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Identification and optimization of parameters for the semi-continuous production of garbage enzyme from pre-consumer organic waste by green RP-HPLC method

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ABSTRACT

Reuse and management of organic solid waste, reduce the environmental impact on human health and increase the economic status by generating valuable products for current and novel applications. Garbage enzyme is one such product produced from fermentation of organic solid waste and it can be used as liquid fertilizer, antimicrobial agents, treatment of domestic wastewater, municipal and industrial sludge treatment, etc. The semi-continuous production of garbage enzyme in large quantity at minimal time period and at lesser cost is needed to cater for treatment of increasing quantities of industrial waste activated sludge. This necessitates a parameter for monitoring and control for the scaling up of current process on semi-continuous basis. In the present study a RP-HPLC (Reversed Phase-High Performance Liquid Chromatography) method is used for quantification of standard organic acid at optimized condition 30 °C column oven temperature, pH 2.7, and 0.7 ml/min flow rate of the mobile phase (potassium dihydrogen phosphate in water) at 50 mM concentration. The garbage enzyme solution collected in 15, 30, 45, 60, 75 and 90 days were used as sample to determine the concentration of organic acid. Among these, 90th day sample showed the maximum concentration of 78.14 g/l of acetic acid in garbage enzyme, whereas other organic acids concentration got decreased when compare to the 15th day sample. This result confirms that the matured garbage enzyme contains a higher concentration of acetic acid and thus it can be used as a monitoring parameter for semi-continuous production of garbage enzyme in large scale.

1. Introduction

Globally pre-consumer type waste generated by food processing industries on a large scale contains 60% of organic matter (Lin et al., 2013). Organic waste is currently a worldwide major issue, its treatment and disposal become more and more important in developing countries. Organic waste poses serious risk to human health and the environment at every stage from generation to transportation and safe disposal (Sinha et al., 2009). On the other hand the emission of greenhouse gases methane and nitrous oxides from the disposal of solid organic waste either in the landfills or from their management by composting. In the atmosphere the ability of heat trapping by greenhouse gas differs, if the heat trapping potential is higher, the gas creates a greater impact on climate changes and the environment (Karl et al., 2009). These obviously become a major environmental, economic and social problem (Gustavsson et al., 2011). In the current society, increasing global population mutually increases the global demand for energy, chemicals and materials. The increasing demands encourage the reuse and management of organic solid waste (Bansal et al., 2012; Chandrasekaran, 2013; Pleissner and Lin, 2013; Zhang et al., 2013). These processes could complement with lower environmental impact strategies and have the potential to generate valuable products for current and novel applications (Zhang et al., 2013; Poeschl et al., 2010). In general organic waste comprised with significant quantities of functionalised molecules like carbohydrates, proteins, triglycerides, fatty acids, phenolic (Pleissner and Lin, 2013; Zhang et al., 2013; Poeschl et al., 2010; Yan et al., 2011; Leung et al., 2010).

In the developing countries, there is gradual increase in waste activated sludge (WAS) produced from the waste water treatment process due to higher industrialization. The WAS need to be stabilized adequately to reduce organic content, pathogen and odour problems before disposal and utilization. Anaerobic digestion is

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one of the best stabilization processes, but the hydrolysis step is major limitations of this process. It can be overcome by various pre-treatment process (Physical (Beszédes et al., 2012), Chemical (Kim et al., 2007; Kavitha et al., 2014a), biological (Kavitha et al., 2014b; Merrylin et al., 2013)).

The garbage enzyme was produced by the fermentation of waste vegetables, fruits, or its peels along with sugar and water (Prakash, 2011; Oon, 2008). The garbage enzyme can function in four categories: decompose, compose, transform and catalysis (Oon, 2008). It has the property to improve wastewater treatment processes by removal of impurities, harmful sludge and bacteria and promotes recycling of waste back into the earth (Prakash, 2011; Tang and Tong, 2011). Nazim and Meera (2013) produced garbage enzyme for treatment of Synthetic Grey water using 5% and 10% Garbage Enzyme Solution. They also characterized the environmental properties (BOD, COD, MPN, etc.) of garbage enzyme and confirmed that the produced garbage enzyme is acidic in nature.

