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Effect of C/N Ratio and Physicochemical Conditions on the Production of Rhamnolipids by *Pseudomonas aeruginosa* LBI

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Abstract: Waste utilization for biosurfactant production is an important approach contributing to the reduction of waste treatment costs, while increasing the economic value of residues and reducing biosurfactant production costs. In an attempt to optimize rhamnolipid production by *Pseudomonas aeruginosa* LBI, the effects of the oil refinery waste as carbon source, C/N ratio, agitation speed, aeration rate, pH control and fed-batch culture conditions on the production of this biosurfactant were assessed in a bioreactor. Maximum production (16.9 g L^{-1}) was attained at a C/N ratio of 23, agitation at 800 rpm and aeration at 2.0 vvm. Controlling the pH and feeding the batch reduced biosurfactant production. The rhamnolipid solution (0.1%, w/v) exhibited surface and interface tensions of 25 mN/m and 0.63 mN/m, respectively. The critical micelle concentration (CMC) was 73.9 mg L^{-1}. The optimal parameters used in this study promoted the formation of a useful biosurfactant with potential for many industrial applications.

Key words: Rhamnolipids % C/N ratio % Soapstock % Aeration rate % Agitation speed

INTRODUCTION

Biosurfactants are secondary metabolites with surface-active properties and are synthesized by a large variety of microorganisms. The domains of these amphiphilic molecules depend on the carbon substrate composition and bacterial strain [1,2].

Microbial surfactants are commercially important compounds with a potential use in environmental protection, petroleum, food, pharmaceutical and other industries [3-6]. These molecules have attracted considerable interest due to their low toxicity, biodegradable nature and diverse structures [1,3,7]. Although biosurfactants have commercially attractive properties and clear advantages over their synthetic counterparts, they have not been employed extensively in industry due to their low yields and high production costs. Thus, in light of the economic constraints associated with biosurfactant production, three basic strategies have been adopted worldwide to make this process cost-competitive: (1) the use of cheaper and waste substrates to lower the initial raw material costs involved; (2) development of efficient bioprocesses, including optimization of the culture conditions and cost-effective separation processes for maximum biosurfactant production and recovery and (3) development and use of overproducing mutant or recombinant strains to enhance product yields [8].

A number of factors can influence biosurfactant production, including the nature of the carbon source, possible nutritional limitations, physical and chemical conditions, such as aeration, temperature and pH [9]. Moreover, the nitrogen source and concentration and the C/N ratio are also reported to have major effects on biosurfactant synthesis [10].

Large amounts of waste are generated by oil and fat industries, such as residual oils, tallow, soapstock and frying oils [11,12]. The use of such low-cost materials as alternative substrates could reduce the economic problem of biosurfactant production, following the development of suitable fermentative processes with high yields.

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The aim of the present study was to determine the best conditions for rhamnolipid production by *Pseudomonas aeruginosa* LBI in a fermenter with soapstock as a carbon source by adjusting the values of C/N, aeration and agitation and to assess the influence of pH control and feeding on production. Surface-active properties and critical micelle concentration (CMC) of the rhamnolipid were also determined.

MATERIALS AND METHODS

Microorganism and Inoculum: *P. aeruginosa* LBI [13] was maintained in a nutrient broth plus 20% glycerol at -20°C. Bacterial growth from a nutrient agar slant incubated for 24 h at 30°C was scraped from the slant tube and suspended in buffer; the optical density (at 610 nm) of the bacterial suspension was adjusted to 0.75. A 4 mL aliquot of this culture was incubated in a 1-L Erlenmeyer flask with 200 mL of a mineral salt medium and 2% (v/v) soybean oil as the carbon source [14]. The 200-mL inoculum was incubated for 24 h at 30°C and 200 rpm in a rotary shaker (New Brunswick, USA).

Culture Conditions

Bioreactor: Rhamnolipid production was carried out in a 5.0 L bioreactor with a working volume of 2.0 L, operating with a defoaming process followed by recycling the liquid into the system. A volume of 200 mL inoculum was added and the culture temperature fixed at 30°C. The carbon source used was soapstock, which is a waste from soybean oil processing.

