Production of Chitin and Chitosan from Shrimp Shell in Batch Culture of *Lactobacillus plantarum*

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Lactobacillus plantarum, as a potential lactic acid and protease producer, was used for biological extraction of chitin from shrimp shell. *L. plantarum* was grown in a batch culture containing shrimp shell powder and date syrup, incubated at 30 °C. The produced organic acids and proteases in *L. plantarum* culture were able to demineralize and deproteinize shrimp shell. Percentages of deproteinization and demineralization were 45 and 54, respectively. In post treatment of the sample, dilute acid and alkali, were implemented to produce the specific chitin. Chitin was converted to chitosan by N-deacetylation with NaOH solution. Percentage of deacetylation based on FTIR spectrum was 83 %.

Key words:
Chitin, chitosan, date syrup, *Lactobacillus plantarum,* shrimp shell

Introduction

Chitin is a homo-biopolymer composed of β-1,4-N-acetylglucosamine. Chitosan is a co-polymer of glucosamine and N-acetylglucosamine which is partially deacetylated chitin.[1,2] These biopolymers can be extracted from yeast, mushroom, cell wall of fungi, crustaceans shell such as crab, shrimp, prawn and squid pens, but up to now shrimp and crab shells are considered the commercial source for chitin production.[3,4] Chitosan has attracted great interest due to its biocompatibility, high charge density, non-toxicity and mucoadhesion. The biological adaptability, biodegradability, hemostatic activity and wound healing properties of chitin and chitosan have been reported, which attracted much attention to their biomedical applications.[5–8] Chitin and its derivatives are widely applied in agriculture due to defensive mechanism in plants, seed coating and time release of fertilizers. They are used in biomedicine for their biocompatible, biodegradable, renewable and film forming characteristics. They are also used in water engineering for metal capturing and dye removal properties, in the food and nutrients industry for antimicrobial, coagulating and film forming ability, and many more applications.[9–12]

Conventional methods for extraction of chitin from crustaceans are chemical processes, which use strong acids and bases for the removal of minerals and proteins.[13–15] Use of strong acid (HCl) may be harmful to the physical-chemical properties of chitin and chitosan. However, biological method along with post mild chemical treatments is an alternative to traditional chitin extraction for the treatment requires a significant amount of alkali and acids.[16] Demineralization (DM) and Deproteinization (DP) are required for the recovery of chitin and chitosan. It has been reported that lactic acid produced by a number of microorganisms such as *Bacillus subtilis,*[17] *Lactobacillus helveticus,*[18] *Pseudomonas aeruginosa, Lactobacillus paracasei, Lecanicillium fungicola* and *Penicillium chrysogenum*[19] is easily utilized for DM. These microorganisms are responsible for the precipitation of organic salts such as calcium lactate, which is easily removed from media by wash out. DP is also carried out with the aid of proteolytic activities of some microorganisms. Thus, deproteinization is carried out in the presence of proteases produced by microorganisms; meaning that proteolysis causes the breakdown of proteins.[17,20–22]

In this research, *Lactobacillus plantarum* was used in date syrup. This microorganism has the ability of DM and DP of shrimp shell powder.[22,23] The purpose of this study was to produce chitin from shrimp waste via biological processes combined with mild chemical post treatment. Low cost carbohydrate sources such as date syrup for the generation of organic acids and proteolytic enzymes were used.

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Materials and methods

Shrimp shell

The shrimp shells used for the experiment were supplied in fresh condition from local market (Tehran, Iran). The shells were separated from the head and legs. The shrimp shells were washed several times with tap water then dried out in a hot room at 70 °C. The dried shells were ground to fine powder.

Date syrup

The date syrup (Sorkhchin) used for the experiment was supplied from market. The results of chemical analysis of the date syrup are shown in Table 1.

Table 1 – *Chemical characteristics of date syrup*

Component	Quantity
Total sugar (g L^{-1})	298
Ash (%)	1.32
Moisture content (%)	16
Protein (%)	0.94

Microorganism and preparation of inoculation

Lactobacillus plantarum 1058 was purchased from Persian Type Culture Collection (PTCC), provided by Iranian Research Organization for Science and Technology (IROST). The MRS (Man Rogosa Sharpe) medium was used for growing *L. plantarum* PTCC 1058. The medium was autoclaved at 121 °C and 101 kPa for 20 min. The inoculated culture was cultivated in an incubator shaker (Stuart, S1500 series, USA) at 30 °C and agitation rate of 180 rpm for 24 hours.

