S. cerevisiae fermentation activity after moderate pulsed electric field pre-treatments

Jessy R. Mattar a,b,⁎, Mohammad F. Turk c, Maurice Nonu a, Nikolai I. Lebovka a,d, Henri El Zakhem b, Eugene Vorobiev a,b

a Équipe TAI EA TIMR 4297, Université de Technologie de Compiègne, Centre de Recherche de Royallieu BP 20529-60205 Compiègne Cedex, France
b Chemical Engineering Department, University of Balamand, Amioun, Lebanon
c Équipe TAI EA TIMR 4297, École Supérieure de Chimie Organique et Minérale, 1 allée du réseau Jean-Marie Backmaster 60200 Compiègne Cedex, France
d Department of Physical Chemistry of Disperse Minerals, Institute of Biocolloidal Chemistry, NAS of Ukraine, 42, Blvr. Vernadskogo, Kyiv 03142, Ukraine

⁎ Corresponding author at: Équipe TAI Laboratoire TIMR 4297, Université de Technologie de Compiègne, Centre de Recherche de Royallieu, BP 20529-60205 Compiègne Cedex, France. Tel.: +33 648110611.
E-mail address: jessy.mattar@gmail.com (J.R. Mattar).

Abstract

The batch fermentation process, inoculated by Pulsed Electric Field (PEF) treated wine yeasts (Saccharomyces cerevisiae Actillore F33), was studied. PEF treatment was applied to the aqueous yeast suspensions ([Y] = 0.012 g/L) at the electric field strengths of E = 100 and 6000 V/cm using the same treatment protocol (number of pulses n = 1000, pulse duration t = 100 μs, and pulse repetition time Δt = 100 ms). Electrical conductivity was increasing during and after the PEF treatment, which reflected cell electroporation. Then, fermentation was run for 150 h in an incubator (30 °C) with synchronous agitation. Electro-stimulation was revealing itself by the improvement of fermentation characteristics, and thus increased yeast metabolism. At the end of the lag phase (t = 40 h), fructose consumption in samples with electrically activated inoculum exceeded that of the control samples by ≈2.33 times for E = 100 V/cm and by ≈3.98 for E = 6000 V/cm. At the end of the log phase (120 h of fermentation), ≈30% mass reduction was reached in samples with PEF-treated inocula (E = 6000 V/cm), whereas the same mass reduction of the control sample required approximately 20 extra hours of fermentation.

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1. Introduction

The fermentation of yeasts, stabilization of their multiplication and enhancement of the process productivity are industrially important [1]. Recently, various emerging technologies were demonstrated to have a stimulating effect on the microbial flora multiplication process. E.g., it was shown that continuously supplied low-power ultrasound (3W) has a stimulating effect on the microbial flora multiplication process. Moreover, electrical stimulation resulted in the improvement of fermentation characteristics, and thus increased yeast metabolism. Application of pulsed electric fields with restrictions of electric field strength and treatment time within certain reasonable ranges allowed preservation of the functionality of cell membranes [4]. PEF techniques have been known to facilitate the efficient transformation of cells [5,6], their fusion (electrofusion) [7], transport of plasmid DNA [8], destruction of the bacterium [9] and electrostimulation of plant protoplast division [10]. The stimulation of living cells under the impact of PEF treatment has recently attracted great attention. For instance, a stress response analysis of S. cerevisiae has shown that PEF-induced expression of the oxidation genes and glutathione played an important role in the stress resistance [11]. The electrical stimulation was verified in order to alter the S. cerevisiae culture cycles and to promote synchrony in division of cells [12]. Moreover, electrical stimulation resulted in smaller size of yeast cell populations. Application of continuous direct current (DC) or alternating current (AC) treatments to a culture broth after inoculation of yeast suspension allowed significant increase in the cell growth and alcohol production rates [13]. The positive role of electrical current (alternating current or pulsed direct current) as a tool for stimulation of microbe reactions (i.e., fermentation) was also reported [14]. However, the effects of PEF treatment on microbial activity, metabolism, and microbe reactions, practically, were not yet studied.

