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The Effect of Different Strains of *Monascus purpureus* on the Color Value and Citrinin in Angkak and Their Similarities using *ctnA* Gene

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Abstract:
The present study was conducted to evaluate the color value, pigment, and citrinin content from red yeast rice produced by six different strains of Monascus purpureus. The difference of the six strains is based on the phenotypic properties of cultures in Malt Extract Agar (MEA) media. Further, the degree of similarity among the tested citrinin-producing strains was analyzed by using DNA sequence from ctnA gene. The color value was analyzed based on CIE system, including lightness (L), redness (a*), and yellowness (b*), whereas the pigment was analyzed by extracting angkak with 75% ethanol and determined spectrophotometrically using UV-Vis to obtain absorbance value (OD). Citrinin was analyzed by using ELISA method. The tested Monascus purpureus strains significantly affected the color value, pigment, and citrinin in angkak. All the isolates yielded various concentration of citrinin ranging from 0.24 ppm to 146.71 ppm. The resulting phylogeny supported the placement of six strains as a single clade with Monascus ruber M7/KT781075.1, M. aurantiacus EU309474.1, and M. purpureus orf1/AB243667.*

Keywords: *strains, color value, citrinin, ctnA gene*

1. Introduction

Angkak, also called Red Mold Rice (RMR) and Red Yeast Rice (RYR), is the fermentation product of ordinary rice with certain mould species of the genus *Monascus*. It is widely known because of multipurpose, such as for natural coloring for many years. The color of angkak is composed of a mixture of pigments produced by *Monascus* sp. The Monascus pigment is composed of azaphilone and furanoisophthalide pigments, which are generally classified into red, purple and yellow compounds based on their optical spectra [1]. Besides producing colorants and beneficial secondary metabolites, unfortunately, most of *Monascus* strains also yield nephro- and hepatoxic mycotoxin. The mentioned mycotoxin, citrinin, is also produced by other species, *Penicillium citrinum* and *Aspergillus* sp. [2].

Citrinin is classified to polyketides group that is synthesized by iterative type I polyketide synthase [3]. Citrinin biosynthesis in *Monascus* initiated from a tetraketide arising from the condensation of one acetyl-CoA molecule with three malonic-CoA [4]. It is evidenced that polyketide synthase (*pkSCT*) and transcriptional activator (*ctnA*) were involved in citrinin biosynthesis [4, 5]. The *ctnA* gene which codes Zn(II)2Cys6 binuclear DNA binding protein is a main activator in citrinin biosynthesis. Consequently, the disruption of *ctnA* gene may lower the citrinin production.

The presence of citrinin may be a potential threat to the acceptance of *Monascus*-fermented product. Although the different perspective on the acceptability of related products appear, Taiwanese and Japanese government has set maximum allowable amount of citrinin content for *Monascus*-fermented product [6].

In the earlier study, *Monascus* sp. from commercial angkak was isolated. Some isolates showed strongly dissimilarity compared to the general properties of *M. purpureus* cultured on MEA media. In fact, the result of molecular identification using ITS1/ITS4 primer demonstrated that all tested isolates were 100% identical with *M. purpureus*.

Therefore, this present study was performed to compare the number of pigments and citrinin content from the different strains of *Monascus* isolated from angkak which possess various phenotypic characters and to evaluate the similarity among *M. purpureus* strains using their *ctnA* gene.

2. Materials and Methods

2.1. Microorganism

Six isolates from commercial angkak in Indonesia were used in this study, namely: *Monascus purpureus WMSCU 2015 JK2*, *M. purpureus WMSCU 2015 JK9A*, *M. purpureus WMSCU 2015 JK9B*, *M. purpureus WMSCU 2015 SB14A*, *M. purpureus WMSCU 2015 YK16B,* and *M. purpureus WMSCU 2015 BT30B*. The phenotypic characters of tested isolates were compared to that of *M. purpureus* LIPIMC 0141 on Malt Extract Agar (MEA) growth medium which was composed by adding powdered malt extract (20 g), peptone (1 g), glucose (20 g), and; agar (15 g) to distilled water and bringing volume to 1 L. Phenotypic recording of the strains refers to the morphological and cultural nature of *Monascus* [7, 8].

