Appl Biochem Biotechnol (2011) 164:1237–1245
DOI 10.1007/s12010-011-9209-z

Improvement of Ethanol Production Using *Saccharomyces cerevisiae* by Enhancement of Biomass and Nutrient Supplementation

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Received: 12 August 2010 / Accepted: 22 February 2011 /
Published online: 4 March 2011

Abstract Optimization of ethanol production through addition of substratum and protein-lipid additives was studied. Oilseed meal extract was used as protein lipid supplement, while rice husk was used as substratum. The effect of oil seed meal extract and rice husk was observed at varying concentration of medium sugar from 8% to 20%. Of the three oil seed meal extracts used, viz. groundnut, safflower, and sunflower, safflower was found to be most efficient. The use of oilseed meal extract at 4% was found to enhance ethanol production by almost 50% and enhanced sugar tolerance from 8% to 16%. A further increase of almost 48% ethanol was observed on addition of 2 g of rice husk per 100 ml of medium. An increase in cell mass with better sugar attenuation was observed. Further optimization was sought through use of sugarcane juice as the sugar source. While 8.9% ethanol yield with 75% sugar attenuation was observed at 20% sucrose concentration, it was found to increase to 12% (*v*/*v*) with almost complete utilization of medium sugar when sugarcane juice was used. Cell weight was also observed to increase by 26%.

Keywords Ethanol · Oilseed meal extract · Rice husk · Sugarcane juice · *Saccharomyces cerevisiae*

Introduction

Increased efficiency of bioethanol production from renewable resources is essential in view of the deepening energy crisis. The advent of high-gravity brewing since 1980s paved the way for higher ethanol yields in less time. It destroyed the myth that *Saccharomyces cerevisiae* can use sugar efficiently only up to 10–12% producing around 5–7% ethanol as yeast is intolerant to higher ethanol concentration. It was found that the same strain of yeast when supplemented with sugar content over 20% and higher assimilable nitrogen content, ergosterol, oleic acid,

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and oxygen can yield up to 15% (*v*/*v*) ethanol without any genetic manipulation or strain improvement [1]. Although high-gravity brewing is preferred for higher ethanol yield, reduced labor, time efficiency, and reduced contamination; stuck fermentation, excess residual sugar, and decreased cell viability due to osmostress from media solids and ethanol are limiting factors. There is a need to evolve high-gravity brewing methods which can minimize residual sugar, optimize cell growth, and yield higher ethanol [2].

A 2^3 full-factorial composite design employing inoculum size, sugar concentration, and temperature as independent variables showed increased temperature has negative effect on cell viability than ethanol production. Hence, optimal condition of 200 g/L of sucrose at 30 °C when pitched with 40 g/L of dry cell mass could yield around 80.8 g/L of ethanol within 4 h of fermentation time [3]. However, drop in viability with massive leakage of intracellular metabolites with increase in ethanol content above 10% [4] could lead to collapse of fermentation and huge economic losses. The sugar utilization is found to be better in growing cells rather than non-growing cells. The rate of attenuation is found to be slowed by as much as 33-fold when cell mass formation ceases during fermentation [5]. It would be more important to have a mechanism which would lead to new cell synthesis over a time period rather than enhanced pitching rates.

Although use of vitamins, amino acids, sterols, or yeast extract can lead to enhanced ethanol production, they also add to the cost of the process, which may not be industrially feasible. Hence, it is vital to develop high-gravity brewing using cheaper additives, agro-wastes, and agents which can enhance cell growth, viability, and demonstrate better ethanol yields. It was earlier observed that addition of substratum such as rice husk can lead to enhancement in cell mass and thereby higher ethanol yield [6]. In the present study, an attempt is made to use oilseed meal, sugarcane juice, and rice husk to generate higher ethanol yield, with better cell growth at 20% sugar concentration. A comparative study of variation in cell growth, residual sugar, and ethanol production is investigated with increase in sugar content from 8% to 20% with addition of soluble oilseed meal and rice husk.