http://dx.doi.org/10.1016/j.bioelechem.2014.08.016
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Please cite this article as: J.R. Mattar, et al., S. cerevisiae fermentation activity after moderate pulsed electric field pre-treatments, Bioelectrochemistry (2014), http://dx.doi.org/10.1016/j.bioelechem.2014.08.016
The main purpose of the present work was to investigate PEF-induced effects on S. cerevisiae growth in synthetic media. PEF pre-treatment was applied to aqueous suspensions of the cells of wine yeast S. cerevisiae. The electric field strengths were \( E = 100 \text{ V/cm} \) and \( 6000 \text{ V/cm} \), the pulse duration was \( \tau_\text{p} = 100 \text{ μs} \), and the number of pulses was \( n = 1000 \). The conditions of inoculation, incubation, and harvesting of cell populations were carefully kept constant in order to obtain identical fermentation properties. Comparative studies of the kinetics of fermentation were carried out in order to characterize the PEF-induced stress responses of S. cerevisiae.

2. Material and methods

2.1. Preparation of yeast suspensions

The wine yeasts, S. cerevisiae, strain Actifiore F33 (Laffort, Bordeaux, France), were used throughout this study. The industrial dry powder (rod-shaped particles [15]) was mixed with distilled water with initial electrical conductivity of 4 \( \mu \text{s/cm} \) at \( 25 \text{ °C} \), the concentration of yeasts was \( |Y| = 0.012 \text{ g/L} \). The yeast suspension was subjected to vortexing (for 2 min, rotation speed 150 rpm and amplitude 4.5 mm) using Top Mix (Bioblock Scientific, Germany). Note that more vigorous mixing can lead to a drop in viability of cells due to their breakage, therefore it was avoided. The initial suspension conductivity after the vortexing was \( \sigma = 18 \pm 3 \mu \text{s/cm} \). Then the suspension was gently agitated (100 rpm) at \( 30 \text{ °C} \) using magnetic agitator. The swelling process was monitored by means of conductivity measurements (Inolab Level 1, Germany) at the frequency of 50 Hz. In PEF treatment experiments, the suspension was initially agitated for 15 min, then treated by PEF (total time of keeping suspension in PEF treatment chamber was 100 s) and agitated again. The total time of agitation of the PEF-treated and untreated suspensions was the same (\( t_\text{ag} = 30 \text{ min} \)). The pH of the samples was also measured before and after PEF treatments using a pH meter (Consort C931, Belgium); it was found to be always constant and equal to 4.16 ± 0.01. After agitation, the treated and untreated yeast suspensions were immediately inoculated into fermentation substrates.

2.2. Fermentation substrate

The fermentation process may reflect the composition of the reaction medium, which is not stable for a natural product that contains a multitude of components (e.g., grape juice). In order to make comparison of the fermentation effects in untreated and PEF treated inocula, a synthetic fermentation medium was used in this work.

The composition of the fermentation medium was the following: 5 g/L of yeast extract (Sigma Aldrich Steinheim), 6.36 g/L of ammonium sulfate (as a source of mineral nitrogen), 6.36 g/L of monosodium phosphate (as a source of phosphoric content), and 142 g/L of sugar (as source of carbohydrates), including 68 g/L of glucose, 73.6 g/L of fructose, and 0.4 g/L of sucrose [1]. For sterilization purposes, all reagents and apparatuses were heated for 15 min at 121 °C in an autoclave (Lequeux, France). Their sterility was maintained throughout the experiments and monitored by microbiological control tests.

