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Full Length Research Paper

Improvement of ethanol production from sugarcane molasses through enhanced nutrient supplementation using *Saccharomyces cerevisiae*

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***Saccharomyces cerevisiae* as a yeast cream was utilized for alcoholic fermentation using sugar cane molasses. In the present study, fermentation was optimized for urea and yeast hydrolysate (YH) dosage and the combined effect was evaluated. Total sugars as inverts (TSAI) composition of molasses were determined by HPLC as 39% (m/v). Urea concentrations of 4, 2 and 3 gl^{-1} showed optimal ethanol production at 30, 35 and 40°C respectively. A YH concentration of 0.5 gl^{-1} resulted in an ethanol yield of 8.7% (m/v) with a fermentation efficiency of 85.12%. Under optimized conditions (35°C) significant improvements were noticed with ethanol yield of 7.8% (m/v) and efficiency of 76.3%.**

Key words: Ethanol, ethanologenic, fermentation, molasses, *Saccharomyces cerevisiae*.

INTRODUCTION

Yeast alcohol is the most valuable product for the biotechnology industry with respect to both value and revenue. Approximately 80% of ethanol is produced by anaerobic fermentation of various sugar sources by *Saccharomyces cerevisiae.* Yeast alcohol technology has resulted in vast improvements during the last decade but profit margins were narrowed. Contamination, limited availability of raw materials and fermentation process design are the major limitations causing reduced alcohol yields and quality. In view of the importance of alcohol as an alternative for liquid fuel, several investigations in ethanol fermentations are currently reported. The price of the sugar source is an important parameter when considering the overall economy of production and it is of great interests to optimize alcohol yields to ensure an efficient utilization of carbon sources (Bai et al., 2008; Carlos et al., 2011).

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Another crucial factor in fermentation is selecting potent microorganisms with the most commonly used microorganisms being yeasts, which can produce ethanol concentrations as high as 18% of the fermentation broth (Balat et al., 2008). Among the yeasts, *S. cerevisiae* still remains the prime species for ethanol production. Previous published reports showed that the ethanol tolerance and sugar utilization efficiency of yeast may be improved by altering the nitrogen sources in fermentation medium (Thomas et al., 1996; Yalçin and Özbas, 2004). Therefore strong economic incentives can be revealed by improving production processes resulting in a substantial growth for the ethanol industry in the near future (Carlos et al., 2011).

Recent studies have focused mainly on the genetic modification of *S. cerevisiae* to improve ethanol yields and efficient bioconversion of various substrates to alcohol (Cao et al., 1996) and are limited to agave bagasses with enzymatic hydrolysis, utilizing magnetically fluidized bed reactor with immobilized cells and fermentation of molasses by *Zymomonas mobilis*

(Herna´ndez-Salas et al., 2009; Chun-Zhao et al., 2009) but there is lack of nutrient supplementation approach towards improved ethanol production. Thus, the present study was carried out to improve the ethanol yield per ton of molasses by optimizing the temperature and nitrogen sources leading to improved fermentation efficiency at one of the largest alcohol plants in South Africa.

MATERIALS AND METHODS

Feedstock collection and characterization

Molasses samples were obtained from Illovo Sugar, Mere bank, Durban, South Africa and stored at -4°C until use.

Quantification of sugars in molasses

Sugars (sucrose, glucose, fructose) were quantified by HPLC (Varian 3400) provided with a Refractive Index (RI) detector at 40°C and an NH_2 column at 90°C with flow rate of 0.5 ml min^{-1}.

Microorganism and maintenance

S. cerevisiae, an industrial strain provided by Illovo Sugar Ltd, Merebank (Durban, South Africa) was used throughout this investigation. The culture was obtained in the form of yeast cream and it was stored at -4°C.

