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Production of single cell protein from *Saccharomyces cerevisiae* as an alternative source of protein diet for fish feed

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Abstract
Production of Single cell protein (SCP) from *Saccharomyces cerevisiae* was investigated. Baker yeast, Saf-Instant *S. cerevisiae* was activated on Peptone Yeast Glucose (PYG) media. Agro industrial waste, cane molasses and brewery waste were used as substrate to produce SCP biomass for fish feed. For optimization, basal media was used for *S. cerevisiae* culture. Optimization was done to find the effectiveness of cane molasses, ammonium source and brewery waste on the growth of the *S. cerevisiae*. The optimization results showed that basal media with 60 brix 10 % molasses, 2g/l-$(NH_4)_2$ SO_4, 5 % brewery waste on 4th day of fermentation period. Under the optimum parameter, batch fermentation process was done for SCP production, the results revealed that maximum protein content for dry biomass was 29 % after 4th day of fermentation period.

Keywords: single cell protein, *S. cerevisiae*, brewery waste, molasses, fish feed

1. Introduction
Globally, aquaculture plays an important role in food security and poverty alleviation. The global population is increasing and in order to maintain at least the current level of per capita consumption, the world will require an additional 23 million tonnes of aquatic food by 2030, which much come from aquaculture. (FAO, 2012) [10].The demand for aqua feeds also continuous to increase. The important aqua feed ingredients, fish meal supply must continue to increase to meet future demand. Tacon *et al*. 2012 [31] predicted aqua feed production may increase to more than 70 million tonnes by 2020. Feed makes up to 50-60 % of total production costs in aquaculture (Rana *et al*., 2009) [24]. The main protein source for fish feed ingredients is fish meal. Challenges for fish meal production is cost and source of supply. The use in aqua feed, continuous search for other protein source for partially or completely replacement of fish meal in aquaculture production. Varity of protein source for fish feed are animal protein, plant protein, single cell microbial protein. Microbial protein source for aqua feed include algae, fungi and bacteria. Depending on the research finding single cell protein can be a possible protein source for replacement of fish meal in aqua feed. Among the microbial protein sources, so far yeast products are commercially available and become new protein source for fish feed due to their low production cost and high protein content 50-55% (Suman G *et al*., 2015) [29].
Nowadays, yeast is widely used as probiotic and larval feed for growth and immunity promotor in aquaculture. Typical dry yeast composition is 93 - 97 % dry matter and can contain from 40-60 % crude protein nitrogen, 35 - 45 % carbohydrates, and 5-9 % lipids. The Amino acid profile of yeast is close to soybean meal and therefore well adapted to animal nutrition; it is rich in glutamic acid and lysine (up to 8 %). Yeast is naturally rich in vitamin B such as biotin, thiamine and folic acid (Tacon, 2013) [30]. *S. cerevisiae* is useful organism because of easy culture, rapid growth and appropriate cell diameter (approximately 5 - 10 um). *S. cerevisiae*, most of the strains of Baker's yeast have been selected for their high fermentative power, particularly useful for bakers. Baker's yeast comes as a pure and primary culture grown on sugar substrate such as molasses. Yeast cell walls produced from Baker's yeast usually have a high content of mannans. They are recognized as good toxin binders. The most popular aquaculture application of Baker's yeast is in hatcheries where it is a major feed source for artemia (Fazeli and Azari-Takami, 2006) [12] and rotifer (Tacon, 2013) [30].
Many raw materials have been considered as substrate for SCP production (Nasseri *et al*., 2011) [20].Variety of substrates such as starch, molasses, fruits and vegetable. Brewery waste also known as brewer's grain or spent grain, is a byproduct of the brewing process. After brewing process, the waste still has protein and fiber-rich leftovers are good and suitable for fish feed. Brewer's waste used as fish feed ingredient, it can be a potential for replacement of protein source for fish feed by conversion to single cell protein form *S. cerevisiae.*
Objective of this study is that fulfilled the requirement of aqua feed industry is finding alternative protein source by using cheap and ecofriendly bio waste substrate incorporated with fermentation technology to produce single cell protein for fish feed. This study was conducted to give information about brewery waste can be fermented using *S. cerevisiae* could improve its protein content.