2.3. PEF-treatment

The PEF generator, Hazemeyer 5 kV 1 kA (Hazemeyer, Saint Quentin, France), providing monopolar pulses of near-rectangular shape, was used in this work. The treatment chamber consisted of a propylene container, into which 2 stainless electrodes (\( A_{\text{electrode}} = 143 \text{ cm}^2 \) separated by 7 mm gap, was installed. The volume of the treatment chamber was 100 mL. The PEF experiments were carried out at two different electric field strengths of \( E = 100 \) and \( 6000 \text{ V/cm} \) using the same protocol of pulses: the number of pulses \( n = 1000 \), pulse duration \( \tau_\text{p} = 100 \mu\text{s} \), and pulse repetition time \( \Delta t = 100 \text{ ms} \). The time of PEF treatment was 0.1 s and the total time of keeping suspension in PEF treatment chamber was 100 s. The current and voltage data were measured and then collected using a data logger and specific software, developed by Service Electronique UTC, Compiègne, France. The temperatures before and after PEF treatment were monitored by a thermocouple. The use of aqueous suspension with low initial electrical conductivity (\( \approx 18 \mu \text{s} \cdot \text{cm}^{-1} \)) allowed avoiding of a noticeable Joule heating during PEF treatment. The initial temperature was 30 °C. The temperature elevation \( \Delta T \), resulting from PEF treatment, was insignificant (\( \approx 0 \)) at \( E = 100 \text{ V/cm} \). In order to avoid overheating the PEF treatment at \( E = 6000 \text{ V/cm} \) was applied using two trains of pulses with pause between them for cooling of suspension. After the first train with \( n = 500 \), the temperature elevation was \( \Delta T \approx 5 \text{ °C} \), then suspension was cooled down to the initial temperature of 30 °C and the next train with \( n = 500 \) was applied. The treatment chamber was disinfected before and after each experiment by 80% ethanol solution.

2.4. Batch fermentation

Batch fermentation experiments were initiated by transferring 10 mL of 0.012 g/L yeast suspension to 300 mL of sterilized synthetic medium. The final concentration of yeasts in the fermentation substrate was \( |Y| \approx 0.0039 \text{ g/L} \). The small-scale fermentations were carried out in six different vessels (4 with PEF treated yeast suspensions and 2 control ones) for 150 h under the controlled temperature (30 °C) with synchronized agitation at 150 rpm (HT Inforsag Bottmingen).

2.5. Analytical methods

Fermentation performance was estimated throughout the fermentation period using periodic (each 6 h) measurements of the mass of the fermentation substrate \( m \) and soluble matter content (“Brix”). “Brix” value was used as an approximate measure of the content of dissolved solids in aqueous solution and was represented as percentage by weight (% w/w). The values of \( m \) were obtained with the help of an analytical balance (Mettler PM6100, Switzerland). The values of “Brix” were measured using a digital refractometer AR 200 (Leica Microsystems Inc., Buffalo, USA). The initial values of mass \( m_0 \) and “Brix” were 310 ± 0.1 g and 140 ± 0.1, respectively.

Samples were also harvested for offline chemical analyses. UV absorption spectra were measured in the spectral range of 190–900 nm by UV spectrophotometer (Spectronic 20 Genesys, Spectronic Instruments, Rochester, NY). The path width of the Suprasil quartz cuvette was 10 mm (Hellma, Mullheim, Germany). The concentration of sugars was determined enzymatically using glucose and fructose analysis kit (Enzyme fluid Glucose/Fructose, R-Biopharm). The concentration of proteins \( |P| / (\text{μg} / \text{mL}) \) was determined using the Bradford procedure [16]. The protein content calibrations were done using measurements of absorbance at 595 nm with Bovine serum albumin (BSA) (Sigma A7030) as reference substance [16].

2.6. Statistical analysis

All the experiments were done in duplicates, and respective analyses were done, at least, in triplicate. Means and standard deviations of data were calculated. One-way analysis of variance was used for statistical analysis of the data using the Statgraphics plus [version 5.1, Statpoint Technologies Inc., Warrenton, VA, USA]. For each analysis, a significance level of 5% was assumed. The error bars presented on the figures correspond to the standard deviations.

3. Results and discussion

During the swelling, the electrical conductivity \( \sigma \) of the inocula grew with time and reached a stable value of \( 25 \pm 5 \mu \text{s/cm} \) after, approximately, 60 min of agitation (Fig. 1).
To investigate the effect of PEF treatment on fermentation of the synthetic media, the same concentrated inocula ([Y] = 0.012 g/L) were treated at an electric field strength of $E = 100$ V/cm and $E = 6000$ V/cm. These values of electric field strength $E$ corresponded to relatively small fields that can induce only limited yeast cell damage. The commonly reported critical field strength, needed for high degree disintegration of the $S.\text{cerevisiae}$ cells, is more substantial (> 7.5 kV/cm) [17–19]. However, it was expected that fields below this critical value can cause noticeable electrical stimulation of $S.\text{cerevisiae}$ cultures.

