Influence of micronutrients on yeast growth and β -D-fructofuranosidase production

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Abstract β -D-fructofuranosidase is one of the most important enzymes of the food industry especially due to its application in preparation of soft centered candies, confectioneries and to produce fructose syrups. Although several bacteria and filamentous fungi are reported for its production, yeasts are the most preferred source for this enzyme. In the present study mineral nutrients were screened for their effect on yeast growth and the enzyme production using Plackett-Burman design. Of the 11 nutrients screened, three variables (KH_2PO_4 , FeCl_3 and CoCl_2) were significantly effecting yeast growth while six variables, i.e. KH_2PO_4 , CaCl_2 , MnSO_4 , Na_2MoO_4 , ZnSO_4 and CoCl_2 had significant effect on enzyme production.

Keywords β -D-fructofuranosidase · *Saccharomyces* species · Submerged fermentation · Plackett-Burman design · Mineral nutrients

Introduction

Yeast cells require a wide range of metals for their growth and metabolic functions. Bioavailability of essential metal ions in media can dramatically influence yeast fermentation performance. Mineral nutrition of yeasts is thus very important in ensuring successful fermentation. Yeast and its enzymes have been used for decades in brewing and baking industries. β -D-fructofuranosidase (FFase) (EC 3.2.1.26, Invertase) which hydrolyzes sucrose, raffinose and related β -D-fructofuranosidase, is produced by bacteria, fungi, higher plants and some animals [1]. However, *Saccharomyces cerevisiae* is the most preferred organism for FFase production due to its high sucrose-fermenting ability. FFase and microorganisms that produce it are used in many ways in biotechnology to process sucrose. It is used traditionally in production of inverted sugars for industry especially in manufacture of candies and preserves, artificial honey, production of lactic acid and ethanol from fermentation of cane sugar molasses. It is used for the manufacture of calf feed and food for honeybees [2, 3]. FFase also has its application as a sensor for continuous sucrose determination [4].

Metal ions act as co-factors for important fermentation enzymes and also as modulators of environmental stress. Some metals inhibit yeast growth and metabolism, either by antagonism with essential metals or through direct toxicity effects. In general, metal ions can impact on yeast growth and metabolic processes during fermentation by influencing several important parameters including yeast growth, viability, enzyme activities, alcohol fermentation, stress tolerance, etc. Therefore it is necessary to screen vital mineral nutrients for production of industrially important enzymes. Statistical methods of screening have several advantages over conventional methods being rapid, reliable, shortlist

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significant nutrients, help understand interaction at various concentrations and reduce the total number of experiments tremendously. Plackett-Burman [5] method offers a design where initial screening of ingredients is done to understand the significance of their effect on product formation and the most suitable ingredient will be selected for optimization to increase the product yields [6–8].

In the present study we describe statistical selection of mineral nutrients for β -D-fructofuranosidase production in submerged fermentation by high β -D-fructofuranosidase producing yeast strain.

Materials and methods

Microorganism and FFase production

Saccharomyces species strain GVT263, a high β -D-fructofuranosidase producing, Ultraviolet (UV) generated mutant is used in the present study. The strain was grown and maintained on yeast malt agar (YMA) obtained from Himedia, Mumbai by routine subculturing. Twenty-five milliliter of sterile fermentation medium containing g/l: sucrose 10 and yeast extract 3 was prepared in 100 ml Erlenmeyer flasks and inoculated with 250 μ l of the 24 h culture (10^8 CFU/ml). Incubation was done for 24 h at 30°C and 100 rpm.