2. Materials and Methods
2.1 Baker yeast
Baker yeast, Saf-Instant (*S. cerevisiae*) was used for fermentation, instant dry yeast culture on peptone yeast extract glucose (PYG) agar plate for 48 hours and a single colony transferred to PYG medium incubated 24 hours culture used as inoculum.

2.2 Brewery waste
Brewery waste samples were collected from Myanmar beer factory, Mandalay. It was dry and grinded to make powder. Biochemical analysis of dry brewery wastes was determined by AOAC [4] methods. Brewery waste hydrolysate were prepared by treating with 10 % Hcl for one hour autoclaving

at 121 °C for 30 minutes (Mondal *et al.,* 2012) [19].

2.3 Molasses
Molasses were collected from sugar factory from Mandalay Industrial Zone. Molasses filtered with no1 filter paper and make dilution with distilled water to desired brix and stored in refrigerator. Biochemical analysis of molasses was determined by AOAC [4] methods.

2.4 Media preparation
The following basal media were used for this experiment. Peptone yeast extract glucose (PYG) medium: peptone - 20 g/l, yeast extract - 10 g/l, glucose - 20 g/l, used for inoculum preparation. Basal medium was used for the fermentation D-glucose - 10 g/l, $(NH_4)_2SO_4$- 5 g/l, KH_2PO_4- 1 g/l, $MgSO_47H_20$ - 0.5 g/l, NaCl - 0.1 g/l, $CaCl_2$- 0.1g/l (Dhanasekaran *et al.*, 2011) [9] throughout the experiment.

2.5 Optimization of molasses concentration
Optimization was performed on basal media with different concentration in 5%, 10% and 15% molasses of 10 brix, 20brix, 30 brix, 40 brix, 50 brix and 60 brix respectively. Basal media without glucose prepared with different concentration of molasses distributed and one loopful of *S. cerevisiae* growth from the surface of the medium added to culture flasks, pH 5.3-5.5 incubated in water bath shaker at 35 °C, 120rpm for 6 days. *S. cerevisiae* cultured on basal medium with glucose as control. Every 24 hours, the growth rate was measured by UV vis-spectrophotometer at 600 nm (Bergman L.W, 2001) [5].

2.6 Optimization of ammonium sulfate concentration
For the optimization of ammonium sulfate $(NH_4)_2$ SO_4 on *S. cerevisiae* growth was determined by using basal medium including 10% 60 brix molasses with 0.1% to 0.5% of ammonium sulfate concentration, pH 5.3-5.5 incubated in water bath shaker at 35°C,120rpm for 6days. Every 24 hours, the growth rate was measured by UV vis-spectrophotometer at 600nm(Bergman L.W, 2001) [5].

2.7 Optimization of brewery waste concentration
Optimization was determined by using viable cell count every 24hours of incubation period. A loopful of *S. cerevisiae* from PYG agar medium cultured on glucose free basal medium including 10% 60 brix molasses, 0.2% $(NH_4)_2SO_4$ with five brewery waste concentrations 1%, 2%, 3%, 4% and 5% respectively, in shaker flasks, pH 5.3-5.5 and incubated in water bath shaker at 35°C,120 rpm for 6days.

2.8 Inoculum preparation
Single colony of *S. cerevisiae* from 24 hours PYG agar plate culture added to the PYG medium incubated at 24 hours.The cell suspension was diluted with sterile distilled water adjustto OD 1 (4×10^6) and it used as inoculum 1:100 for fermentation.

2.9 Fermentation and harvesting
Batch culture aerobic Fermentation was performed for SCP production using molasses basal media (without D-glucose) including $MgSO_47H_20$ - 0.5 g/l, Nacl - 0.1 g/l, CaCl2 - 0.1 g/l, KH_2PO_4 - 1g/l, $(NH_4)_2SO_4$ - 2 g/l supplement with molasses 10 % 60 brix and 5 % brewer waste hydrolysate, at temperature 35°C, pH 5.3 - 5.5. The medium was distributed in shaker flasks and sterilized at 121 °C for 15 minutes. Inoculum was added to each flask incubated at 35 °C, 120 rpm for 6 days in shaking incubator. Every 24 hours of incubation time, biomass was harvested by centrifugation 6000 rpm for 30 minutes, filtration with no1 filter paper and ovum drying at 40 °C. Crude proteins of biomass were analyzed by Kjeldahl method.