Preparation of yeast hydrolysate

Spent yeast cream 14% (w/v) and ethyl acetate (1.5% w/v) was added to the yeast suspension and the pH was adjusted to 5.5. A yeast suspension (250 ml) was transferred to an Erlenmeyer flask and allowed to autolyse at 48°C for 24 h in a rotary shaker at 150 rpm. The autolysate was then heated at 85°C for 30 min to remove ethyl acetate and ethanol and was centrifuged at 5000*g* for 15 min. The pellet was washed with de-ionized water, vigorously stirred and further centrifuged at 5000*g* for 15 min. The two supernatants were freeze-dried for 48 h in Lyo-San unit (Lachute, Canada). The autolysis yield was then calculated as the fraction of solids recovered from the initial yeast solid. The total nitrogen content in the freeze dried yeast hydrolysates was quantified by the Kjeldahl method (Vickery, 1946).

Optimization of urea yeast and hydrolysate dosage at various temperatures

Based on the predetermined total sugar as invert (TSAI) content of molasses, it was diluted accordingly to meet a sugar concentration of 153 gl^{-1} urea (40% m/v) and yeast hydrolysate solution was dispensed into the molasses solutions to give the required concentrations (0.5; 1; 2; 3; 4 and 5 gl^{-1}), molasses without urea and yeast hydrolysate served as the control and was incubated in a shaker at 250 rpm at 30, 35 and 40°C, respectively (Bafrncová et al., 1999). Samples were removed at 3 h intervals and analyzed for growth, sugar consumption and ethanol production using a spectrophotometer, HPLC and GC, respectively as described subsequently.

Combined effect of urea and yeast hydrolysate on ethanol production

The dilute molasses medium was prepared as described earlier. It was supplemented with both urea and yeast hydrolysate (YH) at different concentrations (0.5; 1; 2; 3; 4 gml^{-1} for urea and gml^{-1} for YH). Molasses medium without urea and yeast hydrolysate served as controls. Samples were removed at 3 h intervals and analyzed for growth, sugar consumption and ethanol production using a spectrophotometer, HPLC and GC, respectively as described subsequently.

Analytical methods

Gas chromatography

The alcohol produced after fermentation was quantified by a GC (Varian 3400) with an FID detector at 250°C (Column type: 15QC 2.5/BP 30-0.25; Injector temperature: 230°C; Column temperature: 80°C; Flow rate: 10 $mlmin^{-1}$).

Quantitative analysis of residual sugars

Residual sugars were quantified by HPLC as previously stated and the overall ethanol yield was calculated as follows:

Ethanol yield (%) = Concentration of ethanol produced/ Initial concentration of sugar × 1/0.51 × 100

Where, 0.51 indicates the theoretical ethanol yield (0.51 g ethanol /g hexose)

RESULTS AND DISCUSSION

Quantification of molasses and yeast hydrolysate

The TSAI composition of molasses was 39% (m/v) comprised mainly of sucrose (22%), glucose (12%) and fructose (5%). Due to the low malleability and high osmotic pressures of molasses, microbial growth was absent in the undiluted form. The yeast autolysate powder contained 64% total nitrogen and 11% amino nitrogen.

Optimization of the dosage of urea at different temperatures

The fermentation performance of molasses supplemented with various urea concentrations at the various temperatures revealed that a urea concentration of 4 gl^{-1} gave maximum fermentation efficiency of 85.12% and ethanol yield of 8.7% m/v at 35°C at 30°C, lower fermentation efficiency was (78.07%) and ethanol yields (7.9% m/v) were achieved, while at 40°C, it was 64.57 and 6.6% m/v, respectively. On the other hand a YH concentration of 0.5 gl^{-1} gave a maximum fermentation efficiency of 85.12% and ethanol yield of 8.7% (m/v) at35°C. Subsequently, the fermentation efficiency (79.25