Assessment of Effect of C/N Ratio: The percentage of carbon in the soapstock, based on the composition of waste fatty acids (Table 1) was determined by gas chromatography. The carbon to nitrogen (C/N) ratio was determined in terms of soapstock carbon content divided by $NaNO_3$ concentration (both in grams). The concentration of soapstock was fixed at 20 g L^{-1} (corresponding to 15 g of C), whereas the concentration of the nitrogen source was adjusted to 4 (0.65 g of N) and 7.14 g L^{-1} (1.17g of N), resulting in C/N ratios of 23 and 13, respectively. Two agitation speeds were used, 200 and 250 rpm; the reactor was aerated by sparger at 1 L air/min (0.5 vvm).

Assessment of Aeration and Agitation Speed: The effect of aeration and agitation speed on rhamnolipid production was determined in experiments with 4% of the carbon source. Two agitation speeds were used, 500 and 800 rpm; both were evaluated with two aeration rates of 2.0 L air/min and 4.0 L air/ min.

Table 1: Composition of fatty acids from oil refinery waste used as carbon source by *P. aeruginosa* LBI

Fatty acids composition	% (w/w) Oil wastes (Soapstock)
C16 palmitic	16.66
C18 stearic	3.58
C18:1 oleic	22.90
C18:2 linoleic	50.28
C18:3 linilenic	4.54

Assessment of pH Control and Fed-batch Process: The pH control was carried out at 800 rpm agitation and 2.0 vvm aeration; pH was maintained at 7.0±0.1 by the addition of 1M HCl. Fed-batch fermentation was carried out in the same conditions. The feeding consisted of adding 500 mL of the culture medium with 3% soapstock and 2 g L^{-1} of the $NaNO_3$. The flow rate was maintained at 0.2 mL/min. The feeding was performed after 24 h of fermentation in one experiment and after 48 h in another.

Analytical Measurements

Rhamnolipids: Rhamnolipids were measured in the cell-free broth as rhamnose [15]. Rhamnolipid content was determined by multiplying rhamnose values by 3.4 [16]. Analyses were carried out in triplicate. Error bars represent standard deviation.

Nitrate: Nitrate concentration was determined through ultraviolet spectrophotometric screening methods [17].

Biomass: Cell growth was estimated by counting viable cells (CFU/mL). The spread plate technique was used, serially diluting well-mixed samples in sterile water and spreading the samples on nutrient agar plates using a sterile glass spreader. Plates were counted after 24 h of incubation at 30°C. Only plates with counts of 30-300 were used to calculate the colony-forming units per mL (CFU/mL) for a given sample. Analysis was performed in three independent replicates. Error bars represent standard deviation.

Surface Activity: Surface and interface tension were measured with a Krüss Tensiometer (Krüss Germany) using the ring method. Interface tension was determined against hexadecane. The critical micelle concentration (CMC) was determined by measurements of the surface tension of serial dilutions of an aqueous solution of biosurfactants (0.1 %, w/v).

Rhamnolipid Extraction: The rhamnolipids were extracted from the culture medium after removing cells through centrifugation at 12,000 rpm for 20 min. The supernatant pH was adjusted to 2.0 with 6N H_2SO_4 and an equal volume of $CHCl_3$/CH_3OH (2:1) was added. The mixture was vigorously shaken for 10 min and allowed to settle until phase separation. The organic phase was removed and the operation was repeated again. The rhamnolipid product was concentrated from the pooled organic phases in a rotary evaporator. The viscous yellowish product obtained was dissolved in methanol and concentrated again by evaporation of the solvent at 45°C.

RESULTS AND DISCUSSION

Effect of Carbon to Nitrogen (C/N) Ratio on Rhamnolipid Production: A correlation was found between biosurfactant production, cell growth and nitrate use during the production of rhamnolipid by *P.aeruginosa* LBI cultured in media with various C/N ratios and agitation speeds (Fig. 1).

The cell growth was favored by a C/N ratio of 13, at both agitation speeds (Fig. 1A). The profiles of cell growth at 48 h were very similar, except for the curve representing the fermentation at 250 rpm agitation and a C/N ratio of 13, in which the metabolism was totally driven to increasing the biomass, reaching its maximum at 24 h and decreasing thereafter. This may account for the lower rhamnolipid production (2.03 g LG^{1}) illustrated in Fig. 1B. The increase in cell count after 50 h likely occurred due to a slow carbon source release, as the agitation speed in this case was lesser.