Fermentation

The 100 mL shrimp shell powder broth (5 g)[19,24] containing 20 g L^{-1} carbohydrate in date syrup was sterilized, cooled and inoculated with φ = 5 % of seed culture. Batch fermentation was carried out in a 250 mL flask in the incubator shaker at 30 °C and 180 rpm. The prepared medium was incubated for 6 days. The samples were taken periodically from the culture to monitor optical density, consumption of sugar, and variation of pH.

Preparation of chitin

Solid fraction obtained after fermentation (raw chitin) was treated with 0.5 mol L^{-1} HCl with chitin to HCl solution ratio of 1:25 (g mL^{-1}), for 2 hours at room temperature then washed with distilled water. The acid-treated solid was then treated with 0.5 mol L^{-1} NaOH with chitin to NaOH solution ratio of 1:25 (g mL^{-1}) for 2 hours at room temperature, then washed with distilled water.[16,17] This treatment was carried out in order to obtain more purified chitin. Purified chitin was dried at 55 °C overnight.

Preparation of chitosan

The purified chitin was put into a flask with 55 % NaOH solution with chitin to NaOH solution ratio of 1:25 (g mL^{-1}), in a water bath at 95 °C for 4 hours.[25,26] After that chitosan was washed with distilled water and dried at 55 °C overnight. The FTIR spectrum of chitosan sample was measured and compared with the spectrum of commercial chitosan.

Analytical procedure

The optical density was measured at 620 nm using a spectrophotometer (Unico, 2100, USA) then correlated to standard cell dry mass. For carbohydrate concentration, 1.5 mL samples were collected and the cells were settled by centrifugation at 7000 rpm for 7 min using a micro centrifuge (Hermle model: Z 233 M-2, Germany). The reduced sugar was determined by colorimetric method using 3,5-dinitrosalicylic acid (DNS) method.[27] The pH values of liquid samples were measured using a pH meter (Hanna, Germany). Total titratable acidity (TTA) was determined by titration sample with 0.1 N NaOH to pH 8.4.[28,29] It was calculated using eq. 1, where V_1, V_2 and N are NaOH volume, sample volume, and normality of NaOH solution. For determination of protein content of shrimp shell and chitin, the nitrogen content was first determined by Kjeldahl method (2300 kjettec analyzer unit, Foss Tecator, Sweden). The percentage of crude protein can be calculated by percentage of nitrogen multiplied by a nitrogen-to-protein factor (6.25).[29]

$$TTA\,(\mathrm{g\ L^{-1}}) = \frac{V_1 \cdot N \cdot 90.08 \cdot 1000}{V_2 \cdot 100} \qquad (1)$$

The moisture content was measured by drying samples in an oven (Binder, Germany) at 105 °C until reaching constant mass. Ash content was determined by burning the samples in a crucible at 600 °C in a furnace for 2 hours.[30] Demineralization efficiency (DM/%) was calculated using the following equation, where A_O and A_R are the ash concentrations (g g^{-1}) before and after fermentation; O and R are the mass (g) of original sample and fermented residue, respectively:[28]

$$DM\ (\%) = \frac{A_O O - A_R R}{A_O O} \cdot 100 \qquad (2)$$

Deproteinization efficiency (DP/%) was calculated using the same equation but replacing A_O, A_R in the equation by P_O, P_R, which represent protein concentrations in the original and fermented residues, respectively.

FTIR spectra (Fourier Transform Infrared Spectroscopy) were obtained. The dried powder of chitosan was analyzed with Fourier Transform Infrared (FTIR, Model Vector 22, Bruker, Germany) to define the functional group of produced chitosan. The dry sample powder was mixed with KBr and pressed into pellets under reduced pressure. The FTIR spectra were obtained by scanning between 4000 and 500 cm^{-1}.