3. Results and Discussion
3.1 Biochemical analysis of brewery waste and molasses
Biochemical analysis of brewery waste result is presented in Table.1. Brewery waste is cheap and easily available all year round from beer factory and which can be used as fish feed ingredient. Brewery waste composition depends on the nature of grains, period of fermentation, brewing process and analytical procedures (Senthilkumar 2010) [28].Brewery waste has highly moisture content 80 - 85 % (Mathias *et al*., 2015) [18]. Present study, Brewer's spent grain made powder to avoid contamination for long termed used, moisture content 7.27% and ash 3.12% while Ajanaku *et al*. (2011) [2]mentioned that moisture content was 6.14%. Santos *et al*. (2003) [26] reported that brewery waste ash content 3.12 % was similar with present study. Zhaoxia *et al*. (2012) [34] evaluated range between 3.4% and 4.4% for ash on a dry basis. However, Senthilkumar (2010) [28] mentioned that high total ash and crude protein of brewery waste was 5.76% and 24.34%. In present study, protein content of dry brewery waste was about 14.51 %. Roberson (2010) [25] and Faulds *et al*. (2009) [11] found the protein content to be near 18%. Celus *et al*. (2006) [7] found crude protein value 26.7 %, crude fat value 2.82 % and carbohydrate value 41.09 %. Ajanaku *et al*. (2011) [2] reported value of crude fat 2.79 % and carbohydrate 51.3 %. The crude protein, crude fiber content of brewery waste was 24.34 %, 19.62 %, respectively, on dry matter basis (Mathias *et al*., 2015) [18] (Curtin 1983) [8]. According to the results brewery waste showed low protein, ash, fat compared to reported results. Protein content depends on the raw materials used and processing methods applied (Forssell *et al.,* 2008) [14]. Present study, brewery waste contains high carbohydrate content, it might be energy source for microorganism to support the biomass production.

Nutritional value of cane molasses in presented in Table. 2. Cane molasses are by products of sugar industry. Composition of various molasses differs according to the specific geographic area of production (Sarlin and Philip, 2013) [27]. According to the result, molasses crude protein 4.72 % and ash 4.28 %. This is in agreement with Caldwell (2015) [6] and Olbrich (1963) [21] reported that the molasses content fall in range between, protein 1.2 - 10.6 % and ash 5.9 - 13.4 %. In present study, high value of dry matter 75.9 %, and total sugar 67.42 % was observed when compared to Caldwell (2015) [6] and Olbrich (1963) [21] reported value dry matter 67.5 - 74.6 % and total sugar 46 - 62 %. Reported data indicated that the content of molasses was subject to wide variations. In some cases, depending on origin and processing, cane molasses has contained as much as 10.6 % protein, mostly molasses contains no protein content. 2/3 of sugar consists of sucrose (Leclerc, 2003) [17].

Table 1: Chemical composition of brewary waste.

Sample marked	Brewery waste
Crude protein (%)	14.51
Fiber (%)	27.94
Fat (%)	2.82
Carbohydrate (%)	41.09
Moisture (%)	7.27
Ash (%)	3.12

Table 2: Chemical composition of cane molasses.

Sample marked	Molasses
Crude protein (%)	4.72
Ash (%)	4.28
Dry matter (%)	75.90
Total sugar (%)	67.42

3.2 Effects of molasses concentration on *S. cerevisiae* growth

Fig. 1 illustrates the growth of *S. cerevisiae* in basal media with 5 % molasses at 10 - 60 brix during 6 days fermentation period. Maximum growth was observed on 4th day incubation at 60 brix 5 % concentration. Among the tested concentration control showed minimum growth rate of *S. cerevisiae* on1th day of incubation. Fig. 2 represents the effect of 10 % molasses concentration in different brix 10 - 60 on the growth of *S. cerevisiae* during 6 days fermentation period. The results revealed that the maximum growth rate occurred on 4th day of incubation at 60 brix 10 % concentration. Minimum growth rate of *S. cerevisiae* was recorded on 1st day of incubation at 10 brix 10 % concentration. Fig. 3 shows the effect of 15 % molasses concentration in different brix 10 - 60 on the growth of *S. cerevisiae* during 6 days fermentation period. The maximum growth rate of *S. cerevisiae* was recorded at 60 brix 15% on 4th day of incubation. The minimum growth rate was recorded in control on 1st day of incubation. According to optimization results, among the different concentration (5%, 10%, 15%) of molasses in 10 – 60 brix, the optimum growth rate of *S. cerevisiae* was achieved by 60 brix 10 % on 4th day of incubation.