As illustrated in Fig. 1B, the two best rhamnolipid yields, 13.4 g LG^{1} (200 rpm) and 6.4 g LG^{1} (250 rpm), occurred at a C/N ratio of 23. Other C/N ratios were used in experiments (results not shown), but the C/N ratios of 23 and 13 show more interesting results. According to Guerra-Santos *et al* [10], a C/N ratio of 18 was optimal for maximum biosurfactant production in a continuous culture, reaching a final concentration of around 0.6 g LG^{1}. Wu *et al.* [18] found that the highest rhamnolipid levels (6.8 g LG^{1} and 7.5 g LG^{1}) were produced by *P.aeruginosa* EM1 at C/N ratios of 26 (glucose) and 52 (glycerol), respectively. When compared with these results, those of the present work show that both C/N ratios (13 and 23) provide high rhamnolipid concentration. However, the first ratio favored growth, whereas biosurfactant synthesis was higher in the second ratio. This was probably due to the lower concentration of nitrogen, thereby providing nitrogen-limiting conditions. Hommel *et al.* [19] reported that higher specific growth rates result in reduced biosurfactant production. Guerra-Santos *et al.* [10] provide additional evidence that biosurfactant production is associated with slow cell growth.

It is important to remember that there is a minimal quantity of nitrogen necessary for rhamnolipid production, as a negligible production was observed by Wu *et al.* [18] when carrying out experiments without a nitrogen source or high C/N ratios.

P. aeruginosa LBI produced rhamnolipids throughout the entire cell cycle; the biosurfactant was detected in the culture medium at the beginning of the incubation period. However, production of the compound became faster when the nitrogen source was exhausted in the medium (after 24 h of culture), when the bacterial population reached the stationary phase of the cell cycle (Fig. 1C). Raza *et al.* [20] report that a *P. aeruginosa* mutant produced rhamnolipids in two stages; in the first stage, the production was associated to growth and, in the stationary growth phase, the biosurfactant concentration continued to rise gradually. These profiles confirm the hypothesis of the acceleration of rhamnolipid synthesis under nitrogen-limiting conditions [20,21]. It has been reported [22] that lipogenesis can be stimulated in several microorganisms by depriving them of essential nutrients other than carbon, such as nitrogen; this could lead to the synthesis and excretion of biosurfactants [23]. When the limiting nutrient becomes exhausted in the medium, growth declines, but carbon continues to be transported into the cell, where it may be used for lipid biosynthesis. Thus, lipid biosynthesis may constitute the predominant route for carbon use during the stationary growth phase [23]. A slight increase in nitrogen concentration can be observed in the culture medium, coinciding with cell lysis, as seen in Fig. 1A and 1C. The culture pH increased and remained between 8.0 and 9.0 in all the fermentations studied (Fig. 1d).

Effect of Aeration and Agitation Speed on Rhamnolipid Production: The bacterial growth profiles at different agitation speeds and aeration rates were very similar (Fig. 2A), except in the case of fermentation at 800 rpm agitation and 2.0 vvm aeration, in which a growth peak was seen at 48 h, reaching 1.7×10^{13} CFU/mL. It is likely that the increase in cell number was favored because the nitrogen source ($NaNO_3$) was directed at the cell growth metabolism. At high rates of aeration and agitation, it was not necessary to employ nitrate as an electron acceptor for the respiratory pathways [24], since these conditions