2.2. Inoculum Preparation for Fermentation

The inoculum was prepared prior to use and obtained from stock culture. It was inoculated in 2 cm diameter test tube on sterilized Potato Dextrose Agar (PDA) media and then incubated for 8 to 10 days in a culture room. After incubation, the spore suspensions were prepared by adding 5 mL distilled water to the inoculation tube. Next, the suspensions were scraped off slowly and were then counted microscopically with a hemacytometer. Later, the concentration was adjusted to target concentration up to 1×10^6 spores per mL.

2.3. Angkak Production

A long grain white rice IR64 was purchased from local market. Afterwards, it was washed under tap water and was wind-dried at 40°C until moisture content of 13.5% (according to initial state of moisture content). Fifty-grams of rice was transferred to Erlenmeyer flask and the moisture content was set about 50%. The sterilization of rice was done using oven at 121°C for 15 minutes. After cooling, the substrates were inoculated with 10% of spore suspensions (1×10^5 spores per gram substrate) and then was incubated for 12 days at 30°C. After 12 days, the wet angkak was dehydrated in oven at 45°C for 12 to 14 hours. The dried angkak was weighed to determine the yield (%) and next, it was grounded into fine powder. The above-mentioned procedure was performed in duplicate for 2 batches fermentation.

$$\%\ yield\ = 100\ \text{x}\frac{dry\ product}{50\ \text{gram}}$$

2.4. Moisture Content Measurement

The moisture content was measured according to A.O.A.C method [9]. One gram sample of angkak was oven-dried at 105˚C to a constant weight. The following formula was used to calculate the moisture content.

$$\%\ moisture = 100\ \text{x}\frac{\text{initial weight} - \text{final weight}}{\text{initial weight}}$$

2.5. Color Measurement

The color measurement of sample using Chroma Meter CR-400 followed the manual instructions of the manufacturer (Minolta Co. Ltd., Osaka, Japan). The Chroma Meter was calibrated with white color standard plate CR-400 prior to use. The color space reading included L* (lightness) having a range of 0 (black) to 100 (white), a* (redness) indicating the direction of +/- (red/green) and b* (yellowness) indicating the direction of +/- (yellow/ blue). The hue angle (˚hue) was determined as ($\tan^{-1}$ b*/a*) and the chroma as $\sqrt{(a^2 + b^2)}$ showing the color and saturation of color, respectively [10, 11]. Each sample was measured thrice for each batch replication.

2.6. Pigments Measurement

Pigments measurement was carried out following the method of [12] with slight modification. One g of finely grounded angkak powder was extracted by diluting 75% ethanol at a ratio 1:5 (w/v). The mixture was shaken at 200 rpm for 1 hour and then was sieved through Whatman No. 1 filter paper. The extract was centrifuged at 10,000 rpm for 5 minutes and measured using spectrophotometer (Shimadzu UV - 1280) at wavelength of 400 nm for yellow pigment, 470 nm for orange pigment, and 500 nm for red pigment. More dilution was needed when the absorbance value exceeded a value of 1.00. The following formula determined the color value which expressed pigment unit.

$$\text{Color Unit}\ \left(\frac{\text{AU}}{\text{g}}\right) = \frac{ODx\ volume\ \ extract\ x\ dilution\ factor}{gram\ sample}$$

2.7. Citrinin Quantification

ELISA method was used to quantify the citrinin content according to Ridascreen®Fast Citrinin (r-biopharm) procedure. Citrinin was extracted with 70% methanol (5:12.5) w/v and shook at 150 rpm for 3 minutes. One mL of filtrate was mixed with 1 mL of double-distilled water. The next step was done following Biopharm procedure. Absorbance at wavelength 450nm value was recorded in a micro plate reader (optic ivy men system 2100-C). The limit detection was 15 ppb. The absorbance values were converted to levels of citrinin by using the software of Ridasoft Win.NET - FAST citrinin. The tests were performed twice and the data were presented as a mean ± standard deviation (SD).

