leq0.05$.

Kinetic study

Kinetic variables were studied according to the procedures laid down by Pirt (1975). The values for specific growth rate *i.e.*, μ (h^{-1}) were calculated from the plots of ln(X) versus time of fermentation (Table I). The growth yield coefficient ($Y_{x/s}$) was calculated as the dry cell mass divided by the amount of substrate utilized during fermentation. The product yield coefficients namely $Y_{p/s}$ and $Y_{p/x}$ were determined by using the relationships $Y_{p/s}$ = dP/dS and $Y_{p/x}$ = dP/dX, respectively. The

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

Statistical analysis

Duncan's multiple range tests (Spss-22, version 14.5) were applied under one-way analysis of variance (ANOVA-I) and the treatment effects were compared according to Snedecor & Cochran (1980). Significance is presented in the form of probability (<p>) values.

Plackett-Burman design (PBD) and response surface methodology (RSM)

The significant variables were identified using a two-factorial system i.e., Plackett-Burman experimental design (PBD) and response surface methodology (RSM) (Ahuja *et al*., 2004). Table IIb shows PBD used in this study. The symbols '+' and '−' represent the lower and higher values of the process parameters. The lower values were within a narrow range of their higher levels with the exception of factor D which was related to inoculum optimization (Table IIa)The variables were denoted at two widely spaced intervals and the effect of individual parameters on enhanced FFH production by the mutant yeast strain was calculated by the following equations,

$$E_o = (\Sigma M_+ - \Sigma M_-) / N \qquad \text{Eq. I}$$

$$E = \beta_1 + \Sigma \beta_2 + \Sigma \beta_3 + \beta_{123} \qquad \text{Eq. II}$$

In Eq. I, 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. II, E is the significant parameter, β_1 is the linear coefficient, β_2 the quadratic coefficient and β_3 is the interaction coefficient for process parameters.

Table IIa.- Process parameters of stirred fermentor at two levels used in PBD.

Captions	Process parameters*	Lower level (−)	Higher level (+)
A	Incubation period (h)	48	72
B	Sucrose conc. (g/l)	10	16
C	Initial pH	5	6.5
D	Inoculum size (%)	10	14
E	Soybean meal (mg/ml)	4	6

The optimal process parameters were determined separately for each trial.

Table IIb.- Application of PBD and reverse PBD on various process parameters.

Combinations	Process parameters				
	A	B	C	D	E
1	−	−	−	+	+
2	−	−	+	−	−
3	+	+	−	−	+
4	−	+	−	+	−
5	+	−	+	−	+
6	−	+	−	−	−
7	−	+	−	+	−
8	+	−	+	−	−
Reverse design					
1	−	+	−	+	−
2	+	−	−	−	−
3	−	−	+	−	−
4	−	+	−	−	−
5	−	−	−	−	−
6	+	−	−	−	−
7	−	−	−	−	+
8	−	−	−	−	−

The symbols '+' and '−' represent the lower and higher values of the process parameters.

A central composite design was employed to determine the reverse PBD (Ahuja *et al*., 2004). It was constructed by reversing the signs (− to + and + to −) for all the five combinations that were tested for yeast FFH activity (Table IIb). The two values for which the response parameters were calculated were 40 and 56 h after inoculation, respectively. The low level used in the design (−) was the same as higher level (+) used in PBD.