Yeast cell can grow on a variety of carbon source. Molasses is cheap source of carbon for fermentation and large-scale production of SCP biomass. It contains most of the nutrient required for yeast growth and rich source of minerals calcium, sodium, magnesium, copper, zinc, iron and manganese(Feedipedia, 2017) [13]. In present study, increased growth rate occurred along with increased molasses concentration from 5% to 10% in different brix when concentration increased to 15 % molasses in different brix was showed low growth rate. Sarlin and Philip (2013) [27] found that increased growth of marine yeast with increased molasses concentration however more than9mg/ml molasses concentration was found to have adverse effect and lesser growth. Periyasamy *et al*. (2009) [22] reported that bioethanol concentration increased along with molasses concentration increased 300g/l and further increasing of molasses inhibits the ethanol productivity of *S.cerevisiae*. In present study, better concentration was 10% when compared to 5% and 15%. Among the different brix in 10%, optimum molasses concentration was recorded on 4th day of incubation at 60 brix 10%.Over all, after 4thday of fermentation the growth was likely to decline due to less nutrient and less activity of yeast. Some inhabitation effect occurs during long-term incubation period due to sugar depletion and less nutrient caused growth rate decline. Which mentioned that the consequences result in decrease SCP production.

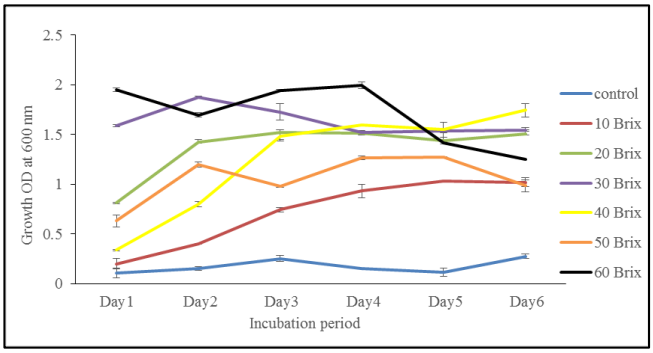

Fig 1: Growth of *S. cerevisiae* in control and basal medium with 10 – 60 brix 5 % molasses concentration for 6 days incubation periods. OD = optical density, the value indicates the means and error bar shows standard deviation, n=2.

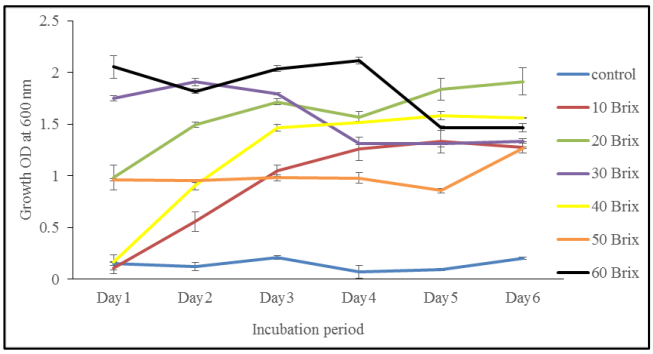

Fig 2: Growth of *S. cerevisiae* in control and basal medium with 10 - 60 brix 10 % molasses concentration in for 6 days incubation periods. OD = optical density, the value indicates the means and error bar shows standard deviation, n=2.

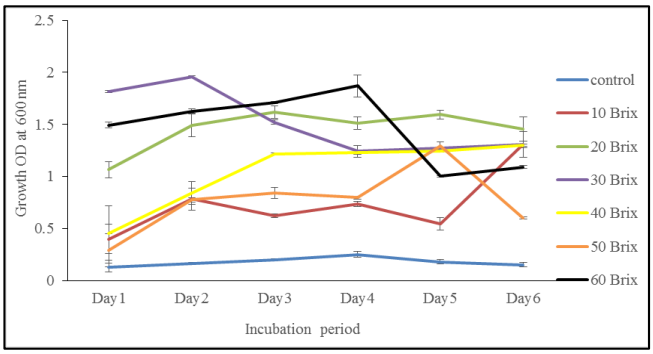

Fig 3: Growth of *S. cerevisiae* in control and basal medium with 10 – 60 brix 15 % molasses concentration for 6 days incubation periods. OD = optical density, the value indicates the means and error bar shows standard deviation, n=2.