The electrical conductivity jumped to some extent (see, gray shading in Fig. 1) during the PEF treatment and continued to increase after the PEF treatment ($t > 16.5$ min). It evidently reflected the leakage of intracellular ionic components, caused by electroporation of $S.\text{cerevisiae}$ cells during and after the PEF treatment [15,20–23]. Noticeable changes of $\sigma$ were observed even after PEF treatment of the sample with lower electric field strength ($E = 100$ V/cm). The $\sigma$ changes were larger after treatment at $E = 6000$ V/cm, however, electrical conductivity reached saturation at $t > 30$ min in this case.

The time evolutions of the relative weight $m/m_i$ and relative soluble matter content "Brix/°Brix" during the process of fermentation ($t = 0–140$ h) are presented in Fig. 2a and Fig. 2b, respectively. The individual $S.\text{cerevisiae}$ cells were maturing and not yet able to divide during the lag phase. This process was accompanied with decrease of the relative weight $m/m_i$ (Fig. 2a), whereas "Brix/°Brix", passed through a minor maximum (Fig. 2b).

At this stage, the difference between PEF-treated and control inocula was insignificant; however it was increasing with time.

During the log (logarithmic or exponential) phase, $S.\text{cerevisiae}$ cells were duplicating with intensive depletion of nutrients. In this phase, the difference between behaviors of $m/m_i$ (Fig. 2a) and "Brix/°Brix" (Fig. 2b) in samples with PEF-treated and control inocula became noticeable. E.g., the mass losses were essentially higher in samples with inocula treated at $E = 6000$ V/cm than in samples treated at $E = 100$ V/cm, or in untreated inocula samples (Fig. 2a). On the other hand, kinetics of "Brix values was approximately the same in samples with inocula treated by PEF at $E = 100$ V/cm and at $E = 6000$ V/cm and noticeably faster than in samples with control inocula (Fig. 2b). Consequently, the process of fermentation in the log phase was significantly affected by PEF treatment.

At the beginning of declined (stationary) phase ($t \approx 120$ h), depletion of essential nutrients and formation of the inhibitory products (i.e. organic acids) resulted in restriction of fermentation. At this time (120 h of fermentation), \approx 30% mass reduction was attained in samples with PEF-treated (E = 6000 V/cm) inocula, whereas the same reduction in control sample required approximately, 20 extra hours of fermentation. In general, it may be concluded that faster kinetics of fermentation in samples with PEF-activated inocula was accompanied by higher consumption of carbon sources in the culture medium.

Fig. 3 presents UV absorption spectra for culture suspensions inoculated by untreated and PEF-treated yeasts at the beginning of the log phase ($t = 40$ h). The spectra have the peaks at $\lambda \approx 260$ nm that is generally attributed to the absorption of nucleic acids [24], presumably RNA [25]. The PEF treatment of inocula resulted in a noticeable increase of spectral line intensities, and approximately the same spectra were observed after PEF treatments at $E = 100$ V/cm and $E = 6000$ V/cm. Absorbance values $A$ at $\lambda \approx 550$ nm may be used for estimation of
PEF-treated inocula (at $E = 6000$ V/cm. during the fermentation was $t = 40$ h). Insert shows the values of absorbance measured at $\lambda = 550$ nm for samples inoculated by control and PEF treated yeasts at different times of fermentation. Fig. 3 shows time dependences of the concentration of proteins on $S.\ cerevisiae$ yeasts at different times of fermentation absorbance measured at $\lambda = 550$ nm for samples inoculated by control and PEF treated yeasts. The time of fermentation was $t = 40$ h. Insert shows the values of absorbance measured at $\lambda = 550$ nm for samples inoculated by control and PEF treated yeasts at different times of fermentation. Fig. 4 shows time dependences of the concentration of proteins on $S.\ cerevisiae$ yeasts at different times of fermentation absorbance measured at $\lambda = 550$ nm for samples inoculated by control and PEF treated yeasts. The time of fermentation was $t = 40$ h. Insert shows the values of absorbance measured at $\lambda = 550$ nm for samples inoculated by control and PEF treated yeasts at different times of fermentation.

Insert in Fig. 3 shows that values of absorbance were systematically higher for culture suspensions, inoculated by PEF-treated $S.\ cerevisiae$ yeasts. It proved that the growth of biomass was accelerated in samples with PEF-Treated inocula. Similar effects were observed in a previous study of the static magnetic field effect on $S.\ cerevisiae$ biomass growth [3], in which magnetized yeasts showed accelerated kinetics of fermentation and higher biomass growth rate.