Materials and Methods

Yeast Culture

S. cerevisiae NCIM 3494, a routine laboratory strain was obtained from National Chemical Laboratory, Pune, India. Slants were maintained on 1% glucose (*w*/*v*), 0.3% yeast extract (*w*/*v*), 0.5% peptone (*w*/*v*), and 2.5% (*w*/*v*) agar. For inoculation of the flasks a loopful of cells were transferred to 10 ml of medium containing 8% sucrose (*w*/*v*), 0.3% yeast extract (*w*/*v*), and 0.5% peptone (*w*/*v*). The inoculum was transferred to 100 ml of the medium after 24 h.

Oilseed Meal Processing

Sunflower, groundnut, and safflower oilseed meal cakes were obtained locally from local oil industry. Twenty grams of oilseed meal was dispersed in 100 ml distilled water and stirred thoroughly for 5 min at room temperature (25 °C). The solution was centrifuged at 1,500 rpm for 5 min and supernatant was added to the fermentation medium as per the experimental requirement prior to medium sterilization. The protein and lipid content of the oilseed meal extract was analyzed.

Rice Husk Processing

Rice husk (2 g) was boiled in water for 3 min and allowed to settle for 30 min. Supernatant was discarded and rice husk was air dried, weighed, and used as substratum.

Fermentation Studies

Medium (100 ml) in 250-ml Erlenmeyer flasks containing 4%, 8%, 12%, 16%, and 20% sucrose (*w*/*v*) with 0.3% yeast extract (*w*/*v*) and 0.5% peptone (*w*/*v*) were used. Six more sets of such flasks containing in addition to the above contents, 10, 15, 20, and 30 ml of 20% (*v*/*v*) soluble oilseed meal extract of sunflower, groundnut and safflower, and 2 g (*w*/*v*) of rice husk were autoclaved for 20 min. Fermentation was initiated by addition of 10% (*v*/*v*) inoculum and carried out for 48 h at 25±2 °C. Residual sugar was measured by phenol-sulphuric acid method and ethanol was measured by potassium dichromate method [7].

High-Gravity Brewing

For further optimization, 100 ml medium containing 16%, 20%, and 25% sucrose (*w*/*v*), yeast extract, peptone with increasing concentration of oilseed meal extract from 5 to 30 ml (*v*/*v*) was added. In another set sugar cane juice with 16%, 20%, and 25% sugar content (*w*/*v*) with similar additions were used. Sugarcane juice contained about 15% to 16% sugar content the remaining sugar was made up by addition of sucrose. Ethanol content was measured.

Five flasks containing 20% sucrose (*w*/*v*), with 0.3% yeast extract (*w*/*v*), 0.5% peptone (*w*/*v*), safflower oilseed meal extract 20 ml (*v*/*v*) and 2 g rice husk was inoculated simultaneously and fermentation stopped at 24, 36, 48, 72, and 86 h. Temperature was maintained at 25±2 °C .All the flasks were subjected to analysis of residual sugar content, cell weight, and ethanol produced. A similar experiment was carried out with 20% sugarcane juice (only 10 ml oilseed meal extract instead of 20 ml was used) and various parameters analyzed. The fermented broth was filtered to remove rice husk and dry cell weight was obtained by method described by Choi et al. [8]

Results

Tables 1 and 2 demonstrates the composition of the oilseed meal and the extracts used. Groundnut oilseed meal extract shows higher nitrogen and lipid content, while sunflower has the lowest. Although safflower has lower protein and lipid content as compared to groundnut oil seed meal extract, it is reported that safflower has higher content of polyunsaturated fats and the highest fiber content [9].

The growth of the yeast with use of different oilseed meal extracts and effect of rice husk was investigated (Fig. 1). The use of safflower oilseed meal extract shows the optimal effect on the cell growth. A further 20–25% increase in the cell weight is observed on addition of rice husk at 20% sugar content. The control cells show enhancement in cell growth up to 8% sugar, beyond which there is a gradual decrease in cell growth. In the presence of oilseed meal extract, the sugar tolerance is seen to increase up to 16% sugar content in all the three oil seed meals and no further increase is observed up to 20% sugar content. While the cells grown with oilseed meal and rice husk show a similar picture but a higher yield in cell growth. In spite of higher protein and lipid content, the addition of groundnut meal extract does not show increase in cell weight beyond 12% sugar content with very little rise up to 16%.