Results and discussion

Effect of pH and total titratable acidity (TTA)

Table 2 presents data for pH and TTA variation during fermentation. As it is evident, pH and TTA show reverse trends. During the fermentation, *L. plantarum* utilized the hydrocarbon source by breaking down its structure, which led to lactic acid production in the solution.[17,23] Gradual reduction of pH value in the culture was due to the organic acids production. As a result, the amount of produced acid (TTA) had an increasing trend.

Table 2 – *Variation of pH and TTA during fermentation*

Time (h)	pH	TTA (g L^{-1})
0	6.80	0.00
24	5.58	4.05
48	4.83	8.58
72	4.50	10.43
96	4.32	12.43
120	4.22	13.50
144	4.22	13.51

Substrate utilization and cell growth

Fig. 1 shows cell growth and date syrup consumption as sole carbon source with respect to fermentation time, duration of 144 hours. After 60 hours of fermentation, about 85 % of carbon source was utilized. Also, the exponential growth phase was observed to 60 hours of fermentation. Maximum cell dry mass (CDM) was obtained in 96 hours.

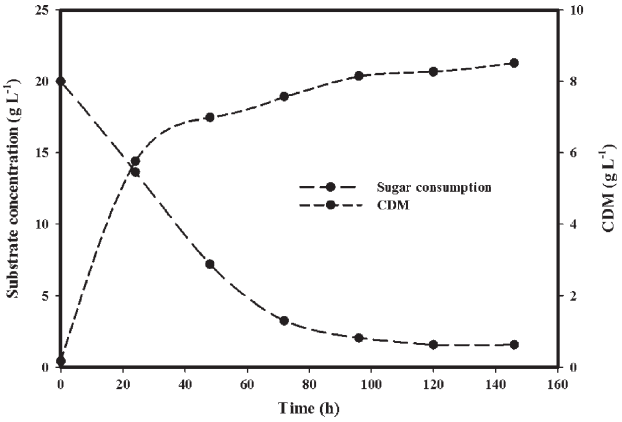

Fig. 1 – *Substrate consumption and cell growth of Lactobacillus plantarum*

Growth kinetics

The first-order differential equation may describe substrate utilization with respect to time as the equation stated follows:

$$-\frac{dS}{dt} = K_S S \qquad (3)$$

Where S is substrate concentration (g L^{-1}), t is incubation time (h) and K_s is the first order kinetic constant in (h^{-1}). Integration of eq. 3 resulted in eq. 4, where S_0 is the initial substrate concentration (g L^{-1}):

$$S = S_0 \exp(-K_S t) \qquad (4)$$

By taking natural log of eq. 4, a linear model is obtained as given below:

$$\ln\left(\frac{S}{S_0}\right) = -K_S t \qquad (5)$$

Fig. 2 illustrates $\ln\left(\frac{S}{S_0}\right)$ versus time, the slope represents the first order kinetic constant. It is

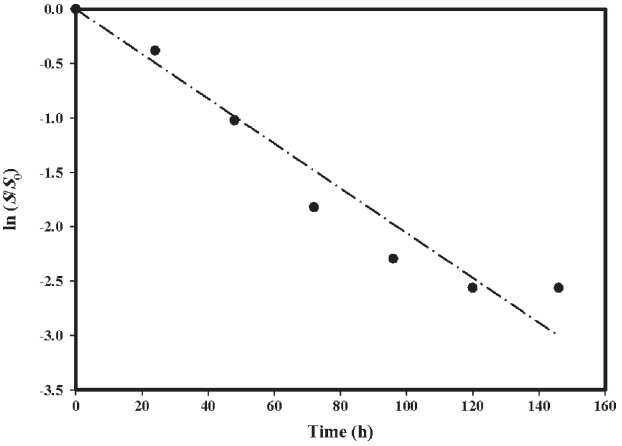

Fig. 2 – *Kinetic model for substrate consumption rate*

shown that the experimental data were fitted well by the first-order rate equation with $R^2 = 0.93$. Malthus law represents cell growth kinetics in a batch system, as follows:[31,32]

$$\frac{dX}{dt} = \mu X \tag{6}$$

Where X is cell growth rate (g L^{-1}), t is incubation time and μ is specific growth rate (h^{-1}). Fig. 3 shows the plotted data for cell biomass with respect to incubation time. The data fitted well with the projected growth model.