PEF treatment of inocula also affected the process of protein synthesis. Fig. 4 shows time dependences of the concentration of proteins $[P]$ during the process of fermentation ($t = 0–140$ h) in samples with untreated and PEF-treated inocula. During the initial log phase ($t < 40$ h), the value of $P$ was nearly constant for samples with untreated or weakly PEF-treated inocula (at $E = 100$ V/cm), however, it noticeably decreased for samples with PEF-treated inocula at $E = 6000$ V/cm. During the lag phase ($t = 40–100$ h), the concentration of produced proteins continuously increased and PEF treatments resulted in a proportional increase of $[P]$ values.

Consumption of sugars (glucose and fructose) during the fermentation ($t = 0–140$ h) in samples with untreated and PEF-treated inocula is presented in Fig. 5. The said sugars are important nutrients, related to the growth of $S.\ cerevisiae$. The faster consumption kinetics was observed for glucose than for fructose. It is typical for fermentation conducted by $S.\ cerevisiae$ and can be explained by higher affinity of hexose transporters (which translocate each sugar into the cell) to glucose than fructose [27,28]. The data show that the fermentation processes were starting at approximately equal concentrations of both monosaccharides ($[C_i] = 68$ g/L for glucose, and $[C_i] = 73.6$ g/L for fructose), however, by $t = 100$ h, practically, all glucose were consumed and the yeasts continued to consume exclusively fructose in the ongoing fermentation (Fig. 5). The fermentation–stimulation effects of PEF treatment resulted in more accelerated sugar depletion. The most evident differences were observed at the end of the lag phase (at $t \approx 40$ h). By this moment, consumption of fructose in samples with electrically activated inocula exceeded that in samples with control inocula by $\approx 2.33$ times and $3.98$ times, for $E = 100$ V/cm and $E = 6000$ V/cm respectively. Highly significant differences between fermentation activities of electrically activated and controlled inocula were observed during the log (exponential) phase.

4. Conclusion

The obtained results clearly prove the positive impact of PEF treatment of wine $S.\ cerevisiae$ yeast inocula on the batch fermentation process. Electro-stimulation was confirmed by the observed increase of electrical conductivity of suspensions. It evidently reflected the leakage of intracellular ionic components, caused by electroporation of $S.\ cerevisiae$ cells. The electrical stimulation of $S.\ cerevisiae$ cultures resulted in a noticeable enhancement of the fermentation kinetics that was seen from time dependencies of mass losses, soluble matter content (Brix, consumption of sugars, and synthesis of proteins. It is remarkable that significant acceleration of sugar consumption was observed at the initial stage of fermentation (in the lag phase).

Please cite this article as: J.R. Mattar, et al., $S.\ cerevisiae$ fermentation activity after moderate pulsed electric field pre-treatments, Bioelectrochemistry (2014), http://dx.doi.org/10.1016/j.bioelechem.2014.08.016
The obtained results may reflect the impact of PEF stimulation on the efficiency of synthesis of RNA and enzymes, frequency of cell division events, the probability of daughter cell survival, tolerance to ethanol, and fermentation capacity. Few possible mechanisms can be proposed for explanation of the observed phenomena. In principle, the killing efficiency of PEF treatment depends on the diversity of cell size and their resistance to electrical stress. Owing to these effects, some portion of yeast subpopulations may be selectively killed and removed from the further fermentation process. It is reasonable to expect that killing effects are much less pronounced at \( E = 100 \text{ V/cm} \) than at \( E = 6000 \text{ V/cm} \).

On the other hand, the increase of \( E \) can result in stimulation of subpopulations of the yeast cells. Previous studies have shown that PEF treatment can influence the rate of survival of the yeast cells, and its maximum was observed at the optimal electric field \( E = 850 \text{ V/cm} \). The mechanism of PEF-induced stimulation of yeasts is still unknown in all details. Note that phenomenon of temporary electroporation with successive resealing can result in modification of the cytoplasmic membrane. It is known that membrane proteins are electrogenic (e.g., they act as membrane ion pumps) and external electric fields can induce protein activity. The electric field can improve transport of useful nutrients through the modified cell membranes due to pore formations or activation of transport proteins. We can conclude that although the potential of the practical application of electrical stimulation for increasing performance of the yeast cells is high, the general mechanisms and optimal procedures of electrically-enhanced fermentation still require further detailed studies in the future.