2.8. DNA Extraction

The extraction of DNA genome for each isolate followed protocol of [13] with slight modification as follows. The strain culture of *Monascus* grown on PDA for 7 days were transferred to 1.5 mL Eppendorf tube with 500 µL of PDB and then incubated for 8 days at 25-30°C. The mycelia were collected and centrifuged at 13,000 rpm for 5 minutes until the pellet formation was done. The pellet was washed by 500 µL of buffer TE and re-pelleted. Buffer TE was decanted and soaked to 300 µL of extraction buffer. The extraction buffer contained 200 mM Tris HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS. The mycelia were grounded by using conical grinder and then 150 µL of Na-acetate 3 M (pH 5.2) was added. The tube was kept for 10 to 15 minutes at -20˚C. The mixture was centrifuged in micro centrifuge for 5 minutes and the supernatant was transferred to a fresh tube with 1 × isopropanol (similar volume) and kept at ambient temperature for 15 minutes. DNA pellet was centrifuged briefly and then washed by 70% ethanol. The pellet was dried and redissolved in 50 µL TE buffer.

2.9. DNA Amplification Using ctnA Gene

The ctnA gene was amplified by polymerase chain reaction (PCR) using the forward primer reg F (aaactacgctgtgacggaca) and the reverse primer reg R (taactgcaccagacgaaacg) [5], The PCR was performed in a 25 µL reaction mixture containing 2 µL DNA (about 50 ng genomic of *Monascus* DNA as a template), 0.2 mM of each primer, 800 mM dNTP, free-RNAse aquabidest and 2 units Taq DNA polymerase. The reaction was performed for 30 cycles: denaturation at 95°C for 1 min, annealing at 50°C for 1 minute, extension at 72°C for 2 minutes, with an initial denaturation at 95°C for 5 min before cycling and final extension at 72°C for 10 min after cycling. An aliquot of the PCR amplification product was electrophoresed in a 0.8% agarose gel, 1X TBE buffer, stained and observed under ultraviolet light to compare with DNA ladder. Automated sequencing was carried out by using two aforementioned primers. The sequences were manually submitted to Gene Bank, National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/).

2.10. Sequence Alignment and Phylogenetic Analysis

The sequences were aligned with CLUSTALW [14] the gap opening cost and the gap extension cost were set at 15 and 30, respectively. All nucleotides were unordered with equal weights for the estimation of the phylogenetic relationship among the strain. Phylogenetic trees were constructed by the maximum likelihood. Bootstrap test were performed with 1000 replications.

2.11. Statistical Analyses

The experimental data was obtained from two batch fermentations. On each batch, three replications were performed. All data for water content, yield fermentation, lightness, hue, chroma, pigment yield at 500 nm, and ratio of 500/400 as well as citrinin content were subjected to Analysis of Variance (ANOVA). Fisher's Least Significant Difference (LSD) test was used as a post-hoc test for mean comparison at a significance level of 5%.

3. Results and Discussions

3.1. Microorganism Characteristics

The observed results of the phenotypic and culture character of the six strains of Monascus are shown in Table 1. *Monascus purpureus* colony was orange in color or orange with white at the margins, plane or sometimes with little aerial growth at the center; bearing abundant aerial mycelium, short to elongated with orange pigmentation at the center and white at the margin [7, 8]. The similar characteristics were also shown by the tested strains JK2, JK9A, and SB14A compared to the referent isolate (LIPIMC 0141). Meanwhile, BT30B colony was slightly different with pale orange in color, whereas JK9B and YK16B strain was white in color (Table 1) which was contrastive with general characteristic of *Monascus purpureus*, *M. pilosus, or M. ruber.* The three species of *Monascus* were traditionally isolated species from red oriental foods [2, 15]. However, the identification result using molecular testing showed that two strains were *Monascus purpureus* (data unpublished).

The identification result was supported by microscopic characteristics of *Monascus* strains which showed that there was no significant difference compared to referent isolate and clearly indicated that the tested isolates were strain of *Monascus purpureus*.

3.2. Yield and Moisture of Angkak

Moisture content of angkak ranged from 8.05% to 9.25% after drying at 45°C for 12 to 14 hours (Table 2). Moisture content is associated with shelf life of the product. The moisture content of commercial angkak product was approximately 10% [16]. Twelve-days fermentation resulted yield of dry mass (%) with a range of 34.75% to 52.66% (Table 2). Other researchers showed that the yield of dry mass from 10-days fermentation of *Monascus purpureus* was 36.4%. In detail, at the end of a 240-hours fermentation, carbon

balance of *Monascus purpureus* was as follows: 23% of carbon was converted to biomass, 35% to CO_2, 15% to ethanol, 1% to acetic acid and 17% remained unused [17]. The yield of dry mass of JK9B and YK16B strain was higher than that of other strains. The substrate degradation by microbe to the formation of fermented products depend on external and internal factors which were microbe types, at both species and strain level.