Table 1 Nutritional values of three oil seed meals

	Component	Groundnut	Sunflower	Safflower
1	Nitrogen	6.4	4.5	5.7
2	Oil	3.82	0.97	1.56
3	Fiber	7.88	23.88	56
4	Organic carbon	55.2	54.3	51.4
5	Ash	0.035	0.054	0.024

All components are measured as percent value

Utilization of sugar from the medium is another essential parameter to be observed in fermentation. It can be seen from Fig. 2 that there is good utilization of sugar (around 65%) in cells grown with oilseed meal till 16% sugar content thereafter residual sugar suddenly shoots up to 50–60%. With addition of rice husk, there is excellent attenuation with almost 80–82% sugar utilization even at 20% sugar content for groundnut and safflower. The control cells do not show complete sugar utilization even at 8% sugar content. They can show total sugar utilization only up to 4% sugar content (results not shown). As compared to groundnut and safflower, addition of sunflower oilseed meal extract showed less sugar utilization similar to the control cells. However, on addition of rice husk the sugar utilization was found to improve.

Figure 3 shows the variation in ethanol content with increasing sugar content. Again while the control cells do not show any significant increase in alcohol content with increased media sugar concentration beyond 8% sugar concentration, cells grown with oilseed meal extract show almost 43% enhancement in ethanol yield. However, it is to be observed that the major enhancement in ethanol yield is up to 16% sugar and beyond that there is minimal increase. The addition of rice husk shows a further 33% enhancement in ethanol yield with a continuous increase in ethanol content even up to 20% sugar. The safflower meal was found better additive than the other two oilseed meals, as it gave better ethanol and cell yield and demonstrated better sugar utilization.

Further optimization with varying concentration of oilseed meal using sucrose and sugar cane juice revealed that while 4% oil seed meal extract was needed to yield optimum ethanol with sucrose as the sugar source, while 2% was sufficient for sugar cane juice (Table 3). It can be observed from Table 3 that maximum optimization is possible up to 20% sugar concentration. However, a 46% enhancement in the ethanol yield was observed on using sugar cane juice.

It can be seen from Fig. 4 that optimum ethanol yield is obtained by 48 h of fermentation. Almost 50% sugar is utilized by 30 h of fermentation while a maximum of almost 75% of sugar is totally utilized with 25% remaining as residual sugar when sucrose is used as the sugar source. However, in case of sugarcane juice (Fig. 5) it can be observed that 50% sugar is utilized by 24 h and by 36 h almost total sugar is utilized. The ethanol yield is higher by 46% with sugarcane juice as compared to when sucrose was used as a sugar source. The cell weight is also higher (4.8 g) as compared to 3.8 g with sucrose as the sugar source.

Table 2 Protein and lipid content of oilseed meal extracts used

Oil seed meal type	Protein (mg/g)	Lipid (mg/g)
Groundnut	54±2.1	42.4±1.3
Sunflower	36±1.8	12.9±0.5
Safflower	44±1.9	18.56±1.8

Protein and lipid content estimated from solubilized extract of 1 g oilseed meal (n=3)