RESULTS AND DISCUSSION

In the present investigation, a total of 105 different strains of *Saccharomyces carlsbergensis* were isolated from different fruits including jaman, plum, peach, date, banana and mango which were collected from different localities of Lahore District. The isolates were identified after Bold *et al*. (1980) and showed following typical characteristics: *S. carlsbergensis* is non-mycelial budding yeast. The buds were globular to elliptical in shape. The scars were more prominant on a single cell. The initial colonies were of white to creamy color with smooth or glabrous surface. The yeast isolates were screened for extracellular β-D-fructofuranosidase fructohydrolase (FFH) production by submerged fermentation (SmF) in 250 ml Erlenmeyer flasks. The enzyme production ranged from 0.115 to 0.8 U/ml/min. Of all the isolates examined, the strain GCBt-99 isolated from plum was selected for batch culture optimizations as it exhibited relatively better enzyme activity (0.8 U/ml/min) when compared with other yeast isolates. This culture was selected for induced mutagenesis through ultraviolet radiations (UV). On exposing the wild yeast strain at 1.2×10^2 J/m^2/s for 45 min, UV induction yielded a mutant (UV-dg4) exhibiting an enzyme activity of 14.34 U/ml/min which was ~17.86 fold improvement. The mutants were made resistant after treating at various 2dg levels. Initially high yielding isolates were obtained at 0.02 µg/ml 2dg; however, their enzyme production phenotype became unstable after approximately 2 weeks. The reason may be the development of tolerance in yeast cells after a small number of generations that permitted a few unstable mutants to thrive. To mitigate this problem, isolates were grown on the medium containing higher 2dg levels. The concentration of 0.065 µg/ml was found optimal, as at this level UV-dg4 gave a consistent FFH yield. The enzyme production rate of this mutant was significantly different ($p\leq0.05$) from that of all other isolates or mutant strains. Although the strain showed a high-level tolerance to 2dg, FFH production was still under catabolite repression. The enzyme produced by the mutant shares similar characteristics to FFH produced by other strains reported previously (Bourgi *et al*., 1986; Gancedo, 1998; Zhang *et al*., 2005).

The kinetic parameters viz. specific growth rate (µ), enzyme production parameters (Q_p, $Y_{p/s}$, $Y_{p/x}$, q_p) and sugar consumption variables ($Y_{x/s}$, Q_s, q_s, Q_x) were compared for the wild-type (GCBt-99) and mutant strain (UV-dg4) of the yeast *S. carlsbergensis* (Table I). The comparison of Q_s (g cells/L/h) for FFH productivity demonstrated that the mutant strain UV-dg4 has a higher value for volumetric rate of substrate consumption (Q_s = 0.286 g/L/h) than the wild-culture GCBt-99. Several fold improvement in terms of volumetric FFH productivity was noted with the mutant UV-dg4 at all the rates examined. Although wild-type GCBt-99 achieved a higher value ($Y_{x/s}$ = 1.116 g yeast cells/g) than the mutant (0.762 g yeast cells/g), however UV-dg4 demonstrated a significant improvement ($p\leq0.05$) in volumetric rate of product formation. In addition, when both of the cultures were monitored for specific rate constant, the mutant UV-dg4 gave higher values for q_p (greater than 9-fold improvement). Hence, the mutant exhibited an over all 8 to 10 fold improvement in the values for Q_p, $Y_{p/x}$, $Y_{p/s}$ and q_p over the parental strain (LSD 0.031) which is highly significant (*HS*) and this was supported by the findings reported by Pirt (1975). Skowronek and Fiedurek (2004) found that nutritional parameters influence the substrate consumption rate, specific growth rate and subsequent productivity of FFH. The kinetic values revealed that the enzyme is an FFH as it has higher affinity for the sucrose rather than polymers or other oligosaccharides. In the present study, the best enzyme producing mutant, *S. carlsbergensis* UV-dg4 was selected for cultural and nutritional studies in 500 ml capacity shake flasks prior to scale up in a stirred fermentor.

Sucrose is considered to be the best sole carbon source for FFH production as the availability of glucose for yeast is dependent on carbohydrate hydrolysis by the enzyme. Therefore, sucrose concentration markedly influences enzyme biosynthesis. In Figure 1 is depicted the effect of different sucrose concentrations (2-30 mg/ml) in the culture medium for extracellular FFH production by *S. carlsbergensis* UV-dg4. Maximum enzyme production (14.69 U/ml/min) was noted at sucrose concentration of 10 mg/ml. DCM and sugar consumption were 1.71 and 2.85 mg/ml,

respectively. The mechanism of glucose and fructose repression by FFH has been clearly reported by Vitolo *et al*. (1995). The enzyme hydrolyses sucrose into fructose and glucose; these sugars in the medium repress further enzyme secretion from yeast cells. In a similar study, Herwig *et al*. (2001) reported carbon catabolite repression of the enzyme, as FFH production was checked by the presence of monosaccharides like glucose and fructose in the medium. Therefore, an optimal sucrose concentration is critical for maximal extracellular FFH production as a higher sucrose concentration acts as repressor as reported by Elorza *et al*. (1977). Further increase in sucrose concentration, however did not enhance extracellular FFH production. In disparity, sugar consumption continued to rise with the course of fermentation while DCM was dropped gradually.