3.3. Citrinin Content

Citrinin content from fermentation of *Monascus purpureus* greatly varied with a range of 0.36 ppm (SB14A) to 146.71 ppm (BT30B) (Table 2). JK9B and YK16B strain also produced citrinin at low level, however, pale in product appearance. JK9B and YK16B colonies were white on surface and orange to pale orangish brown on reverse.

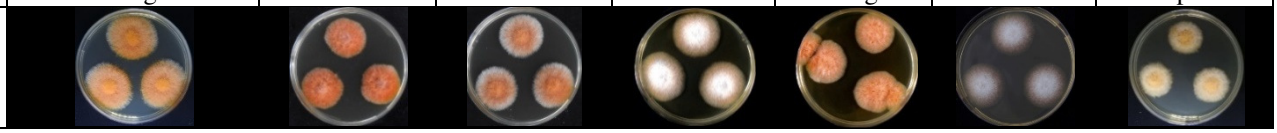
Cultural and phenotypic characters	**LIPIMC0141**	**JK2**	**JK9A**	**JK9B**	**SB14A**	**YK16B**	**BT30B**
ASEXUAL – ALEURIOCONIDIA							
Shape	pyriform	pyriform	pyriform	pyriform	pyriform	pyriform	Pyriform
Colour	hyaline	hyaline	hyaline	hyaline	hyaline	hyaline	Hyaline
Type of conidia chain	straight	straight	straight	straight	straight	straight	Straight
Number of conidia	2 - 4	2	2-3	2	2	2 - 4	2 – 4
Size (µm)	8.8x11.6 – 9.9x13.4	8.2x8.5 – 9.3x11.3	5.4x6.2 – 6.4x8.2	6.1x7.2 – 8.4x11.7	7.1x10.8-9.5x11.1	13.5x14.3 – 14.0x15.4	6.7x10.7 – 9.2x11.4
SEXUAL – CLEISTOTHESIA							
Shape	subglobos	globos	globos	subglobos	globos	subglobos	subglobos
Colour	hyaline	hyaline	hyaline	hyaline	hyaline	hyaline	hyaline
Size, Diameter (µm)	54.6x61.7 – 54.2x65.6	49 - 50	39 - 41	64.4x70.9 – 68.3x75.7	50 - 55	55.16x60.0 – 63.1x73.3	49.4x52.3 – 53.9x64.9
SEXUAL - ASCOSPORE							
Shape	oval	oval	oval	oval	oval	oval	oval
Colour	hyaline	hyaline	hyaline	hyaline	hyaline	hyaline	hyaline
Size (µm)	4.1x5.5 – 4.5x6.1	4.1x5.4 – 4.5x5.6	4.8x5.4 – 4.7x5.8	4.1x5.9 – 5.0x6.2	4.2x5.5 – 3.9x5.6	4.2x5.7 – 5.5x7.0	4.1x5.2 – 4.6x5.3
COLONY							
Diameter (mm)	38	30-32	30	37-38	32	34-36	28-30
Colour	Orange red, white edge	Orange, white edge	Orange, white edge	White opaque, pale orange edge	Orange white	White opaque, pale orange edge	Pale orange, white edge
Shape	Flat, slightly rised in the centre	Flat	Flat	Flat	Flat	Flat	Flat, slightly rised in the centre
Aerial mycelium	Long, orange in the centre, white at the edge, abundant	Long, white, abundant	Long, orange in the centre, white at the edge, abundant.	Short, white, abundant	Long, white, abundant	Long, white, abundant	Long, orange in the centre, white at the edge, abundant
Excudates	orange	-	-	-	orange	-	transparent
Picture							

Table 1: Phenotypic and cultural characters of Monascus purpureus LIPIMC 0141 and Monascus purpureus strains on MEA, 25˚C, 7 days