Screening of trace elements using Plackett-Burman design

Plackett-Burman (PB) design for 11 variables at two levels (+1 and –1) was used for screening of trace elements. Eleven

Table 1 Micronutrients and their levels selected for β -D-fructofuranosidase production and growth of *Saccharomyces* species GVT263

Label	Variable	+Level	–Level
		(% w/v)	(% w/v)
A	MgSO ₄	0.040	0.005
B	NaCl	0.040	0.005
C	KH ₂ PO ₄	0.040	0.005
D	CaCl ₂	0.040	0.005
E	CuSO ₄	0.040	0.005
F	KI	0.040	0.005
G	FeCl ₃	0.040	0.005
H	MnSO ₄	0.040	0.005
I	Na ₂ MoO ₄	0.040	0.005
J	ZnSO ₄	0.040	0.005
K	CoCl ₂	0.040	0.005

trace element sources namely MgSO₄, NaCl, KH₂PO₄, CaCl₂, CuSO₄, KI, FeCl₃, MnSO₄, Na₂MoO₄, ZnSO₄ and CoCl₂ were tested for their significant effect on yeast growth and production of FFase enzyme. Concentration of variables was fixed based on prior experiments and observations (Table 1). Sucrose and nitrogen source, i.e. yeast extract concentrations were fixed at 1.0 and 0.3%, respectively. Physical parameters like temperature, pH and agitation were held constant at 30°C, 6.0 and 100 rpm, respectively.

Enzyme assay

The reaction mixture contained 1.5 ml of 1% sucrose solution prepared in 0.1 M phosphate buffer (pH 6.5) and 500 μ l of culture supernatant obtained by centrifugation at 4,000 rpm. Incubation was done for 30 min at 30°C. Release of reducing sugars was quantified by di-nitro salicylic acid method [9]. Growth was determined as absorbance at 660 nm. One unit of enzyme was defined as the amount of enzyme required to release 1 μ M of reducing sugar per milliliter per minute under assay conditions.

Statistics

All experiments were conducted in triplicates and repeated thrice. Plackett-Burman experimental design and analysis of the results were done using Minitab 13 software.

Results and discussion

Metal ions have been shown to significantly effect production of various industrially important products like vitamins, biomass, organic acids, enzymes, etc. [10–13]. Production of extracellular enzymes is greatly influenced by the presence of various components in the media that include inducers, carbon and nitrogen substrates and trace elements. Yeasts require a range of metals for optimal growth, metabolism and fermentation performance. The requirement for metal ions varies, so widely with the different strains that it is necessary to adjust the composition of the medium to avoid the inhibitory effects of others. Effect of eleven trace elements has been investigated in the present study on yeast growth and FFase production in submerged fermentation. A 20 run Plackett-Burman design was selected to screen these variables (Table 2).

Effect of metal ions on yeast growth

Growth was found to be highest (2.001 OD₆₆₀) in combinations as per run 5 and 7 and least (1.895 OD₆₆₀) in

Table 2 Plackett-Burman design for screening of micronutrients for growth of *Saccharomyces* species GVT263 and β -D-fructofuranosidase production (FFase) [Factors: 11, Replicates: 1, Design: 20, Runs: 20, Center points: 0]

Run	A	B	C	D	E	F	G	H	I	J	K	Growth	Enzyme
1	+	–	+	+	–	–	–	–	+	–	+	1.942	239.9
2	+	+	–	+	+	–	–	–	–	+	–	1.964	209.7
3	–	+	+	–	+	+	–	–	–	–	+	1.914	374.5
4	–	–	+	+	–	+	+	–	–	–	–	1.970	248.8
5	+	–	–	+	+	–	+	+	–	–	–	2.001	202.5
6	+	+	–	–	+	+	–	+	+	–	–	1.954	228.4
7	+	+	+	–	–	+	+	–	+	+	–	2.001	218.7
8	+	+	+	+	–	–	+	+	–	+	+	1.913	332.4
9	–	+	+	+	+		–	–+	+		–+	1.895	279.2
10	+	–	+	+	+	+	–	–	+	+	–	1.971	244.1
11		–+		–+	+	+	+		–	–+	+	1.980	274.6
12	+		–+		–+	+	+	+		–	–+	1.934	356.3
13		–+	–	+	–	+	+	+	+	–	–	1.996	117.6
14	–	–	+	–	+	–	+	+	+	+	–	1.975	285.1
15	–	–	–	+	–	+	–	+	+	+	+	1.947	276.2
16	–	–	–	–	+	–	+	–	+	+	+	1.966	234.3
17	+	–	–	–	–	+	–	+	–	+	+	1.960	285.2
18	+	+	–	–	–	–	+	–	+	–	+	1.953	199.2
19	–	+	+	–	–	–	–	+	–	+	–	1.941	339.7
20	–	–	–	–	–	–	–	–	–	–	–	1.974	190.1