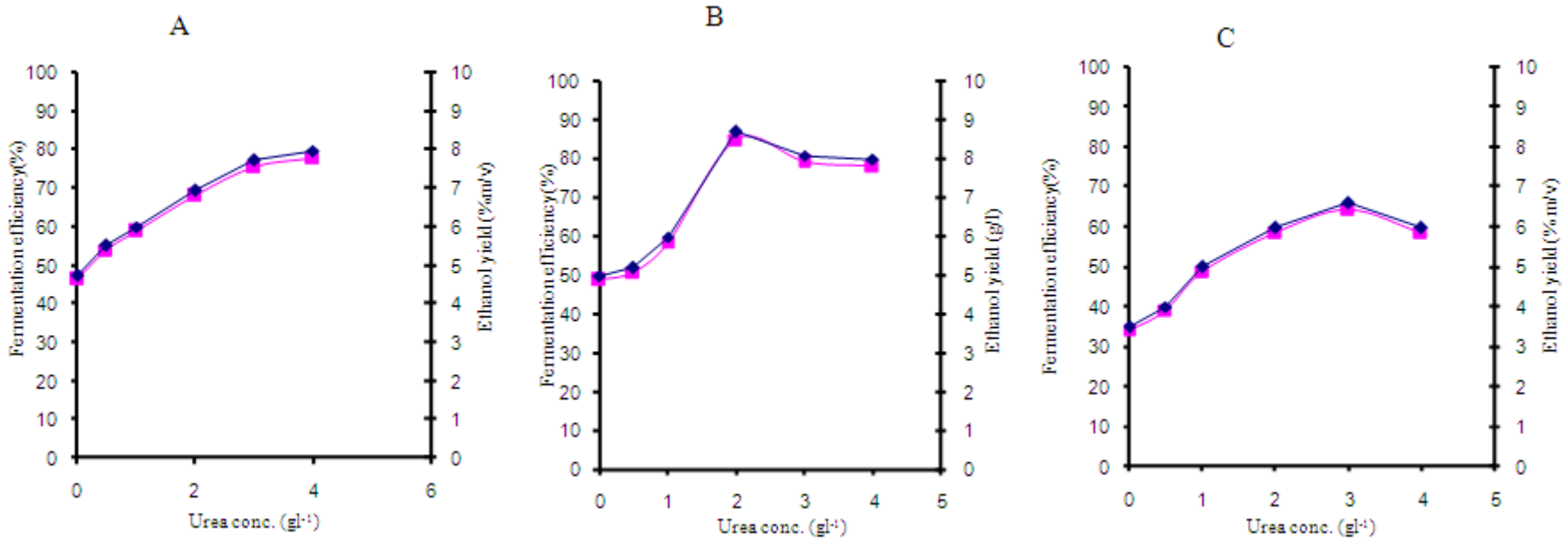

Figure 1. (A-C) Graphical representation of ethanol yield (gl^{-1}) (♦) and % fermentation efficiency (■) on molasses medium supplemented with urea concentrations between 0-4 gl^{-1} at 30°C (A), 35°C (B) and 40°C (C).

and 68.49%) and ethanol yields (8.1 and 7%; m/v) at 30 and 40°C were achieved (Figures 1 and 2). The aforestated results are similar to the findings of Bafrncová et al. (1999), wherein nitrogen was imperative for growth, ethanol tolerance and ethanol productivity of yeasts. Since urea is widely used as a nitrogen source for ethanol fermentation, therefore the effects of urea dosage were examined.

Effect of urea concentration on ethanol fermentation at different temperatures

After 30 h fermentation on a dilute molasses medium supplemented with urea at concentrations between 0 to 4 gl^{-1} at 30°C, the final ethanol yields and fermentation efficiencies are 7.9% (m/v) and 78.07% respectively (Figure 1A). The fermentation efficiencies were calculated on the basis of the ratio of the ethanol yield achieved to the maximum achievable yield. The results indicated a linear relationship between urea concentration and an ethanol yield of between 0 and 3 gl^{-1} urea; therefore ethanol yields did not increase at urea concentrations higher than 4 gl^{-1}. Hence, a concentration of 3 gl^{-1} urea was found to be the optimal for ethanol production. At this concentration, only 2.4% (m/v) of sugar was left unfermented (Table 1) and a 7.7% (m/v) final ethanol yield was achieved representing 76% of the apparent theoretical maximum (Gough et al., 1996). However, a urea dosage concentration showed no significant ($p \geq 0.05$) effect on average growth rate and doubling time (Table 1).