In our previous work the antimicrobial activity, bio catalytic activity and consequently the stabilization of dairy waste activated sludge using crude garbage enzymes were studied and reported to check whether the garbage enzyme produced from preconsumer is capable of stabilizing the waste activated sludge or not. The result obtained confirmed that garbage enzyme solution has antimicrobial activity, bio catalytic activity and it also has the capability to stabilize the waste activated sludge (Arun and Sivashanmugam, 2015).

Till now the garbage enzyme production is being performed in batch fermentation process for a period of 3 months. The semi-continuous production of garbage enzyme in large quantity in minimal time period and at lesser cost is needed to cater for treatment of increasing quantities of industrial waste activated sludge. This necessitates a parameter for monitoring and control for the scaling up of current process on semi-continuous basis. In many fermentation processes, monitoring of organic acid in the product is used as a major parameter for process control and quality testing.

Various methods like spectrophotometric with and without bio catalyst, electrophoretic and chromatographic methods have been reported for determining organic acid (Mato et al., 2005). The ion exchange column HPLC protocol justifies lower environmental impacts and running costs but the ion exchange column is more expensive compared to C-18 HPLC column. Another common method used to determine organic acid is gas chromatography (GC), but the GC method for the quantification of organic acid was found to be unsuitable because the procedure was very tedious. Kerem et al. (2004), reported that reversed-phase HPLC (RP-HPLC) method was found to be very simple, reliable and stable for the quantification of organic acid among all other the chromatographic methods.

Organic solvents such as acetonitrile and methanol are generally used in RP-HPLC for separation. Kelebek et al. (2009), used sulphuric acid with acetonitrile as solvent to determine the concentration of organic acid in orange juice and organic wine using HPLC method. But these organic solvents are significantly hazardous to human health and very expensive with respect to the disposal of solvents (Wei et al., 2011). Thus, RP-HPLC techniques without using organic solvents are now preferred (Moldoveanu and David, 2012; Nour et al., 2010; Koel and Kaljurand, 2010). The HPLC method using water as the mobile phase is commonly referred as a green HPLC method. Sánchez-Machado et al. (2008) reported the HPLC method for determination of organic acid in fermented shrimp waste using water as the mobile phase with pH = 2.1. Nour et al. (2010) determined the organic acid in citrus fruit using potassium dihydrogen orthophosphate buffer as the mobile phase with pH 2.8. The above cited works were only confined with citrus fruit extracts, fermented shrimp waste, etc. to determine the concentration of organic acid in RP HPLC and till now no attempt has been made to determine organic acid in garbage enzyme solution using RP HPLC method.

In the present work an attempt has been made

- To quantify the organic acid (Acetic, oxalic, citric, malic, and lactic acid) in garbage enzyme solution using potassium dihydrogen orthophosphate buffer as mobile phase using RP-HPLC method.
- Chromatographic parameters like pH, temperature, concentration of the mobile phase and flow rate were optimized.
- The optimized chromatographic environments were used to investigate the concentration of organic acid during the production of garbage enzyme at different time interval.

2. Materials and methods

2.1. Chemicals and reagents

Acetic acid, citric acid, malic acid, lactic acid, oxalic acid, orthophosphate, phosphoric acids and HPLC water were purchased from Merck Ltd. All reagents were of analytical grade and were used without further treatment.

2.2. HPLC equipment

Shimadzu prominence binary gradient HPLC system (Japan) with a LC-20AD pump, SIL-20A HT auto sampler (Shimadzu, Japan), CTO-20AC column oven (Shimadzu, Japan) and a PDA detector (Shimadzu, Japan) were used for the present study. Separations were accomplished using a C18 column (5 μm particle diameter and 120 Å pore diameter, Shimadzu, Japan).

2.3. Organic acid standard preparation

A standard stock solution of 1050 g/l acetic acid, 1 g/l citric acid, 2 g/l malic acid, 0.3 g/l oxalic and 1210 g/l lactic acid was prepared. The stock solution are used to prepare corresponding dilutions using ultrapure water and stored at low temperature (4 °C) in dark places.