Angkak	Yield of dry mass (%)	Moisture (%)	Citrinin (ppm)
JK2	37.93 ± 1.84	8.66 ± 0.22	2.74 ± 0.37 [a]
JK9A	39.54 ± 2.74	8.19 ± 0.05	82.80 ± 10.97[b]
JK9B	50.04 ± 8.02	9.25 ± 0.08	0.24 ± 0.04[a]
SB14A	34.75 ± 1.26	9.05 ± 0.92	0.36 ± 0.12[a]
YK16B	52.66 ± 0.85	8.67 ± 0.83	0.82 ± 0.52[a]
BT30B	40.37 ± 1.48	8.05 ± 0.64	146.71 ± 2.85[c]

Table 2: Yield fermentation, moisture and citrinin content of angkak produced by strains M. purpureus

3.4. Color value and pigment

The pigment composition which was produced during fermentation determined the color value of angkak. Hue angle for six strains of *Monascus* ranged from 24.54 (JK9A) to 56.88 (JK9B). The lower hue angle causes the redder *Monascus*-fermented product. In this present study, the hue angle of product was about 30. The hue angle of JK9B and YK16B strain was more than 30 (Table 3). Lightness (L*) of both strains was also high (more than 70) which was nearly similar to the lightness of rice prior to fermentation (approximately 82-83). The hue angle JK2 strain was less than 30, but the lightness was relatively high. It indicated that JK2 was brighter than JK9A. This indication was also supported by pigment yield data as shown in Table 4. Angkak produced by JK2 strain had ratio of 500/400 less than 1 as it tended to be orange. The composition of lightness, hue angle, and chroma showed very dark red or purplish red in color for angkak produced by JK9A strain, while showed very pale orange for angkak produced by JK9B strain. Angkak produced by SB14A strain had ratio of 500/400 greater than 1 which indicated that the production of red pigment was more than that of yellow and orange pigment. Due to low total pigment, this study suggested that SB14A strain were not a promising isolate for pigments production, as well as BT30B strain.

Angkak	Lightness (L*)	Redness (a*)	Yellowness (b*)	°Hue	Chroma
JK2	42.36 ± 13.53[b]	26.66 ± 0.19[c]	14.23 ± 2.06	28 ± 3.60[ab]	30.28 ± 0.80[d]
JK9A	24.18 ± 0.76[a]	25.99 ± 1.08[c]	11.85 ± 0.04	24.54 ± 0.83[a]	28.56 ± 1.00[c]
JK9B	78.02 ± 2.66[d]	10.13 ± 2.85[a]	15.13 ± 0.31	56.88 ± 6.93[d]	18.34 ± 1.83[a]
SB14A	52.86 ± 4.22[c]	23.55 ± 0.11[c]	14.49 ± 0.06	31.60 ± 0.02[b]	27.65 ± 0.13[c]
YK16B	73.56 ± 4.03[d]	15.53 ± 2.27[b]	14.79 ± 0.71	43.89 ± 2.83[c]	21.47 ± 2.12[b]
BT30B	53.27 ± 10.68[c]	23.92 ± 1.79[c]	14.42 ± 0.63	31.22 ± 2.99[b]	27.97 ± 1.21[c]

Table 3: Color value of angkak produced by strains *M. purpureus*
Values expressed are means ± standard deviation; *a-d,*
means in the same column with different superscripts were significantly (p≤0.05) different

3.5. The Similarity among strains of Monascus purpureus using ctnA gene

The amplification result of *ctnA* gene from all tested strains is shown in Figure 1. This clearly evidenced that all tested strains of *Monascus* were able to produce citrinin. The gene *ctnA* is a main activator in citrinin biosynthesis. The similarity among strains of *Monascus* using their *ctnA* gene were shown in Figure 2.