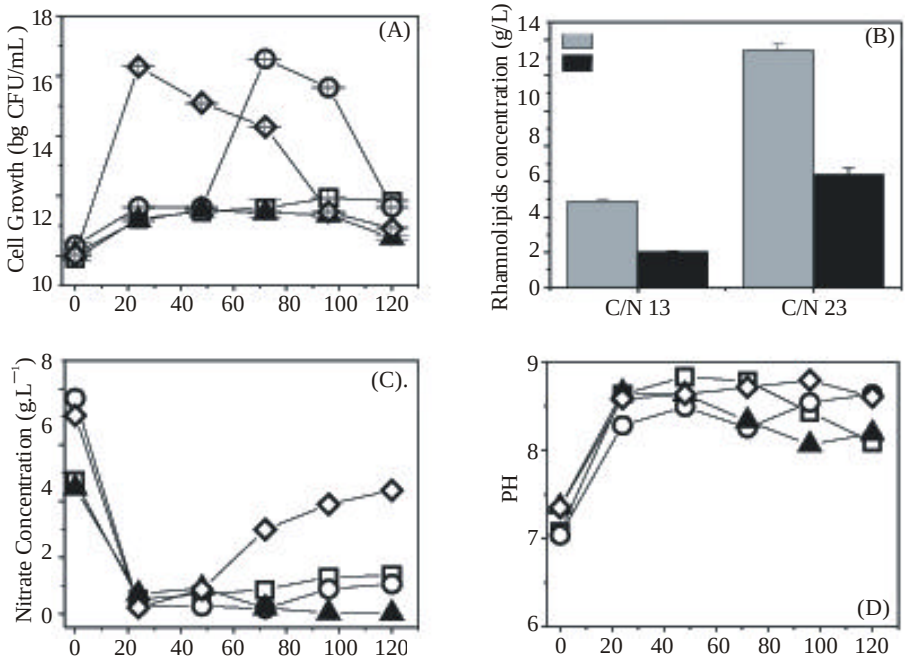

Fig. 1: Effect of varying C/N ratio on rhamnolipid production by *P. aeruginosa* LBI at various agitation speeds. (A) Cell growth (log CFU/Ml); (B) Rhamnolipid production (Gl^{-1}); (C) Nitrate consumption (Gl^{-1}) and (D) Culture pH. Symbols: —○— C/N of 13, 200 rpm; —◇— C/N of 13, 250 rpm; —□— C/N of 23; 200 rpm; —▲— C/N of 23; 250 rpm

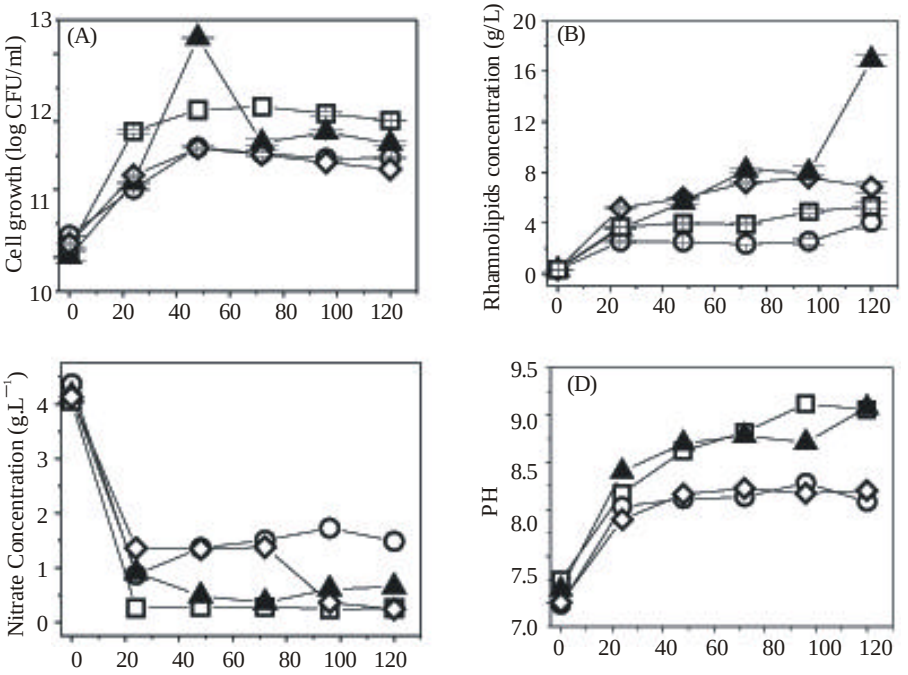

Fig. 2: Effect of agitation speed and aeration rate on rhamnolipid production by *P. aeruginosa* LBI. (A) Cell growth (log CFU/Ml); (B) Rhamnolipid production (Gl^{-1}); (C) Nitrate consumption (Gl^{-1}) and (D) Culture pH. Symbols: —○— 1.0 vvm, 500 rpm; —◇— 1.0 vvm, 800 rpm; —□— 2.0 vvm; 500 rpm; —▲— 2.0 vvm, 800 rpm