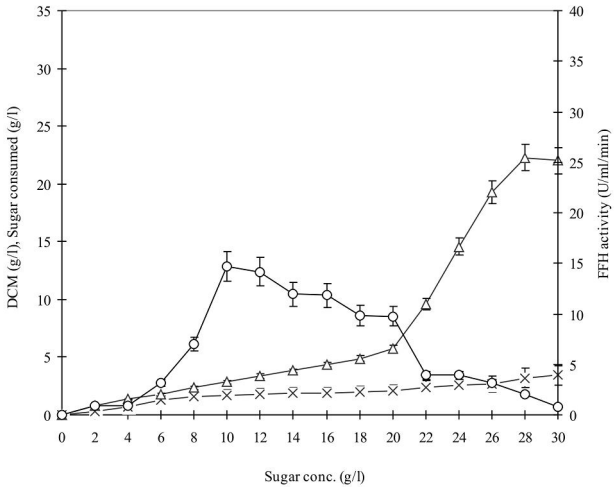

Fig. 1. Effect of different concentrations of sucrose on extracellular FFH production by *S. carlsbergensis* UV-dg4 in stirred fermentor (DCM -×-, Sugar consumed -Δ-, FFH activity -○-). Y bars show standard deviation among the three parallel replicates which differ significantly at 5% level of significance (p≤0.05). Temperature 30ºC, agitation rate 240 rpm.

Appropriate incubation period is of critical import for FFH synthesis as longer incubation can cause feedback repression of the enzyme. In batch wise fermentation, the enzyme production starts after a lag phase of 12 h and reaches maximum at the onset of stationery phase. Afterwards, enzyme activity declined due to decrease in nutrients availability in the medium, or carbon catabolite repression, as the expression in yeast is checked by the presence of monosaccharides like glucose and fructose as reported by Samia (2008). Thus proper incubation time is very important for maximal enzyme production. The rate of extracellular FFH production by *S. carlsbergensis* UV-dg4 was also investigated. Incubation was carried out from 12-96 h after inoculation. The results are given in Figure 2.

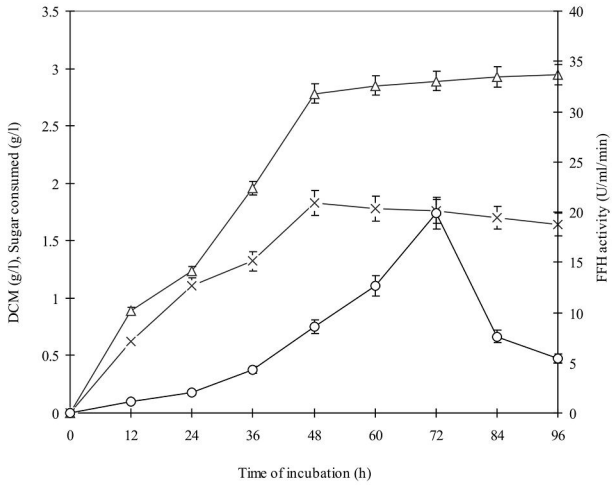

Fig. 2. Rate of extracellular FFH production by *S. carlsbergensis* UV-dg4 in stirred fermentor (DCM -×-, Sugar consumed -Δ-, FFH activity -○-). Y bars show standard deviation among the three parallel replicates which differ significantly at 5% level of significance (p≤0.05). Temperature 30ºC, agitation rate 240 rpm.

An enzyme activity of 1.12 U/ml/min was obtained in 12 h which was gradually increased by further increasing the time of incubation but up to a certain extent. The maximum enzyme production (19.89 U) was achieved in 72 h. DCM and sugar consumption were found to be 1.756 and 2.89 mg/ml, respectively. As monosaccharide concentration in the medium decreased to a value below 0.2 mg/ml, carbon catabolite repression was halted resulting in a faster rate of enzyme biosynthesis as reported by Roitsch *et al*. (2003). Further increase in the incubation period did not increase enzyme production rather it was sharply declined possibly

due to the decrease in the amount of available nitrogen in production medium, the age of organism, the addition of inhibitors produced by yeast itself and the protease production characteristic of decline phase. Other workers have reported FFH production by *S. carlsbergensis* culture medium incubated for a longer period from 84-120 h (Vitolo *et al*., 1995).