3.3 Effects of ammonium sulfate and brewery waste concentration

Fig. 4 reveals that 60 brix 10 % molasses supplement with basal media containing five ammonium sulfate concentration tested as a nitrogen source on the growth of *S. cerevisiae* for 6 days fermentation periods. *S. cerevisiae* growth results showed that the maximum growth rate occurred at the concentration 0.20 % on 4th day and the minimum growth rate occurred at the concentration 0.50 % on 4th day. SCP production depends on substrates type and media composition. The yeast cell can grow on the variety of nitrogen source. The optimum ammonium sulfate concentration was 0.20 % on 4thday of fermentation period.

The ammonium sulfate concentration increased to 0.50% the growth rate decreased. This result agreement with Hoda *et al.* (2010) [15] reported that excess amount of nitrogen source associated with ammonium ion may retarded growth rate.

Fig. 5 presents that effect of brewery waste concentration on the growth of *S. cerevisiae*. This study performed that the basal medium supplement with 60 brix 10 % molasses, 0.20% ammonium sulfate and different concentration (1%-5%) of brewery waste for 6 days fermentation. Brewery waste is locally available agricultural wastes product from beer factory the whole year. The optimum brewery waste concentration was 5% on 4th day of incubation and the growth rate continuous decreased to 6thday.High carbohydrate content of brewery waste supports the *S. cerevisiae* growth. The same finding in fungus *Aspergillus terreus* showed maximum protein yield at 5% concentration of the substrate Eichomia and banana peels (Jaganmohan *et al.*, 2013) [16] Generally, high growth rate of *S. cerevisiae* occurred on 1th day of incubation in all concentration. This finding was revealed that *S. cerevisiae* inoculum size (4×10^6) might be higher. Similar finding by A.O. Ojokoh and Uzeh (2005) [3] reported that viable cell count of *S. cerevisiae* in papaya extract medium were found to decrease after one or two days dueto disproportionate increase in growth and autolysis is likely to be increased with high initial inoculums (Reade and Gregory1975) [1].The minimum growth rate of *S. cerevisiae* by brewery waste concentration was 5% on 6th day of incubation. It was indicated that prolong incubation time affected on *S. cerevisiae* growth.

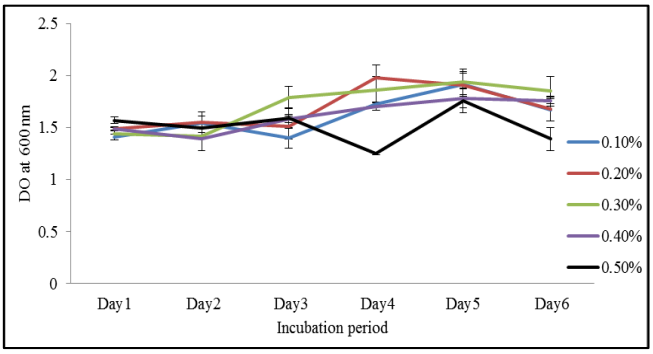

Fig 4: Growth of *S. cerevisiae* at 0.10 - 0.50 % ammonium sulfate concentration in basal medium supplement with molasses (60 brix 10%) for 6 days incubation periods. OD= optical density, the value indicates the means and error bar shows standard deviation, n=2.