Table 3 Effect of metal ions on growth of *Saccharomyces* species strain GVT263 and β -D-fructofuranosidase (FFase) production

Term	Growth			FFase production		
	Effect	<i>P</i>	Significance	Effect	<i>P</i>	Significance
Constant		0.000			0.000	
MgSO ₄	0.00350	0.613	*	–10.36	0.373	*
NaCl	–0.01290	0.089	*	1.14	0.919	*
KH ₂ PO ₄	–0.02390	0.007	Significant	70.10	0.000	Significant
CaCl ₂	0.00070	0.919	*	–28.66	0.031	Significant
CuSO ₄	–0.00430	0.537	*	24.09	0.059	*
KI	0.01030	0.161	*	11.22	0.336	*
FeCl ₃	0.02270	0.009	Significant	–19.76	0.109	*
MnSO ₄	–0.01190	0.112	*	26.88	0.040	Significant
Na ₂ MoO ₄	0.00490	0.483	*	–49.12	0.002	Significant
ZnSO ₄	0.00850	0.238	*	26.35	0.043	Significant
CoCl ₂	–0.03430	0.001	Significant	56.70	0.001	Significant

*Insignificant.

run 9 (Table 2). Three variables, KH₂PO₄, FeCl₃ and CoCl₂ were significantly effecting yeast growth as represented by their '*P*' values which were <0.05. FeCl₃ had a positive effect on yeast growth, while KH₂PO₄ and CoCl₂ had a negative effect indicating their inhibitory effect at higher

concentration. Other variables had no significant effect on this parameter with their '*P*' values >0.05 (Table 3).

Potassium, magnesium, calcium and zinc are cationic nutrients that play essential structural and functional roles in yeast cells. Potassium is the most abundant cellular cation

in yeast, consisting 1–2% of yeast cell dry weight and is the main electrolyte essential for osmoregulation, charge balancing of macromolecules [14]. Fe^{2+} ions are used for heme and cytochromes synthesis, cobalt ions for vitamin B_{12} (cyanocobalamine). Therefore these ions are quite obvious to show their effect on yeast growth. Calcium was insignificant for yeast growth as its requirements are very low [15, 16]. Calcium antagonizes uptake of magnesium and can block essential magnesium-dependent metabolic processes, therefore high levels would be detrimental [17]. The presence of excess calcium in fermentation medium can inhibit yeast growth [18] and fermentative activity [19]. Sodium present in high concentrations may prove to be toxic to yeast, possibly by antagonizing essential potassium-dependent functions.

Effect of metal ions on enzyme production by yeast

β -D-fructofuranosidase production was found to be maximum (374.5 U/ml) when the variables were at levels indicated by run 3 and least (177.6 U/ml) in run 13 (Table 2). Analysis of the data indicates that six variables, i.e. KH_2PO_4 , CaCl_2 , MnSO_4 , Na_2MoO_4 , ZnSO_4 and CoCl_2 had significant

effect on enzyme production with ' P ' value <0.05 (Table 3). However the effect of CaCl_2 and Na_2MoO_4 was negative indicating that they had inhibitory effect at higher concentrations and should be used at the lower level (–1).

Shafiq et al. [20] studied the effect of mineral nutrients on FFase production by *S. cerevisiae* and KH_2PO_4 was identified as one of the important sources of phosphate. In the present study it was observed that this variable had significant effect on both yeast biomass formation and FFase production. Potassium acts as a major cofactor for enzymes involved in oxidative phosphorylation, protein biosynthesis and carbohydrate metabolism. Zinc is a co-factor for numerous important biosynthetic and metabolic enzymes including, various glycolytic enzymes and alcohol dehydrogenase. In addition it plays critical regulatory roles through the action of zinc finger DNA binding proteins, and affects yeast-yeast flocculation. Zinc is also known to modulate yeast stress responses, mainly due to its role as a co-factor for the antioxidant enzyme, superoxide dismutase. Furthermore, zinc ions are thought to have some effects on stability and dynamics of cell membranes, which may lead to downstream effects on cell permeability and signaling systems [21].