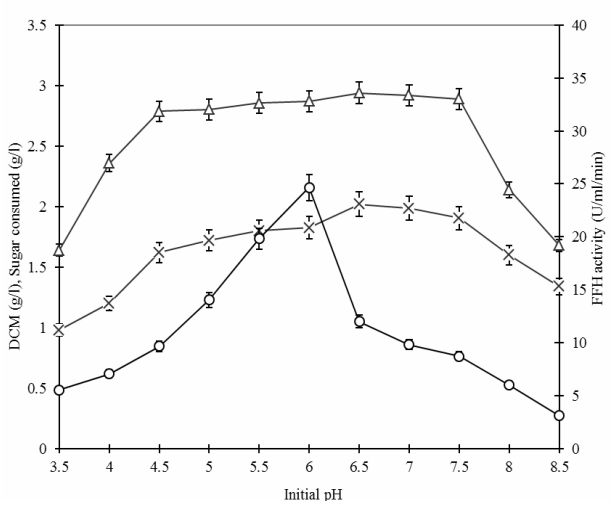

Fig. 3. Effect of different initial pH of medium on extracellular FFH production by *S. carlsbergensis* UV-dg4 in stirred fermentor (DCM -×-, Sugar consumed -Δ-, FFH activity -○-). Y bars show standard deviation among the three parallel replicates which differ significantly at 5% level of significance ($p \leq 0.05$). Temperature 30ºC, agitation rate 240 rpm.

The enzyme FFH is largely dependent on pH of the fermentation medium. Figure 3 shows the effect of different initial pH (3.5-8.5) on extracellular FFH production by *S. carlsbergensis* UV-dg4. The maximum enzyme production (24.65 U/ml/min) was obtained when initial pH of the medium was kept at 6. The DCM and sugar consumption were 1.825 and 2.87 mg/ml, respectively. Less enzyme activity, accompanied by a decrease in dry cell mass and sugar consumption, was noticed at pH other (less or more) than the optimal. Although significant growth rate was observed at pH 6.5; however maximum product rate was noted at initial pH of 6. It means that although growth was more favored at pH 6.5, but as far as extracellular FFH production was concerned, pH 6 was the best. It was noted that during SmF by *S. carlsbergensis*, final pH of the reaction mixture was more than initial pH; besides, extent of the increase in pH was proportional to the enzyme activity. The reason for this relationship was that extracellular FFH production accompanied the secretion of some anions and basic proteins, or selective uptake of cations as reported by Aranda *et al.* (2006).

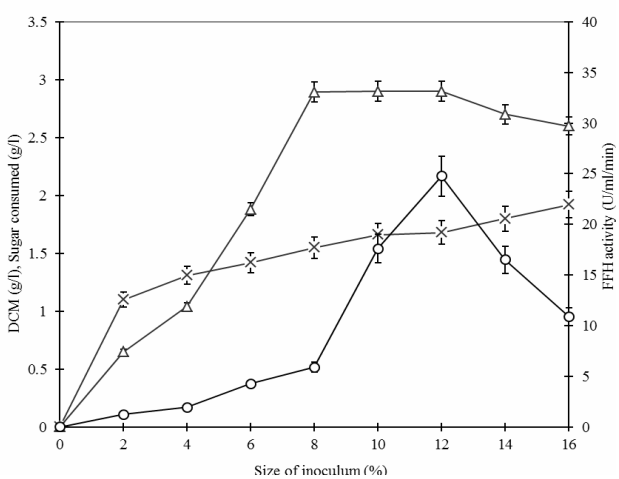

Fig. 4. Effect of inoculum size on extracellular FFH production by *S. carlsbergensis* UV-dg4 in stirred fermentor (DCM -×-, Sugar consumed -Δ-, FFH activity -○-). Y bars show standard deviation among the three parallel replicates which differ significantly at 5% level of significance ($p \leq 0.05$). Temperature 30ºC, agitation rate 240 rpm.