Fermentations at high temperatures (>30°C) results in sluggish and stuck fermentations, however the average final ethanol yield increased from 6 to 7% m/v at 35°C (Figure 1B) which supports some related findings (Yalçin and Özbas, 2004; Dhaliwal et al., 2011).

Supplementation with urea at 2 gl^{-1} gave the best ethanol yield (8.7% m/v and 85% fermentation efficiency); a relatively high growth rate (7 h^{-1}) and low doubling time after 1 h

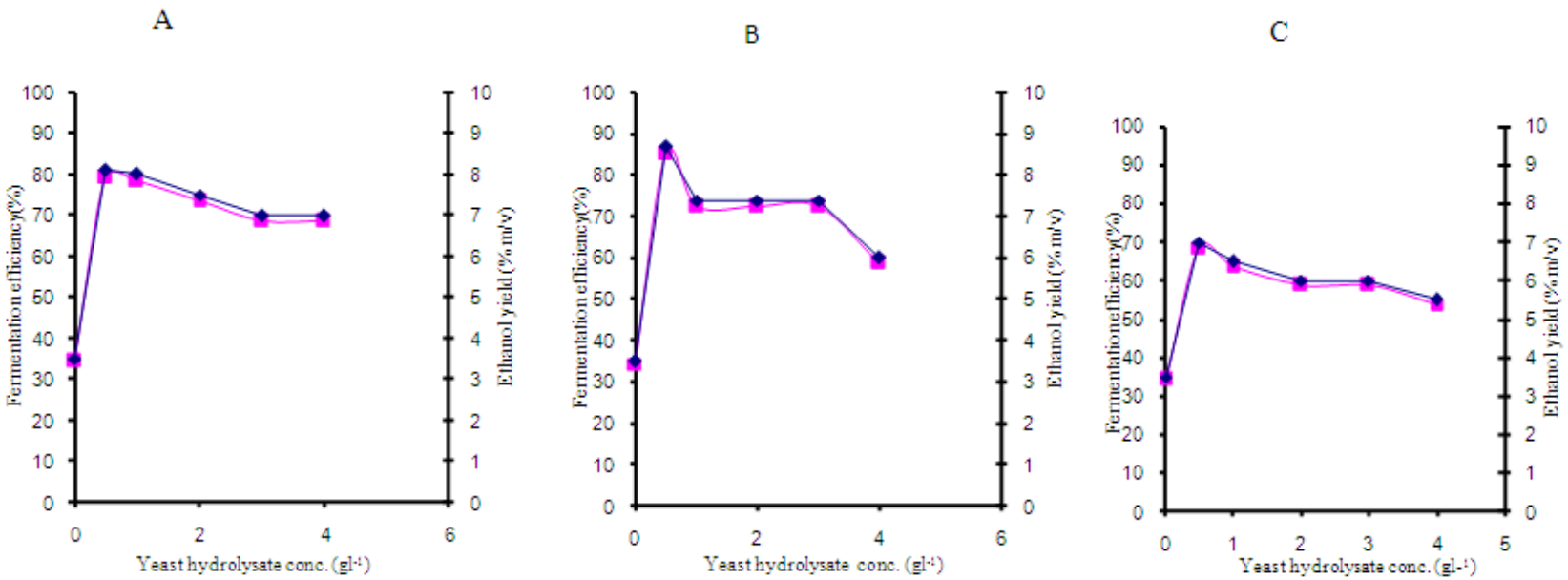

Figure 2. (A-C) Graphical representation of ethanol yield (gl^{-1}) (♦) and fermentation efficiency (■) on molasses medium supplemented with yeast hydrolysate concentrations between 0 to 4 gl^{-1} at 30°C (A), 35°C (B) and 40°C (C).