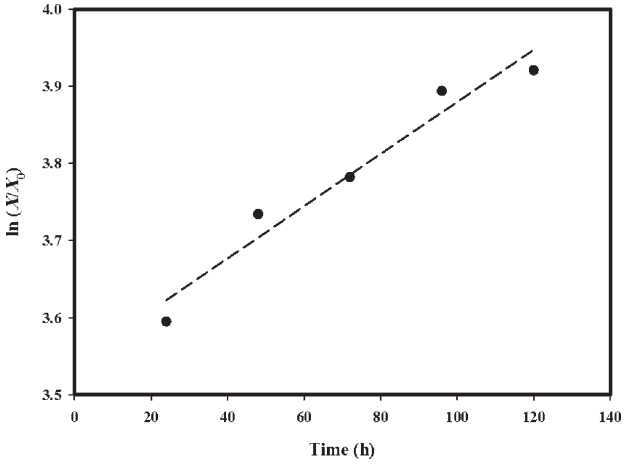

Fig. 3 – *Cell growth kinetics presented by Malthus law*

The Monod kinetic model is also known as a simple unstructured model, which is related to microbial growth to substrate concentration through two constants, the maximum growth rate constant μ_{max} (h^{-1}) and the substrate saturation constant or the substrate affinity constant, k_s (g L^{-1}). This model is expressed by the following equation:[33]

$$\mu = \frac{\mu_{max} \cdot S}{k_S + S} \tag{7}$$

According to Lineweaver-Burk linearized Monod kinetic model (eq. 8), a plot of $\frac{1}{\mu}$ versus $\frac{1}{S}$ with $\frac{1}{\mu_{max}}$ intercept and $\frac{k_S}{\mu_{max}}$ slopes is shown in Fig. 4. The obtained kinetic parameters are summarized in Table 3 with $R^2 = 0.94$.

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{k_S}{\mu_{max}} \cdot \frac{1}{S} \tag{8}$$

Another interesting growth model is known as Logistic model; which is one of the distinguished models for growth kinetics prediction. The predicted specific growth rate by logistic model is shown as follows:[32]

$$\mu = \mu_{max}\left(1 - \frac{X}{X_{max}}\right) \tag{9}$$

Where, X_{max} presents the maximum cell dry mass concentration (g L^{-1}). To obtain cell dry mass concentration, eq. 9 was incorporated into eq. 7 and integration resulted with the following equation:

$$X = \frac{X_0 \exp(\mu_{max} t)}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - \exp(\mu_{max} t))} \tag{10}$$

Fig. 5 depicts the experimental data were well fitted to logistic model.

A few growth-related kinetic models such as, Monod non-linear, Westerhoff and Moser, simulate cell growth using date syrup as sole carbon source;

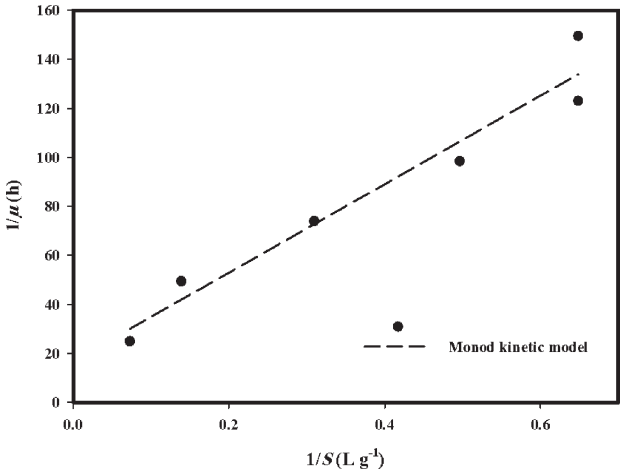

Fig. 4 – *Monod kinetic model for experimental data*

Table 3 – *Kinetic parameters*

Substrate consumption rate	
K_S (h^{-1})	0.02
R^2 (%)	0.93
Monod kinetic model	
μ_{max} (h^{-1})	0.06
k_S (g L^{-1})	10.72
R^2 (%)	0.94
Logistic kinetic model	
μ_{max} (h^{-1})	0.319
X_{max} (g L^{-1})	7.9
R^2 (%)	0.97