2.4. Preparation of garbage enzyme samples

About 300 g of organic wastes (Tomato, Cauliflower, and Pineapple, Orange and Mango peels) were mixed with 100 g of molasses (carbon source) and a litre of water in 2 l air tight containers. The fermentation was carried out for 3 months in dark at room temperature. The solutions from the container were collected at regular interval of 15 days and centrifuge at 3000 rpm for 30 min .The supernatant was diluted to 1:10 with ultrapure HPLC water and filtered using membrane filter (0.45 μm) before injection into HPLC for the organic acid determination.

2.5. RP-HPLC method optimization

To optimize RP-HPLC condition for determination of the organic acid in the garbage enzyme solution, effects of concentration of the mobile phase, the pH of the mobile phase, temperature of column oven and flow rate of the mobile phase of separation with a constant wavelength (214 nm) were investigated. The experimental designs are shown in Table 1.

The standard curves of 5 organic acids were obtained at proposed optimal condition. With these standard curves as reference,

Table 1
Experimental design for optimization of HPLC method.

Factors	Concentration of KH_4PO_2 (mM)	pH of mobile phase	Temperature of column oven	Flow rate (ml/min) of mobile phase
Effect of concentration of mobile phase	10,30,50,70	2.8	35	0.8
Effect of pH of mobile phase	50	2.5,2.6,2.7,2.8	35	0.8
Effect of Temperature of column oven	50	2.7	20,25,30,35	0.8
Effect of Flow rate of mobile phase	50	2.7	30	0.5,0.6,0.7,0.8

the concentration of organic acid in garbage enzyme was estimated.

3. Result and discussion

3.1. Effects of concentration of the mobile phase

The mobile phase concentration is one of the most important variables in the control of retention time in RP-HPLC. In this proposed process the different concentration of the mobile phase (10, 30, 50, 70 mM) was prepared by dissolving potassium dihydrogen phosphate (KH_2PO_4) in water and the pH value was adjusted to pH = 2.8 with phosphoric acid. Fig. 1 presents the effect of concentration of the mobile phase on separation of organic acid using HPLC method. From this figure, it is observed that the value of capacity factor (K') is increasing for all acids from 10 to 50 mM KH_2PO_4 concentration and start decreasing when the concentration of the mobile phase increasing from 50 to 70 mM. Therefore, 50 mM KH_2PO_4 is considered as the optimum mobile phase concentration. However oxalic acid has K' factor less than one in this concentration of KH_2PO_4 which can be improved by preparing the mobile phase in advance to remove some ionic interaction of inorganic compounds in a sample. Ding et al. (2006), reported a similar observation, when determining the organic acid concentration in soil.

3.2. Effects of pH on mobile phase

The mobile phase pH can have a strong effect on the selectivity of a separation. In the present study 50 mM concentration of the mobile phase was prepared by dissolving potassium dihydrogen phosphate in water and the pH value was adjusted to 2.5, 2.6, 2.7 and 2.8 with phosphoric acid. Effect of pH on mobile phase of separation of organic acid in HPLC method is presented in Fig. 2. From Fig. 2, it is observed that the K' for all organic acid is in increasing trend with increasing pH of mobile phase up to the pH 2.7. Above pH 2.7, the retention factor for all organic acid decreases with increasing pH of the mobile phase. The reason for the reduction of K' is due to increase in the degree of dissociation for the organic acid and changes in retention of the alkyl group on the surface of the column. According to the result obtained, it is concluded that the mobile phase of 50 mM KH_2PO_4 with pH 2.7 is suitable for determination of the organic acid concentration in the garbage enzyme solution.

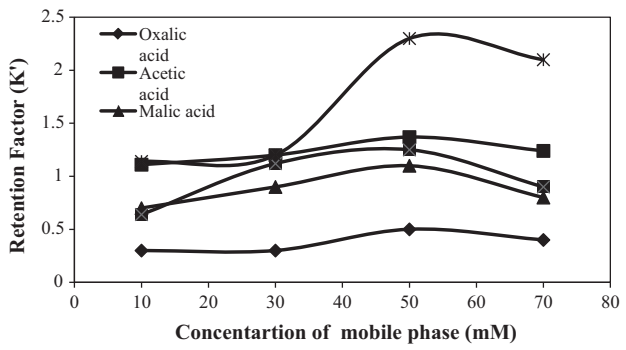

Fig. 1. Effect of concentration of the mobile phase on k' factor at constant temperature 35 °C and 0.8 ml/min flow rate of mobile phase.