The effect of different size of inoculum on extracellular FFH production by *S. carlsbergensis* UV-dg4 was also undertaken. It was ranged from 2-16% (v/v) of basal medium. The results are shown in Figure 4. The enzyme production was not encouraging at low levels of inoculum. It was due to a small number of yeast cells which remained incapable of utilizing the fermentation medium efficiently to secrete enzyme. In addition, the non-compatible ratio between sucrose concentration and the number of yeast cells acted as a repressor. However, the maximum enzyme production (24.76 U/ml/min) was achieved at an inoculum size of 12% (v/v) *i.e.*, 12 ml of inoculum used to seed 100 ml of

production medium. DCM and sugar consumption were 1.68 and 2.9 mg/ml, respectively. Enzyme production was decreased when inoculum size was changed other than the optimal. Inoculum size larger than optimal along with nutrient limitation resulted in less enzyme production as reported previously by Luis *et al*. (2007).

The type and concentration of a nitrogen source is very important and plays a vital role in extracellular FFH production (Tammi *et al*., 1987). The effect of different nitrogen sources such as urea, peptone, soybean meal, $(NH_4)_2SO_4$, NH_4NO_3 and NH_4Cl was studied on extracellular FFH production by *S. carlsbergensis* UV-dg4. The results are shown in Figure 5A. Among all the nitrogen sources tested, soybean meal supported maximal enzyme production (29.657 U/ml/min). The DCM and sugar consumption were 1.827 and 2.87 mg/ml, respectively. Soybean meal is an enriched source of nutrients containing proteins 42%, carbohydrates 29.9%, fats 4%, calcium 0.25%, magnesium 0.25%, phosphorous 0.63%, potassium 1.75% and sulphur 0.32% resulted in a better extracellular FFH production in the fermented broth. The effect of different concentrations of nitrogen in soybean meal (1-8 mg/ml) on extracellular FFH production by yeast was also studied. The results are depicted in Figure 5B. Maximum enzyme production (38.16 U/ml/min) was achieved when 5 mg/ml of nitrogen was added into the production medium. DCM and sugar consumption were 1.824 and 2.868 mg/ml, respectively. Nitrogen concentration in soybean meal higher than optimal also gave less amount of extracellular FFH probably due to catabolite repression. Less nitrogen concentration was not enough to fulfil the nutritional requirements of the yeast resulting in less enzyme production as reported Marianna *et al*. (2005).

A two way factorial experimental method i.e., 2k-Plackett-Burman design was applied to determine the significant process parameters for extracellular FFH production by the mutant strain of *S. carlsbergensis* UV-dg4 (Eq. I & II). The effect of the optimal variables on the response was calculated which was the difference between the average response for the combinations having a higher level (+) of a given component and that for the ones have its lower level (−) [23]. The parameters and their

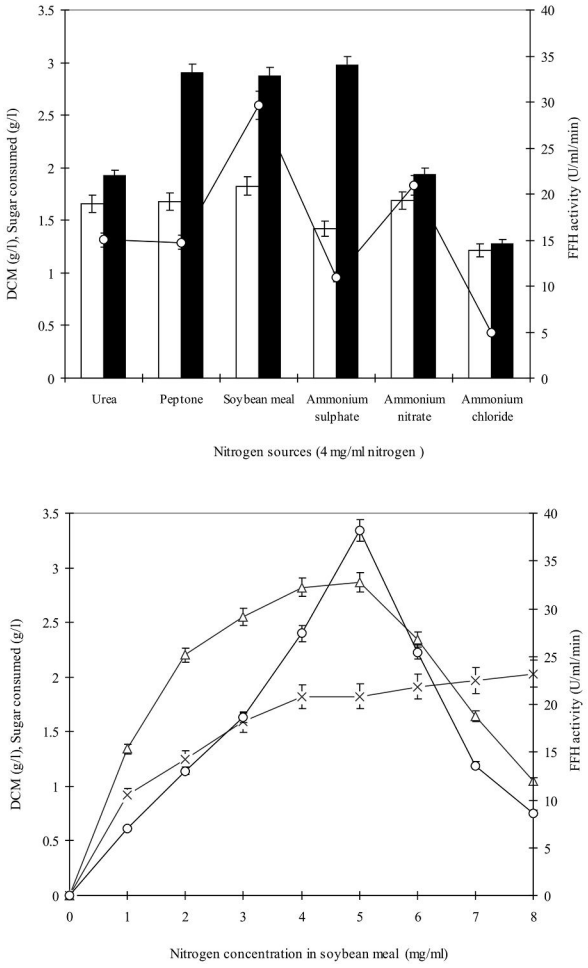

Fig. 5. Effect of different nitrogen sources (A) and concentrations of soybean meal (B) on extracellular FFH production by *S. carlsbergensis* UV-dg4 in stirred fermentor (DCM -×-, Sugar consumed -Δ-, FFH activity -○-). Y bars show standard deviation among the three parallel replicates which differ significantly at 5% level of significance ($p \leq 0.05$). Temperature 30ºC, agitation rate 240 rpm.