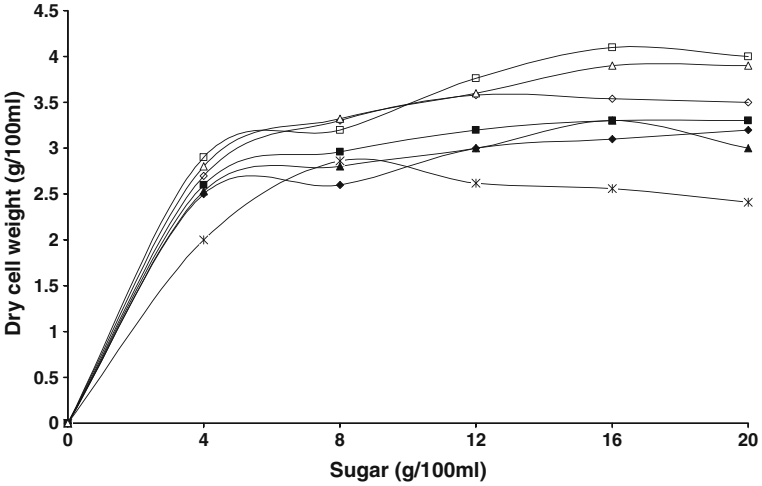

Fig. 1 Variation in cell weight with increasing media sugar in control (*asterisk*) cells and cells grown in oil seed meal extract of groundnut (*black diamond*), sunflower (*black triangle*), safflower (*black square*), and with groundnut+rice husk (*white diamond*), sunflower+rice husk (*white triangle*) and safflower+rice husk (*white square*)

Discussion

Nutrient supplementation with protein in the form of wheat mash [10] or soy flour [11] has shown enhancement in ethanol yield. Lipid and fatty acid additions are also known to increase fermentation performance [12].

Apart from nutrient supplementation, many osmoprotective agents have also demonstrated better fermentation output. The use of glycine in the fermentation medium has demonstrated improved ethanol yield and yeast growth [13]. Likewise, use of horse gram

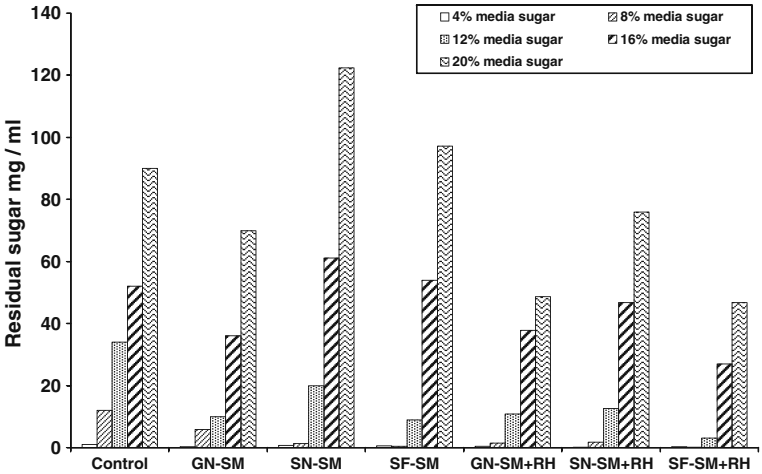

Fig. 2 Amount of residual sugar in medium in control and with meal extract of groundnut (*GN*), sunflower (*SN*), safflower (*SF*), and with groundnut+rice husk (*GN+RH*), sunflower+rice husk (*SN+RH*) and safflower+rice husk (*SF+RH*)

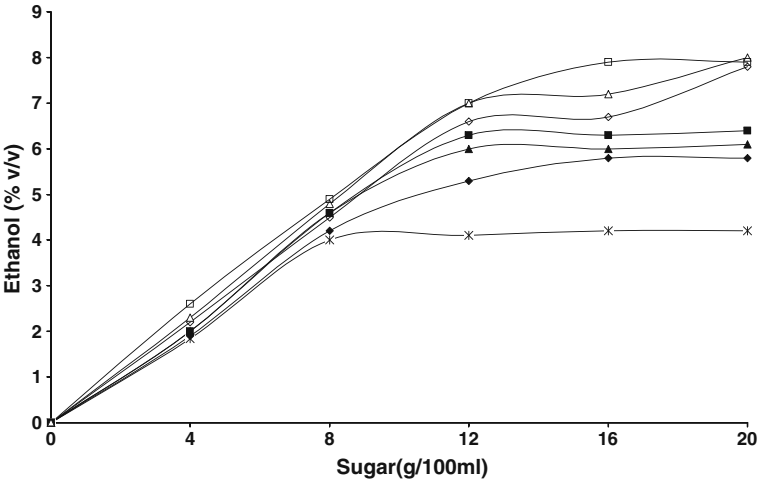

Fig. 3 Ethanol (percentage of *v*/*v*) produced in medium in control (*asterisk*) cells and cells grown in oil seed meal extract of groundnut (*black diamond*), sunflower (*black triangle*), safflower (*black square*), and with groundnut+rice husk (*white diamond*), sunflower+rice husk (*white triangle*) and safflower+rice husk (*white square*)

flour has shown improved cell viability and better ethanol tolerance and acts as a protectant [14]. A combined supplement of 12 g yeast extract, 0.3 g cell walls, 3 g glycine, 20 g soy flour with 300 g sugar/L yielded 103 g/L ethanol in 28 h of fermentation [15].