(Figure 1B and Table 1). The inhibitory effects of increased temperature became more pronounced when the fermentation was run at 40°C. The overall fermentation efficiency decreased to 64.57% from that obtained at 30°C (78.07%). Although this study did not monitor the viability of the yeast cell populations, this decrease in fermentation performance may be attributed to the inhibition of yeast growth and increased the loss of yeast cell viability which occur under conditions of alcoholic and osmotic stress. Although, it is not completely effective, supplementation with 3 gl^{-1} urea improves fermentation efficiency from 34 to 65%

Effect of yeast hydrolysate concentration on ethanol fermentation

The effects of yeast hydrolysate supplementa-tion on growth and ethanologenic fermentation per-formance were studied (Figures 2A-C and Table 2). The results obtained from this study conducted at 30°C showed that 0.5 gl^{-1} concentration of yeast hydrolysate to be ideal (Figure 2A). It was noted that further increase in the hydrolysate concentration to 1 gl^{-1} resulted in inhibition of the ethanol yield (Figure 2) and an increase in growth (Table 2). This was highly contradictory to the findings of (Sato et al., 1992) where they reported the effect of yeast extract and vitamin B12 on ethanol production by *Clostridium thermocellum* on 10% optimum yeast extract concentration.

Combined effects of yeast hydrolysate and urea concentration on growth

A combination of 2 gl^{-1} urea and 0.5 gl^{-1} yeast hydrolysate supplementation were evaluated for its effect on growth and ethanol production performance as presented in Figure 3. The resultant fermentation efficiencies of 73.4% at 30°C, 76.3% at 35°C, 68.5% at 40°C and

Table 1. The effect of urea concentration on growth of *Saccharomyces cerevisiae* and sugar conversion at different temperatures.

Urea concentration (g ml^{-1})	Residual sugar after 30 h (% m/v)			Overall growth rate (h^{-1})			Doubling time (h)		
	Temperature (°C)								
	30	35	40	30	35	40	30	35	40
0	7	6.2	11.2	0.4	0.5	0.2	2	1	3
0.5	5	4.8	10.2	0.6	0.7	0.3	1	1	2
1	4	4.2	8.2	0.6	0.7	0.1	1	1	1
2	2.2	2.9	6.2	0.7	0.8	0.3	1	1	1
3	2.4	2.1	6.4	0.7	0.8	0.4	1	1	1
4	2	2.3	6.1	0.7	0.7	0.4	1	1	1

Table 2. The effect of YH concentration on growth of *Saccharomyces cerevisiae* and sugar conversion at different temperatures.

YH concentration (g l^{-1})	Residual sugar after 30 h (% m/v)			Overall growth rate (h^{-1})			Doubling time(h)		
	Temperature (°C)								
	30	35	40	30	35	40	30	35	40
0	7	6	5	0.5	0.5	0.2	1	1	4
0.5	4	4	5	0.7	0.9	0.5	1	1	2
1	4	5	7	0.2	0.3	0.1	3	2	7
2	5	6	8	0.2	0.3	0.1	3	2	7
3	5	6	8	0.2	0.3	0.1	3	2	7
4	6	8	9	0.1	0.1	0.1	6	6	7