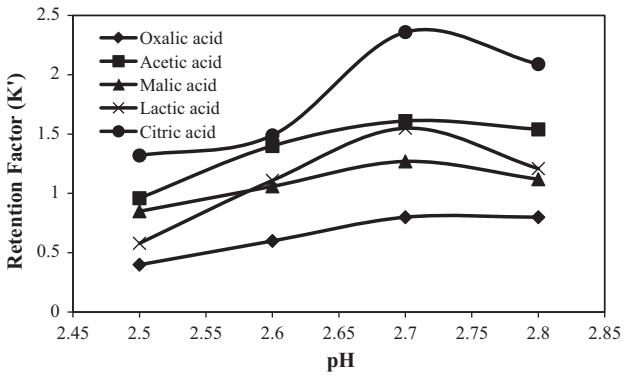

Fig. 2. Effect of pH of the mobile phase on k' factor at constant temperature 35 °C, 0.8 ml/min flow rate of 50 mM KH_2PO_4 mobile phase.

3.3. Effect of temperature on column oven

The effect of temperature on reverse phase HPLC separation showed drastic reduction in retention time and it showed the changes in peak spacing. Solute retention in RP-HPLC is influenced by temperature through changes in solvent viscosity. In the current study the column oven temperature is increased from 20–35 °C with the increment of 5. When the column oven temperature is at 30 °C, oxalic acid, citric acid, lactic acid, acetic acid and malic acid were eluted at minimum retention time of 4.06, 11.85, 7.12, 8.25 and 6.71 min respectively when compared with column oven temperature 20 °C and 25 °C (Table 2). At the column oven temperature (35 °C), the retention time got reduced and causes the decrease in separation factors due to changes in peak spacing. A Similar observation was reported by Qiu (1999), when determining the concentration of organic acid in tobacco using HPLC method. From the result obtained, it is concluded that the 30 °C column oven temperature is suitable for determination of the organic acid concentration in the garbage enzyme solution.

3.4. Effect of the mobile phase flow rate

The effect of the mobile phase flow rate on separation of organic acid in HPLC column is presented in Table 3. From the table it is observed that the retention time of organic acid was decreased with increasing flow rate from 0.5 to 0.7 ml/min. A higher flow rate

Table 2
Effect of column oven temperature at constant pH 2.7 and 50 mM KH_2PO_4 as mobile phase.

Organic acids	Temperature of column oven °C			
	20	25	30	35
Retention time (min)				
Oxalic acid	7.91	4.17	4.06	3.97
Citric acid	13.67	11.97	11.85	11.82
Lactic acid	9.81	7.92	7.12	7.04
Acetic acid	9.47	8.53	8.25	8.23
Malic acid	8.59	6.82	6.71	6.69

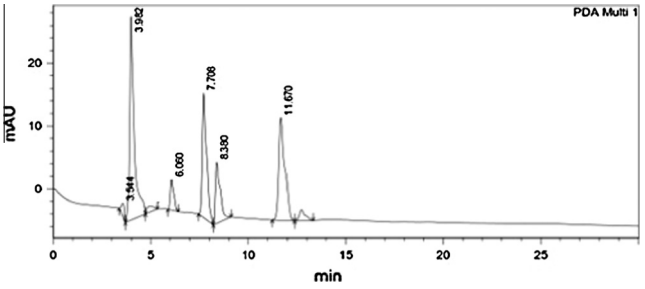

Fig. 3. Chromatogram of standard organic acid mixture at 30 °C column oven temperature, pH 2.7, and 0.7 ml/min flow rate of 50 mM KH_2PO_4 mobile phase.

may adversely affect the quality of the chromatography by not giving sufficient time for organic acids to interact with the stationary phase, as a result the retention time is reduced when increasing the flow rate. Among the standard organic acid used, oxalic acid was eluted first, and its retention time decreased from 8.07 min to 4.21 min when the flow rate of mobile phase increased from 0.5 to 0.7 ml/min. Malic acid was eluted last, and its retention time was decreased to 6.90 min from 9.28 min when the flow rate of mobile phase increased from 0.5 to 0.7 ml/min. The retention time was slightly decreased when the flow rate increased to 0.8 ml/min from 0.7 ml/min. When the flow rate increased from 0.7 to 0.8 ml/min, the degree of separation of organic acid got reduced assessing the solid stationary column. Therefore, the optimal flow rate proposed is 0.7 ml/min. A similar observation was reported by Shen et al. (1999) when determination of organic acid in root exudates using HPLC.