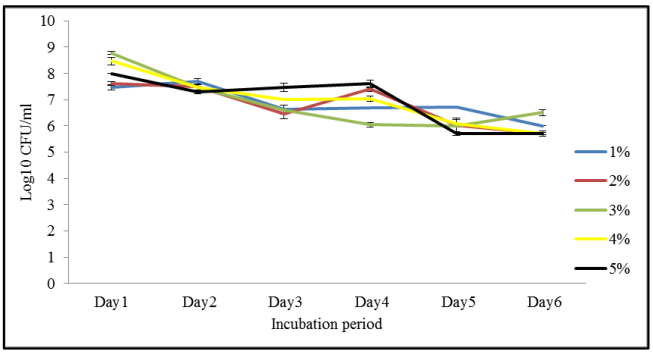

Fig 5: Growth of *S. cerevisiae* at different brewery waste concentration 1 - 5 % in molasses (60 brix 10%) basal medium with 0.2% $(NH_4)_2SO_4$for 6 days incubation periods. CFU = Colony Forming Unit, the value indicates the means and error bar shows standard deviation, n=2.

3.4 SCP biomass production

Mean protein content and weight of SCP biomass are shown in Table. 3. Production of SCP biomass was performed by under optimization parameter, *S. cerevisiae* in basal medium with selected molasses concentration 60 brix 10 %, ammonium sulfate concentration 0.20 % and brewer waste concentration 5 %. Nutritional value of SCP depends on nature of substrate, organism and processing (Suman G *etal.*, 2015) [29]. Some finding revealed that SCP production from *S. cerevisiae* with different fruit waste such as orange, cucumber pineapple, papaya for animal feed (Mondal *et al.*, 2012; Dhanasekaran *et al.*, 2011;A.O. Ojokoh and Uzeh, 2005) [19, 9, 3]. Papaya medium from dry biomass protein content showed 35.5% (A.O. Ojokoh and Uzeh, 2005) [3]. The highest crude protein 53.4% produced from cucumber peel by *S. cerevisiae* and orange peel with *S. cerevisiae* had 30.5%(Mondal *et al*., 2012) [19]. In present study, maximum dry biomass was 4.61g with 24% protein content was observed on 1st day of fermentation due to *S. cerevisiae* could not degrade substrate completely well and it didn`t enhance the protein content. The present finding was agreement with (Viva and Sinha, 2005) [33] reported that the study of SCP production by *Trichodarma harzianum* showed that high biomass on day 1 was occurred. The highest protein content of SCP biomass was 29% with 3.83g on 4th day of fermentation this is due to fully degrading substrate as energy and carbon source by *S. cerevisiae.* Maximum protein content and dry biomass of *S. cerevisiae* were recorded on 3thand 7th days of fermentation at 5% concentration of pineapple waste (Dhanasekaran *et al*., 2011) [9]. More reduce fermentation substrate the greater the oxygen demand for growth and metabolism (Vasey and Powell, 1984) [32]. The protein content of SCP dry biomass gradually declined to26% with less biomass 3.38g on 6days of fermentation period. Increased fermentation period did not enhance protein content and weight of SCP biomass in batch fermentation due to limited amount of substrate concentration. Progressive decline of substrate has considered for energy requirement of organism for growth (Pirt, 1975) [23]. It's also meant that the protein of *S. cerevisiae* depleted and spoilage after 4 day of incubation period.

Table 3: Mean crude protein content and weight of SCP dry biomass in different fermentation periods.

Incubation period	Biomass dry weight gram in 100 mL	Protein content (%)
day1	4.61	24
day2	4.33	26
day3	3.68	27
day4	3.83	29
day5	3.40	26
day6	3.38	26

4. Conclusion

The growth of microorganism depends on many factors: pH, temperature, nutrient composition and incubation periods (Vasey and Powell, 1984) [32]. This experiment was carried out to study the effect of substrate concentration, carbon, nitrogen source for SCP production. Higher molasses (15%) and $(NH_4)_2SO_4$ (0.5%) concentration showed inhabited the growth of *S. cerevisiae*. The increased in growth rate of *S. cerevisiae* were observed when increased brewery waste to 5% concentration. In this study to set media designed showed

that high carbon and low nitrogen ratio supplemented with some mineral. According to the optimization results, the following concentrations 60 brix 10 % molasses, 0.20 % ammonium sulfate and 5% brewery waste on 4th days of fermentation are recommended for SCP production. The best time for SCP production with 29% high protein content on 4th day of fermentation. The dry biomass was good texture and smell for fish feed, it can be used as appetizer that attract to fish. So that it has many potentials for fish feed ingredients. This experiment revealed that SCP can be produce from *S. cerevisiae* with brewery waste, it can be partially or fully replacement of high cost fish meal in aquaculture feed industry.

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