Table 4 – *Kinetic parameters of non-linear models*

Models	Non-linear models	Linear models	Parameters	R^2
Monod	$\mu = \frac{\mu_{max} S}{k_S + S}$	$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{k_S}{\mu_{max}} \cdot \frac{1}{S}$	μ_{max} = 0.12 h^{-1} k_S = 27.98 g L^{-1}	0.96
Westerhoff	$\mu = a + b \ln S$	$\mu = a + b \ln S$	$a = A$ = 2.15e^{-4} h^{-1} b = 1.33e^{-2} h^{-1}	0.88
Moser (n = 2)	$\mu = \frac{\mu_{max} S^2}{k_S + S}$	$\frac{1}{\mu} = \frac{k_S}{\mu_{max}} \cdot \frac{1}{S^2} + \frac{1}{\mu_{max}}$	μ_{max} = 0.04 h^{-1} k_S = 20.59 g^2 L^{-2}	0.81

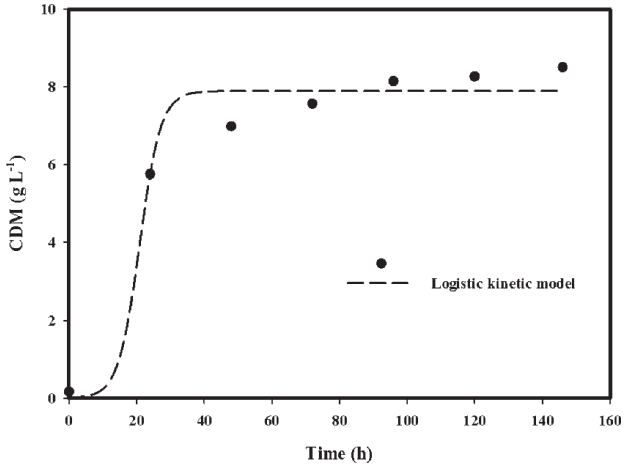

Fig. 5 – *Logistic kinetic model fitted with experimental data*

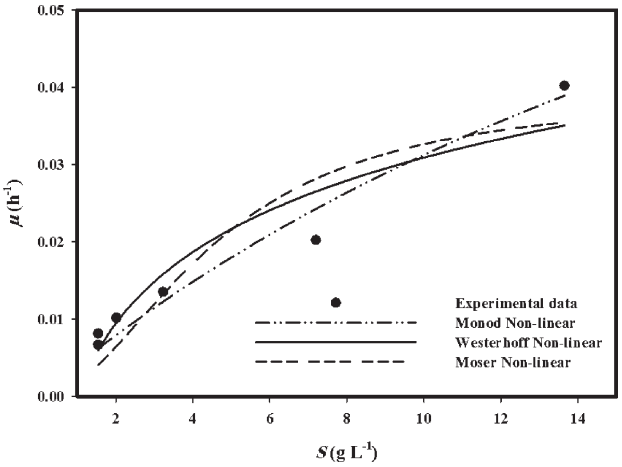

Fig. 6 – *Comparison of specific growth rates obtained from various models with non-linear regression*

the biokinetic data are summarized in Table 4. Westerhoff model proposed a linear model for the cell specific growth rate. The Moser kinetic model with n = 2 is a modified model of Monod equation for the substrate.[34] Fig. 6 shows experimental data fitted with these three non-linear models.

Chitin and chitosan characteristics

Analysis of ash and protein content of samples before and after fermentation are summarized in Table 5.

Table 6 compares obtained data with previous work for chitin extraction with several microorganisms and reported substrate concentration. In addition, high DM and DP were reported; that was probably due to high substrate concentration (10–15 %). In the present work, with low substrate

Table 5 – *Characterization of shrimp shell and raw chitin*

Shrimp shell powder characterization (%)		Raw chitin characterization (%)	
Moisture	13	Moisture	11.3
Ash	30.4	Ash	16.4
Protein	44	Protein	28.8

Table 6 – *Demineralization and deproteinization for chitin extraction with several microorganisms and various substrates*