3.5. Determination of organic acid in garbage enzyme at different intervals of production

The mixture of organic acid (acetic, lactic, oxalic, malic and citric acid) was prepared and the same was analyzed using

Table 3
Effect of mobile phase flow rate at constant at temperature 30 °C, pH 2.7, 50 mM KH_2PO_4 as mobile phase.

Organic acids	Flow rate of mobile phase (ml/min)			
	0.5	0.6	0.7	0.8
Retention time (min)				
Oxalic acid	8.07	5.98	4.21	4.05
Citric acid	13.54	12.87	12.41	12.32
Lactic acid	10.16	8.15	7.89	7.04
Acetic acid	9.96	8.14	8.64	8.37
Malic acid	9.28	6.52	6.90	6.75

Table 4
Equation of the calibration graphs and R^2 value for standard organic acids.

Organic acid	R^2	Equation
Oxalic acid	0.998	$Y = 176149X - 440628$
Citric acid	0.997	$Y = 231235X - 831250$
Lactic acid	0.994	$Y = 111538X - 109139$
Acetic acid	0.997	$Y = 10684X - 123271$
Malic acid	0.998	$Y = 114779X - 331342$

RP-HPLC method at the optimized condition (i.e.) at 30 °C column oven temperature, pH 2.7, 50 mM concentration and 0.7 ml/min flow rate of the mobile phase (potassium dihydrogen phosphate in water). Fig. 3 presents the chromatogram of standard organic acid at proposed optimal conditions. From Fig. 3, it is observed that the retention time of the oxalic acid, malic acid, lactic acid, acetic acid and citric acid are 3.982, 6.060, 7.708, 8.380 and 11.670 respectively.

Different concentrations (20, 40, 60, 80,100 mg/ml) of organic acid were prepared from their respective stock solution of acetic, lactic, malic, citric, oxalic acids. The standard graph was obtained using the peak area of different acids as in the *y* axis vs. concentration of organic acid as in the *x* axis and is presented in Fig. 4.

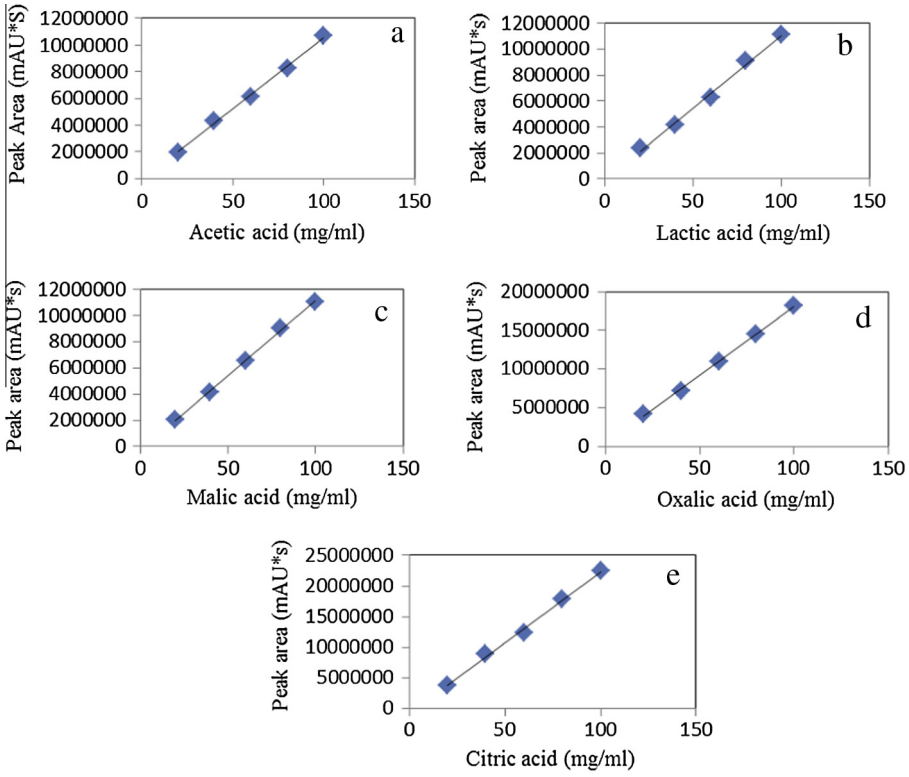

Fig. 4. Standard linear graph obtained at different concentration of (a) acetic acid, (b) lactic acid, (c) malic acid, (d) oxalic acid (e) citric acid.