Acknowledgments

The authors appreciate the financial support from the National Council for Scientific Research, CNRS-Lebanon. Authors thank Dr. N. S. Pivovarova for her help with preparation of the manuscript.

References


Jassy Mattar has graduated in 2011 with a Masters in Food Science and Technology from the University of Balamand (Lebanon) in collaboration with University of Greenwich (UK), University of Valenciennes (France) and University of Milano-Bicocca (Italy). She is currently pursuing her PhD studies at the “Université de Technologie de Compiègne” (France) and the University of Balamand (Lebanon). Her research interests are the emerging electro-technologies in food processes, particularly microbiology studies.

Mohammad Turk is an assistant Professor at the “Ecole Supérieure de Chimie Organique et Minérale”(ESCOM) France and a permanent member in the laboratory for Agro-Industrial Technologies at the “Université de Technologie de Compiègne” (UTC) France. He received his PhD degree in Process Engineering (2010, France). His main research focuses are on mass transfer phenomena (solid/liquid separation), innovative food technologies (especially electrotechnologies) and the relationship between innovative processes and the biochemical aspect of agri-food products. He has published 5 peer-reviewed papers since 2006.
Maurice Nonus is Research Engineer of the Chemical Engineering Department at the Université de Technologie de Compiègne (UTC), France. He received his PhD degree in Industrial Microbiology (1982). His main research interests are focused in fermentation and down-stream processing on microbial population development and control in industrial processes in food, biotech, and environment. He is regularly involved in industrial process development and improvement in research programs. Expert for international and national administration and agencies (UNDP, UNIDO, MEN, OSEO...). He was appointed as research team leader in a private company (88–92) and head of the Chemical Engineering Department at (UTC) 2011–2013.

Nikolai I. Lebovka is a professor of physics in the Physics Department Taras Shevchenko National University of Kiev. He is also a head of the Department of Physical Chemistry, Biocolloid Chemistry Institute, Kiev. He obtained his PhD degree in molecular physics at Taras Shevchenko National University (1986) and HabDr. Science in physics of colloids at the Institute of Biocolloid Chemistry (1995). His research interests encompass the electric field effects in bio and food materials, colloidal suspensions, biocolloids, theory and applications of nanocomposites, computation physics and percolation phenomena. He has been involved in a number of international and national funded projects in these topics. He is co-author of the books “Enhancing Extraction Processes in the Food Industry”, “Electrotechnologies for Extraction from Food Plants and Biomaterials”, “NMR spectroscopy of water in heterogeneous systems” and “Simulation of physical systems: receipts and computer programs”.

Henri El Zakhem is the chairman of the Chemical Engineering Department at the UOB since 2007. He graduated from the University of Technology of Compiegne (UTC) with a PhD in Chemical Engineering in 2006. He was recruited in UTC as a researcher and lecturer in 2006–2007. He worked on various research projects for CNRS France. He joined the UOB in September 2007 to enhance the establishment of the Chemical Engineering Program in terms of curriculum and laboratories. His research interests are Emergent Electrotechnologies in Food Processing, Renewable Energy, and Nanotechnology. He is also a technical reviewer for several international journals.

Eugene Vorobiev is a Professor of the Chemical Engineering Department and a head of the laboratory for Agro-Industrial Technologies at the Université de Technologie de Compiègne (UTC), France. He received his PhD degree in Food Engineering (1980, Ukraine) and his Dr Habil. in Chemical Engineering (1997, France). His main research interests focus on mass transfer phenomena, theory and practice of solid/liquid separation, and innovative food technologies (especially electrotechnologies). He is a member of an editorial board in several journals (“Separation and Purification Technology”, “Food Engineering Reviews”, “Filtration”) and the President of the Scientific Council of IFTS (“Institut de la Filtration et des Techniques Séparatives”). He was awarded by Gold Medal of the Filtration Society (2001) and he is a Laureate of the Prize for the Innovative Technique for the Environment (Ademe, 2008). He was a chairman of several international conferences.

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