PBD combinations along with the respective reverse designs are given in Table IIa and IIb. A positive sign for the effect meant that the component would increase the response if employed at a higher level, and vice versa. It suggested that the interactions among the process parameters existed. The validation of the model was investigated under the conditions predicted against the responses obtained for enhanced enzyme production. A slightly different

Table IIIa.- Application of PBD at various process parameters (designated by different captions) for extracellular FFH activity from mutant *S. carlsbergensis* UV-dg4.

Process parameters at two-factorial design					Protein content (mg/ml)	Extracellular FFH activity (U)	
Incubation period (h)[A]	Sucrose conc. (g/L)[B]	Initial pH[C]	Inoculum size (h)[D]	Soybean meal conc. (mg/ml)[E]		Observed	Predicted
48	16	5	8	3	41.54	8.65	14.24
60	8	5.5	10	4	94.08	16.38	29.15
72	10	6	12	5	129.25	38.72	45.14
72	12	6	14	6	126.42	22.16	30.56
84	14	6.5	14	7	112.96	14.42	24.64

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

Table IIIb.- Statistical analysis of PBD at various significant process parameters for extracellular FFH production from mutant *S. carlsbergensis* UV-dg4.

Significant process parameters	Sum mean values	F-value	Degree of freedom	Probability < p >
A	16.05	3.26	1	0.108
B	35.42	17.02	1	0.085
C	98.12	9.64	2	0.066
D	1.625*E*+0025	8.98	3	0.042
E	65.32	7.19	2	0.024
Correlation	0.516*E*+0025			

CM – 19.24; R^2 – 0.238. The letters represent significant process parameters (incubation period, sugar level, initial pH, size of inoculum, evaluation of nitrogen sources and effect of soybean meal concentration) for enzyme production.

correlation was observed between the observed and predicted values. The data is given in Table IIIa. The optimal levels of the significant process parameters for improved FFH production in shaking culture were sucrose concentration (10 g/l), incubation period (72 h), initial pH (6), inoculum size (12% v/v) and soybean meal as a nitrogen source (5%, v/v). The protein content varied from 41.54 to 129.25 mg/ml. Another important parameter while performing a statistical design is the choice of the response parameter. The statistical analyses of the responses for enzyme production were also performed and are represented in Table IIIb. The correlation (0.516*E*+0025), A, B, C and D for E values depicts that the model is highly significant ($p \leq 0.05$). 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 (Ahuja *et al.*, 2004; Burkert *et al*., 2006). The incubation period and addition of pre-grown yeast cells as an inoculum (degree of freedom 3) were found necessary for better enzyme activity. It is important to note that the experimental design used in the present study which combined PBD and its fold over, used the minimum number of combinations required to distinctly evaluate the major effects. According to these results, the mutant strain of *S. carlsbergensis* UV-dg4 can be considered as a hyper-producing organism of FFH.

CONCLUSIONS

In the present study, the locally developed *Saccharomyces carlsbergensis* (mutant strain UV-dg4) is a promising organism for extracellular FFH production which was produced in a partially

constitutive manner. This is perhaps the first report in which novel yeast was used. The culture gave a 75% improvement in FFH yield (38.72 U/ml/min) compared to wild isolate. 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 values of kinetic variables, notably Q_p (0.826 U/ml/h), $Y_{p/s}$ (2.845 U/ml/g) and q_p (0.102 U/g yeast cells/h) demonstrated that the mutant has a faster growth rate and subsequently a higher enzyme production capability (LSD 0.031, *HS*). The process parameters particularly nature of the carbon-limiting substrate, medium composition and growth temperature were demonstrated using PBD and RSM for enzyme activity. The correlation (0.516*E*+0025), A, B, C and D for E values depicts that the model is highly significant ($p \leq 0.05$).

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Conflict of interest declaration

The authors have declared no conflict of interest.

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