Although use of amino acids and sterols have demonstrated higher ethanol yield, at the industrial level the cost of the raw material is inhibitory. The use of the oil industry by-product—oilseed meal is found to provide the nitrogen and lipid supplement. Although it can be seen from Table 1 that groundnut oilseed meal extract shows the highest content of protein and lipid and is a preferred as a cattle feed, the ethanol yield is higher with use of safflower oilseed meal extract. Moreover, safflower oilseed meal is not useful as cattle feed owing to its high fiber content [16]; hence it can be optimally used for ethanol fermentation. Safflower is also known to possess higher levels of

Table 3 Ethanol produced with sucrose and sugarcane juice as source of sugar and various concentration of solubilized safflower oilseed meal extract

Media sugar	Ethanol produced (% *v*/*v*)[a]				
	5 ml SF	10 ml SF	15 ml SF	20 ml SF	30 ml SF
16% sucrose	7	7.4	7.8	8.6	8.6
20% sucrose	7.3	7.5	7.9	8.8	8.7
25% sucrose	7.7	7.9	8.3	8.8	8.8
16% cane juice	9.6	10	10	10	10
20% cane juice	11.6	12	12	12	12
25% cane juice	11.6	12	12	12	12

SF solubilized safflower oilseed meal extract (*v*/*v*)

[a] 2% rice husk was added in all the flasks

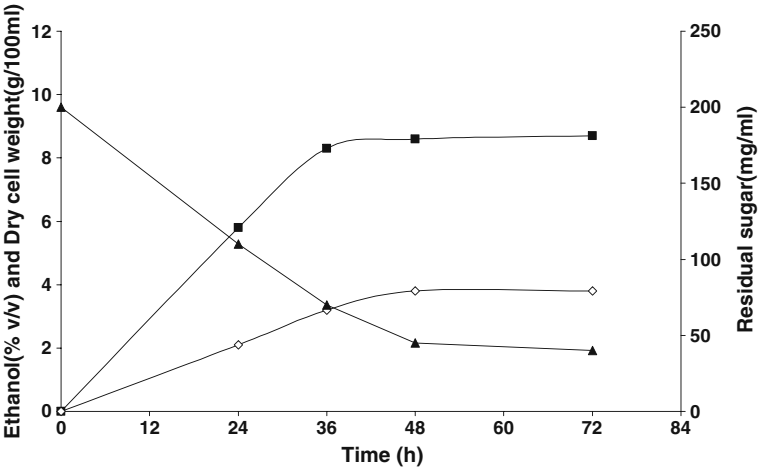

Fig. 4 Fermentation parameter viz. ethanol yield (*black square*), cell weight (*white diamond*), and residual sugar (*black triangle*) with increasing time in 20% sucrose, 4% safflower oilseed meal extract and 2% rice husk

polyunsaturated fats [9]. The use of unsaturated fatty acids and oleic acid is shown to reduce the ethanol stress [17, 18] which may also be the reason for higher ethanol yield. The nitrogen and lipid supplement from oilseed meal is found to enhance ethanol yield by 50%. Based on our earlier observation [6], the addition of rice husk which acts as a substratum can further enhance ethanol yield by almost 45%. A similar observation in enhancement of ethanol yield by addition of hot trub which is particulate in nature is also made [19]. The use of direct oilseed meal extract instead of the solubilized portion can also be made giving similar results to addition of rice husk. However as we have observed that the cells adhere to the substratum during the fermentation period, it was difficult to separate the cells from the oil seed meal due to its small particulate size, we