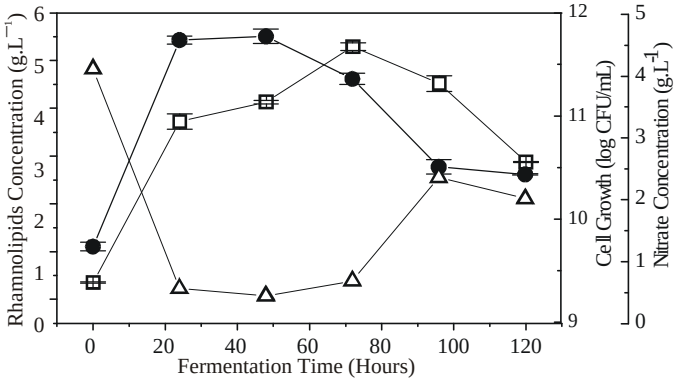

Fig. 3: Effect of pH control on rhamnolipid production by *P. aeruginosa* LBI, at various agitation speeds

stimulate aerobic respiration, in which energy yield is greater.

Raising the agitation and aeration rates had a positive influence on biosurfactant production, as shown by the productions obtained: 4.1 g L^{-1} (500 rpm and 1.0 vvm), 5.3 g L^{-1} (500 rpm and 2.0 vvm), 7.6 g L^{-1} (800 rpm and 1.0 vvm) and 16.9 g L^{-1} (800 rpm and 2.0 vvm) (Fig. 2B). Yeh *et al*. [4] found that an increase in aeration rate from 0.5 to 1.5 vvm and agitation from 200 to 300 rpm favored surfactin production by *B. subtilis*, although a further rise resulted in a drop in the synthesis rate.

The results demonstrate that an increase in agitation from 500 to 800 rpm promoted an increase in productivity from 0.03 to 0.08 g L^{-1} h^{-1} at 1.0 vvm of aeration. At twice the aeration rate, this enhancement was from 0.04 to 0.138 g L^{-1} h^{-1} (500 to 800 rpm). A significant rise in rhamnolipid yield (120 and 220% with 500 and 800 rpm of agitation, respectively) also occurred when the aeration rate increased from 1.0 to 2.0 vvm. Sabra *et al.* [26] proposed that *P. aeruginosa* produces rhamnolipids to reduce the oxygen transfer rate in order to protect itself from oxidative stress. Increasing the aeration and agitation enhances the oxygen transfer rate and the microorganism reacts by synthesizing the tensoactive compound, which binds to the air bubbles, thereby reducing the adverse effect of oxygen.

In the fermentation with the best production (16.9 g L^{-1}), most of the rhamnolipid production occurred in the stationary growth phase of the bacterium (between 96 and 120 h). In fact, in all fermentations, product synthesis was enhanced after the drop in nitrogen concentration (Fig. 2C). The same was observed by Kim *et al.* [21] during glycolipid production by *Candida* sp.

Effect of pH Control and Batch Feeding on Rhamnolipid Production: The culture pH became alkaline (8.5 and 9.0) in fermentations aerated at 2.0 vvm and reached about pH 8.0 at 1.0 vvm (Fig. 2D). According to Robert *et al.* [27], there is an increase in the pH of the culture medium during the production of rhamnolipids by *P. aeruginosa* 44T1, likely due to the metabolism of the nitrogen source ($NaNO_3$).

Controlling the pH resulted in a reduction in rhamnolipid production (Fig. 3). The yield was three times smaller (5.3 g L^{-1}) than in the fermentation with no pH control (16.9 g L^{-1}). After 48 h of the culture, there was a decline in cell growth, coinciding with a rise in nitrogen concentration. The increase in nitrogen concentration can be attributed to the carbon source, which is a complex waste and probably contains some nitrogen source. The ideal pH range for bacterial growth was around neutral. However, there is the possibility that this ideal range is not suitable for rhamnolipid synthesis by *P. aeruginosa* LBI. Robert *et al.* [27] found that 27°C was the best temperature for cell growth, while 37°C was better for product formation and the same may be true of pH.