Microorganism	Inoculum size, %	Substrate content	DM/%	DP/%	Reference
Lactobacillus plantarum along with acetic acid	6.7	Glucose- 5 %	86	75	[23]
Pseudomonas aeruginosa F722	5	Glucose- 10 %	92	63	[19]
Lactobacillus paracasei strain A3	10	Glucose- 10 %	61	77.5	[29]
Pediococcus acidolactici CFR2182	5	Glucose- 15 %	72.5	97.9	[20]
Lactobacillus plantarum	5	Date syrup –2 %	54	45	Present study

Table 7 – *FTIR functional groups*

FTIR absorption bands		Functional group	Degree of deacetylation (DDA/%)	
produced chitosan	commercial chitosan		produced chitosan	commercial chitosan
3490	3489	OH stretching		
2920	2925	C–H symmetrical stretching		
1644	1644	C=O in amide groups (amide I band)	83	86
1548	1548	NH_2 bending vibration in amino group (-NH_2)		
1030	1027	C–O group (-C–O) in amide group		

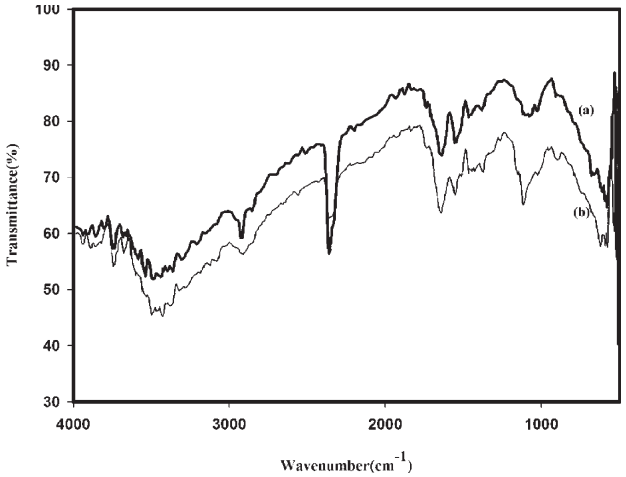

Fig. 7 – *FTIR of (a) commercial and (b) synthesized chitosan*

concentration (2 %), maximum DM and DP were 54 and 45 %, respectively. Even at low substrate concentration (20 g L^{-1}) the percentage of DM and DP were significant (see Table 6).

Fig. 7 represents FTIR spectrum of (a) commercial and (b) synthesized chitosan. The FTIR spectra identified functional groups of the product. The x-axis expressed wavelength in cm^{-1} and the y-axis represents the light transmittance (%) through the sample. Most of the important functional groups are summarized in Table 7, showing good accommodation between synthesized and commercial chitosan.[35]

Conclusion

The obtained results indicate that, *Lactobacillus plantarum,* along with utilization of date syrup as low cost carbon source and appropriate substrate, was able to demineralize and deproteinize shrimp shell. Post treatment of chitin with mild acid and alkali, 0.5 mol L^{-1}, lead to the extraction of chitin with more desirable properties, with 79.5 % demineralization and 71 % deproteinization. The extracted chitin, as desired product was easily converted to chitosan by deacetylation in NaOH solution. In the process of chitin extraction, the Logistic and Monod kinetic models were adequately compatible with the experimental data. The final product, chitosan, reached a good degree of deacetylation of about 83 %.

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Nomenclature

A_O – ash concentration of the original sample, g g^{-1}
A_R – ash concentration of the fermented residue, g g^{-1}
CDM – cell dry mass, g L^{-1}
DM – demineralization, –
DP – deproteinization, –
K_S – first order kinetic constant, h^{-1}
k_S – substrate saturation constant (Monod constant), g L^{-1}
O – mass of original sample, g
P_O – protein content of the original sample, g g^{-1}
P_R – protein content of the fermented residue, g g^{-1}
R – mass of fermented residue, g
S – substrate concentration, g L^{-1}
S_0 – initial substrate concentration, g L^{-1}
t – incubation time, h
TTA – total titratable acidity, g L^{-1}
X – cell dry mass concentration, g L^{-1}
X_0 – initial cell dry mass, g L^{-1}
X_{max} – maximum cell dry mass, g L^{-1}

Greek symbols

μ – specific growth rate, h^{-1}
μ_{max} – maximum specific growth rate, h^{-1}

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