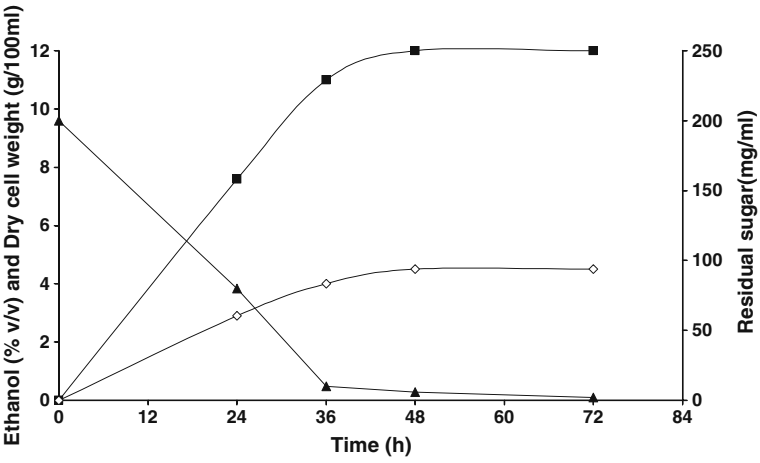

Fig. 5 Fermentation parameter viz. ethanol yield (*black square*), cell weight (*white diamond*), and residual sugar (*black triangle*) with increasing time 20% sugarcane juice, 2% safflower oilseed meal extract and 2% rice husk

have used rice husk as it is easy to separate it after fermentation to account for the cell weight.

Since oilseed meal extract addition showed good attenuation of sugar even at 16% further optimization was attempted by varying the content. The use of sugarcane juice, the raw material for sucrose was also studied. The use of 15% sugarcane juice with yeast immobilized on sugarcane pieces has been shown to yield 8.9% ethanol [20]. It was observed that sugarcane juice yielded almost 46% higher ethanol content in spite of having the same sugar content as sucrose implying that there must be certain micro nutrients present in the cane juice that may contribute to this effect. It was found that while sucrose medium needed 4% oilseed meal to give optimum ethanol, sugarcane juice needed just 2%. Furthermore, the addition of oilseed meal extract and rice husk to sugarcane juice showed almost complete utilization of sugar by 36 h and optimum ethanol production by 48 h. It also needs to be mentioned that the present study was carried out using a regular laboratory strain of *S. cerevisiae*. Use of a brewery distillery strain with higher ethanol and sugar tolerance can yield even better results. In a contrary report, 12% ethanol yield was obtained from sugarcane juice adjusted to 28% sugar concentration with sucrose, 0.5% peptone and 0.3% yeast extract in 60 h [21]. However, we have observed that this enhancement can be obtained only with protein lipid and substratum addition even at 20% sugar concentration. In the absence of protein lipid and substratum additives, we had observed about 5.8% ethanol (results not shown).

In conclusion, as can be observed from Table 4, the same yeast strain which can normally tolerate up to 8% sugar content yielding 4.2% ethanol can produce 6.4% ethanol on addition of 4% oilseed meal. Further addition of rice husk that does not contribute in any way as a nutrient source show almost doubles the ethanol yield. It is likely that the addition of the substratum may lead to reduction in stress which may contribute to such a phenomenon. Interestingly, using sugarcane juice as the medium the same yeast strain can produce 7.2% ethanol on addition of just 2% soluble oil meal extract and further almost three times the volume of ethanol on addition of rice husk. Furthermore, it is essential to study exactly which component from sugarcane juice demonstrates this effect. Thus addition of a substratum and protein and lipid supplements, enhances the ethanol yield and improves both the sugar tolerance and ethanol tolerance of the yeast strain. This was achieved using cheap agro by-products such as oilseed cake and rice husk that would be industrially beneficial in reducing the cost of ethanol production.

Table 4 Ethanol produced in 20% sucrose and 20% sugar cane juice media with different supplements

	Ethanol produced (% *v*/*v*)	
	20% sucrose[a]	20% sugar cane juice[b]
A	4.2	4.2
B	6.4	10
C	8.8	12

A control (no addition of safflower meal and rice husk), *B* control+safflower oilseed meal extract, *C* control+safflower oilseed meal extract+rice husk

[a] 4% solubilized meal extract

[b] 2% solubilized meal extract

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