Hold values: NaCl : 0.005 KH_2PO_4 : 0.005 CaCl_2 : 0.005 CuSO_4 : 0.005 KI : 0.005 FeCl_3 : 0.005 MnSO_4 : 0.005 NaMo : 0.005 ZnSO_4 : 0.005

Fig. 1 Surface plot showing the interactive effect of MgSO_4 and CoCl_2 on β -D-fructofuranosidase production by *Saccharomyces* species strain GVT263.

Other variables MgSO_4 , NaCl , CuSO_4 and KI had no significant effect either on yeast growth or its FFase production (Table 3). MgSO_4 had no effect on FFase production at both the levels studied, while increasing the concentration of CoCl_2 lead to an increase in enzyme production. The interactive effect of these two variables on enzyme production is observed in Fig. 1. Their interaction indicates that MgSO_4 at its lower concentration can

have an additive effect when CoCl_2 is used at its higher concentration (Fig. 1). FFase production was more at lower concentration of CaCl_2 than its higher level. Increasing the concentration of CaCl_2 lead to decrease in enzyme production drastically which could be due to its low requirement for yeast growth and metabolism and high concentration leads to antagonistic effect [15–18]. Enzyme production increased with increase in MnSO_4 concentration

Table 4 Analysis of variance (ANOVA) for growth of *Saccharomyces* species GVT263 (A) and β -D-fructofuranosidase production (B)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
(A)						
Main effects	11	0.014023	0.014023	0.0012748	5.75	0.010
Residual error	8	0.001774	0.001774	0.0002217		
Total	19	0.015797				
(B)						
Main effects	11	69927	69927	6357.0	10.57	0.001
Residual error	8	4809	4809	601.2		
Total	19	74736				

DF: Degrees of freedom; Seq SS: Sum of squares; Adj SS: Adjusted sum of squares; Adj MS: Adjusted mean sum of squares; F: Variance ratio; P: Probability.

Hold values: MgSO_4 : 0.005 NaCl : 0.005 KH_2PO_4 : 0.005 CuSO_4 : 0.005 KI : 0.005 FeCl_3 : 0.005 NaMo : 0.005 ZnSO_4 : 0.005 CoCl_2 : 0.005

Fig. 2 Surface plot showing the interactive effect of CaCl_2 and MnSO_4 on β -D-fructofuranosidase production by *Saccharomyces* species strain GVT263.

Fig. 3 Surface plot showing the interactive effect of KH_2PO_4 and MnSO_4 on β -D-fructofuranosidase production by *Saccharomyces* species strain GVT263.

Fig. 4 Surface plot showing the interactive effect of ZnSO_4 and CoCl_2 on β -D-fructofuranosidase production by *Saccharomyces* species strain GVT263.

in a linear fashion (Fig. 2). The interaction between these two variables indicate that presence of both variables, i.e. CaCl_2 at –level (lower concentration) and MnSO_4 at +level (higher concentration) will result in higher enzyme production than when either used alone at those levels. Increasing the concentrations of KH_2PO_4 had a sharp increase in enzyme production compared to that of MnSO_4 . Enzyme production was observed to be more when these two variables were used at higher concentrations (Fig. 3) and were therefore significant for enzyme production (Table 3). CoCl_2 and ZnSO_4 also showed a similar pattern of effect on enzyme production with both variables having a individual linear effects on FFase production. However combination of both variables at +level (higher concentration) leads to higher enzyme production than both at their individual higher levels (Fig. 4). Analysis of variance (ANOVA) was highly significant for both FFase production and growth with ‘*P*’ values 0.001 and 0.01, respectively (Table 4).

Conclusion

Mineral nutrients should be given careful attention, because efficient conversion of carbon sources to desired product by fermentation depends not solely on the available fermentable carbon but also on the bioavailability of essential metal ions. The present study focused on the screening of mineral elements for FFase production. Two elements KH_2PO_4 and CoCl_2 were found to have significant affect on both yeast growth and FFase production. Optimization of the concentrations of these metal ions in fermentation medium would enable to obtain high yields of biomass and FFase enzyme.

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