The interaction between certain culture conditions and pH values caused inhibition in biosurfactant production. It is known that the RhlR protein regulates rhamnolipid synthesis, reacting to environmental stimuli [28]. Therefore, a group of physicochemical conditions stimulate RhlR synthesis and, consequently, biosurfactant production, whereas other conditions cause inhibition.

Batch feeding had a negative effect on rhamnolipid production (Fig. 4). The production profiles were identical until the feeding was initiated (after 24 h of fermentation). From this point onward, a decline in synthesis occurred, settling at a maximum yield of 4.0 g L^{-1}. The same occurred when the feeding was started at 48 h of fermentation. The best production was obtained without feeding and reached 16.9 g L^{-1}. The feeding had an

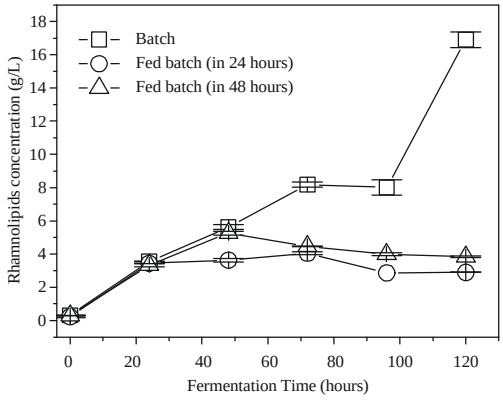

Fig. 4: Effect of batch feeding on rhamnolipid production by *P. aeruginosa* LBI cultured in a soapstock (4%); agitation 800 rpm, aeration 2.0 vvm, temperature 30°C

inhibitory effect on the bacterial metabolism due to the excess of the carbon source. Syldatk *et al.* [29] found that yield dropped when excess glycerol was used. Adamczak and Bednarski [30] found that fed-batch conditions had a negative effect on *Candida antarctica* biosurfactant production after the addition of the carbon source, decreasing the yield from 45.5 to 28.0 g L^{-1}.

The search for waste for biosurfactant production is very important, potentially contributing to a reduction of waste treatment costs, while increasing the economic value of residues and reducing biosurfactant production costs. Previous studies have achieved a rhamnolipid concentration of 11.7 g L^{-1} with *P. aeruginosa* LBI grown in soapstock [31] and 9.9 g L^{-1} in Brazil nut oil [32]. Costa et al. [12] grew P. aeruginosa LBI, LMI 6c and LMI 7a in soybean frying oil, respectively obtaining 5.0; 6.1 and 7.7 g L^{-1} of biosurfactant production. Rahman *et al.* [33] report that *P. aeruginosa* DS10-129 and GS9-119 in soybean oil produced 4.31 g L^{-1} and 1.75 g L ổf rhamnolipid, respectively. Maximum production (16.9 g. L^{-1}) reached in the present study was very similar that found by Benincasa *et al.* [13], who used waste from cottonseed oil processing as a carbon source. The results show that the waste from oil refineries used as carbon source and the values of the physicochemical parameters used were very effective and appropriate.

Characterization: Surface tension of a 0.1% (w/v) solution of rhamnolipids was 25.0 mN m^{-1}. The interface tension against hexadecane decreased from 40 mN m^{-1} to 0.63 mN m^{-1}, exhibiting excellent surface-active properties and this biosurfactant could be applied in bioremediation of oil-polluted areas and as an emulsifying agent for hydrocarbons and oils.

A wide range of CMC values have been reported for rhamnolipids: e.g. 53 mg L^{-1} [34] and 230 mg L^{-1} for a mixture of seven rhamnolipid homologues [35]. The CMC value for the rhamnolipids synthesized by *P. aeruginosa* LBI from a soapstock residue was 73.9 mg L^{-1}.

CONCLUSIONS

The present study demonstrated that the C/N ratio plays an important role in rhamnolipid production by *P. aeruginosa* LBI. The best C/N ratio was 23 and resulted in high rhamnolipid production (13.4 g L^{-1}). The best agitation and aeration values were 800 rpm and 2.0 vvm respectively and resulted in an excellent yield (16.9 g L^{-1}). The control of pH control and batch feeding had negative effects on rhamnolipid production. The rhamnolipids showed excellent reduction of surface tension (65%) and interface tension (98%) and exhibited a low CMC (73.9 mg L^{-1}).

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