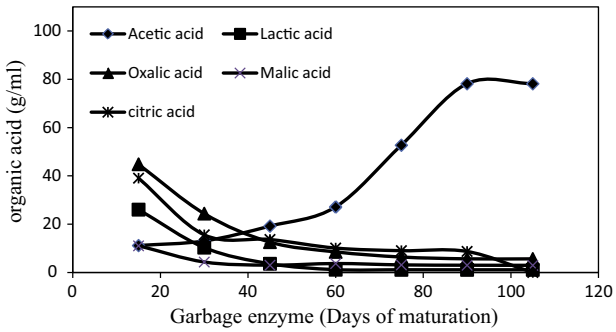

Fig. 5. Determination of organic acids in garbage enzyme at different intervals of production at 30 °C column oven temperature, pH 2.7, and 0.7 ml/min flow rate of 50 mM KH_2PO_4 mobile phase.

The standard curves obtained with a correlation coefficient above 0.99 were used as reference to estimate the concentration of organic acid in garbage enzyme (Table 4). The garbage enzyme solution collected in 15, 30, 45, 60, 75 and 90 days were used as sample to determine the concentration organic acid using proposed optimized HPLC method.

An organic acid in garbage enzyme at different time intervals of production was estimated and presented in Fig. 5. From Fig. 5 it is observed that the garbage enzyme obtained at 15th day showed the concentration of acetic, lactic, oxalic, malic and citric acid was 11.12, 26.02, 44.81, 11.05, 39.05 g/l respectively. When determining the concentration on the 90th day, the matured garbage enzyme solution showed that the concentration of acetic acid got increased to 78.14 g/l and become saturated whereas all other organic acid concentration was decreased. The reason for higher concentration of acetic acid is due to the anaerobic production of garbage enzyme. In anaerobic environment, during hydrolysis the complex organic compounds are converted into simple molecules, which resulted in the production of low molecular weight acetic acid. A similar trend was reported by Dhanalakshmi Sridevi et al. (2012) while demonstrating the conversion of carbohydrate content in vegetable waste to biogas in the single stage anaerobic reactor. This result confirms that the matured garbage enzyme contains a higher concentration of acetic acid. Also, the result obtained confirms that the variation in concentration of organic acid during the production of garbage enzyme, thus it can be used as a parameter for monitoring the different stages of the garbage enzyme fermentation process while going for semi-continuous production.

4. Conclusion

RP-HPLC method was performed for the determination of organic acids in the garbage enzyme solution. The effects of concentration of mobile phase, pH of the mobile phase, Temperature of column oven, the flow rate of the mobile phase on separation were investigated.

The concentration of organic acid was determined using RP-HPLC method at the optimized condition (i.e.) at 30 °C column oven temperature, pH 2.7, and 0.7 ml/min flow rate of the mobile phase (potassium dihydrogen phosphate in water) at 50 mM concentration.

The garbage enzyme obtained at 15th day showed a concentration of 11.12, 26.02, 44.81, 11.05, 39.05 g/l of acetic, lactic, oxalic, malic and citric acid respectively. The concentration of acetic acid in the garbage enzyme was found to be increased from 11.12 to 78.14 g/l at the 90th day of maturation from 15th day, whereas all other organic acids concentration was found to be decreased. This observation confirms the variation in concentration of organic acid during the production of garbage enzyme and it can be used as a parameter for monitoring the different stages of garbage enzyme fermentation process while going for semi continuous production.

The semi-continuous production of garbage enzyme solution in large quantity can be used as solution for solid and liquid waste treatment, fertilizer, antimicrobial agents, etc. Consequently garbage enzyme solution helps to produce a sustainable and pollution free environment.

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