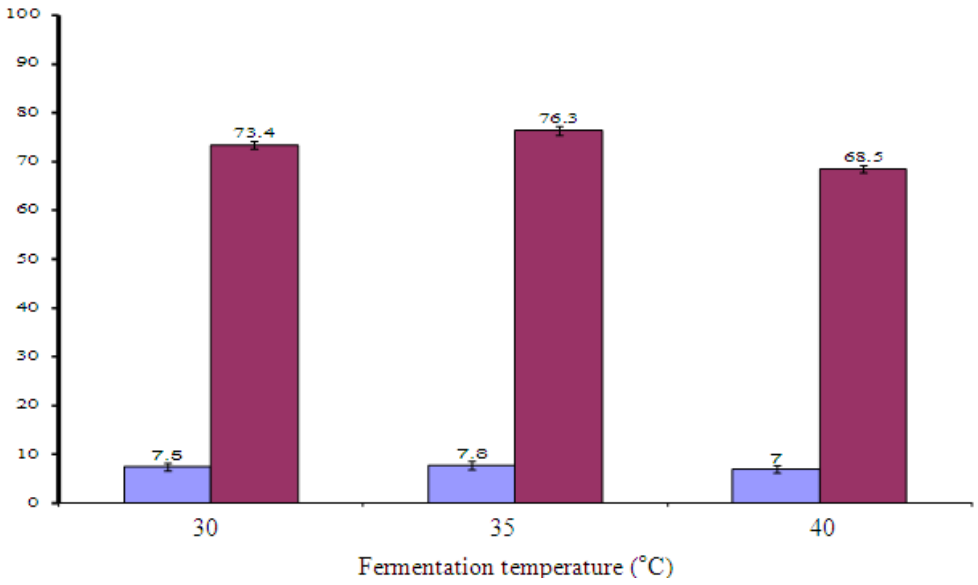

Figure 3. Graphical representation of ethanol yield (g/l) (♦) and fermentation efficiency (■) during shake flask at 30°C (A), 35°C (B) and 40°C (C).

respective ethanol yield (% m/v) of 7.5, 7.8 and 7 were achieved respectively showing clear augmentation. As compared to the fermentation with urea alone, the average ethanol yields are improved significantly (Figure 3). The potential for nitrogen feed rate adjustment to counteract the negative impacts of temperature variation on ethanol productivity in the ethanol fermentation industry was investigated in the present work. Sugars to

ethanol conversion efficiencies as defined by distilleries include values in the range higher than 95% of the theoretical maximum (Kadam and Newman, 1997).

In the present study, we have evaluated the strategies of nutrient supplementation and it was found that all supplementation strategies could be used for enhancing the yield and fermentation efficiency. From our studies, it was clearly demonstrated that switching the nitrogen feed (urea) rate could be an effective method for economical ethanol production when environmental conditions are elevated to inhibitory temperature during fermentation. It was shown that higher levels of urea in the molasses medium, could result in much higher ethanol yields than the urea feed currently applied in industry (0.5 gl^{-1}). In the fermentation trial conducted at 40°C, it was revealed that although this temperature inhibited the overall ethanol yields compared to lower temperatures, the supplementation of the molasses medium with 3 gl^{-1} urea resulted in a fermentation efficiency of 65% which was 30% higher than at urea concentration of 0.5 gl^{-1}. This helped to promote ethanol production significantly (Figures 1 and 2) and was more beneficial for growth of *S. cerevisiae*.

Although some sugars would have been utilized during such studies for biomass production, a 100% efficiency would never be achieved (Gough et al., 1996). It can be noted that none of urea concentrations examined in the present study resulted in the latter, which may be due to the fact that other limiting factors (waste accumulation) exist under laboratory scale. Unlike fermentation at 30°C, urea concentrations higher than 2 gl^{-1} may have inhibited fermentation performance. Most of the industrial applications of this yeast rely on its ability to efficiently ferment sugar into ethanol even under aerobic conditions and this has developed several sensing and signaling mechanisms to repress alternative carbon source utilization favoring the production of ethanol (Badotti et al., 2008).

Previously, ethanol tolerance was thought to be independent of the nutritional conditions but now it is known that there is possibility to increase ethanol yield and survival of yeast at high concentrations of ethanol by altering nutritional conditions (Casey et al., 1983).

The present study has focused on some key factors leading to higher ethanol productivity of ethanol-tolerant strains of *S. cerevisiae* per fermentation run that were not assessed in combination. Recent reports on *S. cerevisiae* and *Z. mobilis* with starchy feedstock's (Bai et al., 2008) and their attenuation was also noteworthy along with the zinc supplementation to the industrial yeast (Byung and Rex, 2007; Zhaoa et al., 2009). There are several reports on fermentation of molasses (Herna´ndez-Salas et al., 2009; Chun-Zhao et al., 2009; Xin-Qing et al., 2011; Dhaliwal et al., 2011) but there is fewer reports about integration towards nutritional supplementation as reported in this study. Our findings will certainly affect commercial distilleries positively, where there is a major focus for reducing significant temperature variations on the economics of the process, but it depends on further feasibility and cost analysis studies.

Our emphasis here was on the improvement of nutritional parameters for higher better yield which can be further optimized for economic feasibility. This was a preliminary study to report on the investigation of nutritional supplementation as a possible method to enhance industrial ethanol production efficiency during fermentation of indigenous molasses in South Africa.

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