Angkak	OD400 (AU/g)	OD470 (AU/g)	OD500 (AU/g)	Total (AU/g)	Ratio 500/400
JK2	169.60 ± 44.80	100.35 ± 3.05	151.35 ± 8.25	421.30 ± 33.50	0.97
JK9A	460.78 ± 116.14	288.23 ± 49.52	480.9 ± 61.6	1229.90 ± 227.27	1.08
JK9B	16.83 ± 4.23	8.72 ± 2.78	11.58 ± 1.02	37.13 ± 5.98	0.75
SB14A	47.19 ± 4.91	33.68 ± 3.92	59.2 ± 10.2	140.07 ± 19.03	1.25
YK16B	34.50 ± 2.82	21.75 ± 0.75	31.23 ± 0.69	87.48 ± 4.26	0.91
BT30B	55.55 ± 10.55	32.14 ± 2.46	54.65 ± 3.51	142.34 ± 16.52	1.01

Table 4: Pigment yield of angkak produced by strains M, pupureus

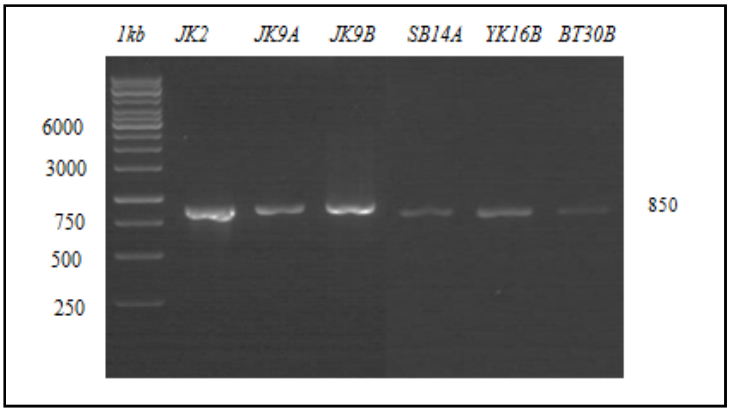


Figure 1: PCR amplification of ctnA gene for DNA genome of the investigated strains of M. purpureus

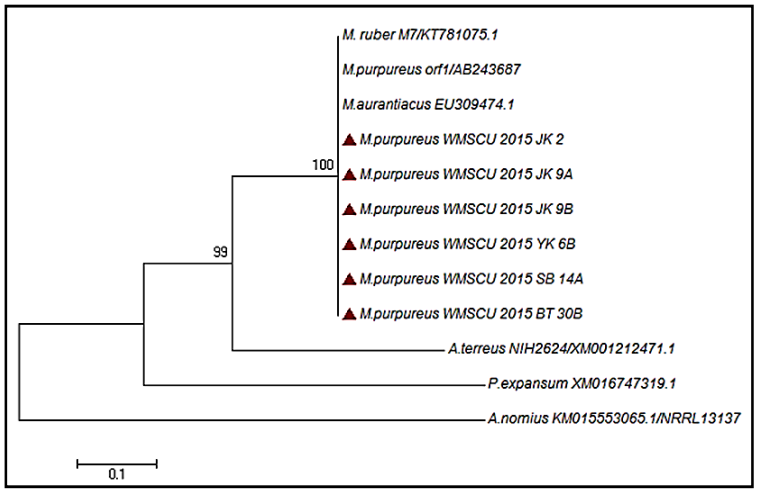


Figure 2: Phylogeny of Monascus purpureus strains

All tested samples were successfully amplified and sequenced using primer reg F dan reg R. The isolated sequence of DNA has a length of 802 bp. Alignment using ClustalW showed no various sequence of DNA for all samples. The base composition of the sequence was A (21.3%), T (21,7%), G (28,1%), and C (28.9%).

The similarity searching of DNA sequences used NCBI molecular database (https://blast.ncbi.nlm.nih.gov) with Basic Local Alignment Search Tool (BLAST) showed that the number of hits found on *Monascus purpureus* was only 1, 2 hits on *M. ruber,* and 1 hit on *M. auranticus.* The degree of similarity for all samples reached 99%, whereas the degree of similarity for other species, *Penicillium expansum*, *Aspergilllus terreus* and *A. nomius* was 90%, 88% and 83%, respectively. The findings indicated that the DNA sequence of citrinin in *Monascus purpureus* had a very high similarity with that of citrinin in *M. ruber* and *M. auranticus.*

4. Conclusion

The conclusion of this study is that the strains type of *Monascus purpureus* highly influenced the color value, pigment, and citrinin of angkak. The high similarity of *M. purpureus* with *M. ruber* and *M. aurantiacus* presumed the citrinin production from related angkak products.

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