CHAPTER 4

RESULTS AND DISCUSSION

4.1 Validation of Analysis methods

4.1.1 Development and optimization of Chromatographic conditions

The amount of monacolin K in red fermented rice sample preparation was determined by HPLC–PDA with the UV spectra as shown in appendix 1. The peak of monacolin K was identified by comparing both the retention times and the UV spectra with the standard. Monacolin K showed a characteristic mountain-like UV spectrum with three maximum absorptions at 229, 237, and 246 nm, respectively (Endo, 1979).The column of Waters Symmetry C_{18} (250mm × 4.6mm i.d., 5 μm) was chosen as the stationary phase. The isocratic elution was applied using acetonotrile/ phosphoric buffer pH 2.5 (70:30) as the mobile phase. The amount of 20 μl sample was eluted with at 1.0 ml/min flow rate, and column temperature was controlled at 25°C. The total analysis time was 15 min. The photo-diode array (PDA) detector was set at 237 nm for detection. This procedure was used as a developed method for monacolin K analysis with retention time of 8.173 min.

The amount of citrinin in red fermented rice sample preparation was determined by HPLC–PDA with the UV spectra as shown in appendix 1. The peak of citrinin was identified by comparing both the retention times and the UV spectra with the standard. Citrinin possessed a UV spectrum with the maximum absorbtion of 330 nm. The column of Waters Symmetry C_{18} (250mm × 4.6mm i.d., 5 μm) was chosen as the stationary phase and an isocratic elution at 0.75 ml/min of acetonotrile/ formic acid

buffer acid pH 2.5 (70:30) was applied. The analysis time was 10 min. Photo-diode array (PDA) was set at 330 nm for. The column temperature was set at 25 ∘C, and the injection volume was 20µl. This procedure was used as a developed method for citrinin analysis with retention time of 6.020 min.

As described in 3.2.3.1 and 3.2.4.1, the extraction processes for monacolin K and citrinin are different. The reason is that amount of citrinin detected in samples is usually significantly lower than monacolin K so that citrinin needs to be concentrated in the extraction to be able to be detected by the developed technique. It can also explain why sometimes citrinin are lower than the detection limit of some methods.

4.1.2 Precision and accuracy determination

The results shown in table 4.1 are %recovery of mevinolin and citrinin standards analyzed by the optimized conditions. They showed the accuracy of the developed methods as % recovery of mevinolin and citrinin are 98.81 and 101.73 respectively.

The precision of the analysis method is demonstrated by Relative Standard Deviation (%RSD). The data of six replicate injections indicated a repeatability of 4.5295 and 1.2538 %R.S.D for mevinolin and citrinin respectively.

4.1.3 Linearity

The calibration curves were established by plotting the peak areas against standard concentrations using linear regression analysis. Eight mevinolin standards were prepared at 0, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0 and 75.0 ppm and injected to

HPLC/PDA with the optimum chromatographic conditions. The correlation coefficient for this curve was 0.9984.

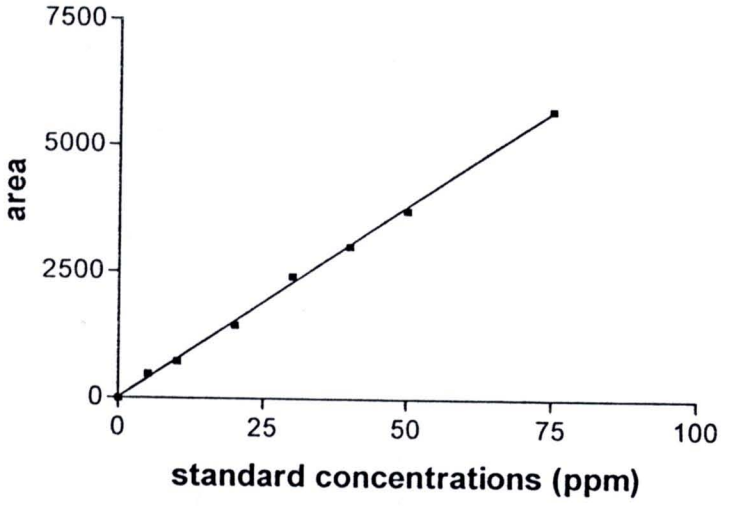
Similarly, eight citrinin standards were prepared at 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 and 5.0 ppm. and injected to HPLC/PDA with the optimum chromatographic conditions. The correlation coefficient for this curve was 0.9978.

4.1.4 Limit of detection and limit of quantitation determination

A series of diluted standard preparations were injected with the volume of 20 µl. The limit of detection (LOD) and limit of quantitation (LOQ) were measured as the concentrations corresponding to signal-to-noise ratio of 3:1 and 10:1 respectively. The LOD for monacolin K and citrinin were 0.5 and 0.08 ppm respectively. The LOQ for monacolin K and citrinin were 1.5 and 0.25 ppm respectively. At the quantitation limit of 1.5 ppm for monacolin K and 0.25 ppm for citrinin, the data of six repeated injections indicated repeatability with a deviation of 4.5295 and 1.2538 %(RSD) respectively.

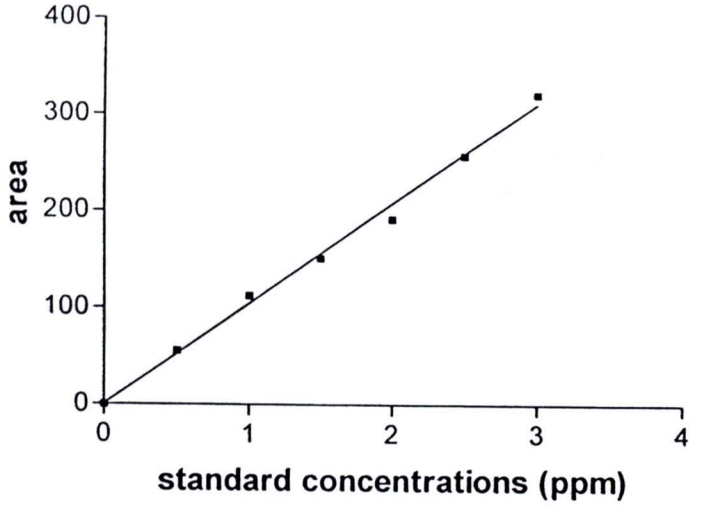
Table 4.1 Limit of detection and limit of quantitation determination

metabolites	LOD	LOQ	%RSD	%recovery
Monacolin K	0.50 ppm	1.50 ppm	4.5295	98.81
citrinin	0.08 ppm	0.25 ppm	1.2538	101.73



r^2 0.9984

Fig 4.1 Standard curve of monacolin K



r^2 0.9978

Fig 4.2 Standard curve of citrinin

4.2 Optimization of monacolin K production

4.2.1 Effects of Monascus strains and cultivation temperatures on monacolin K production

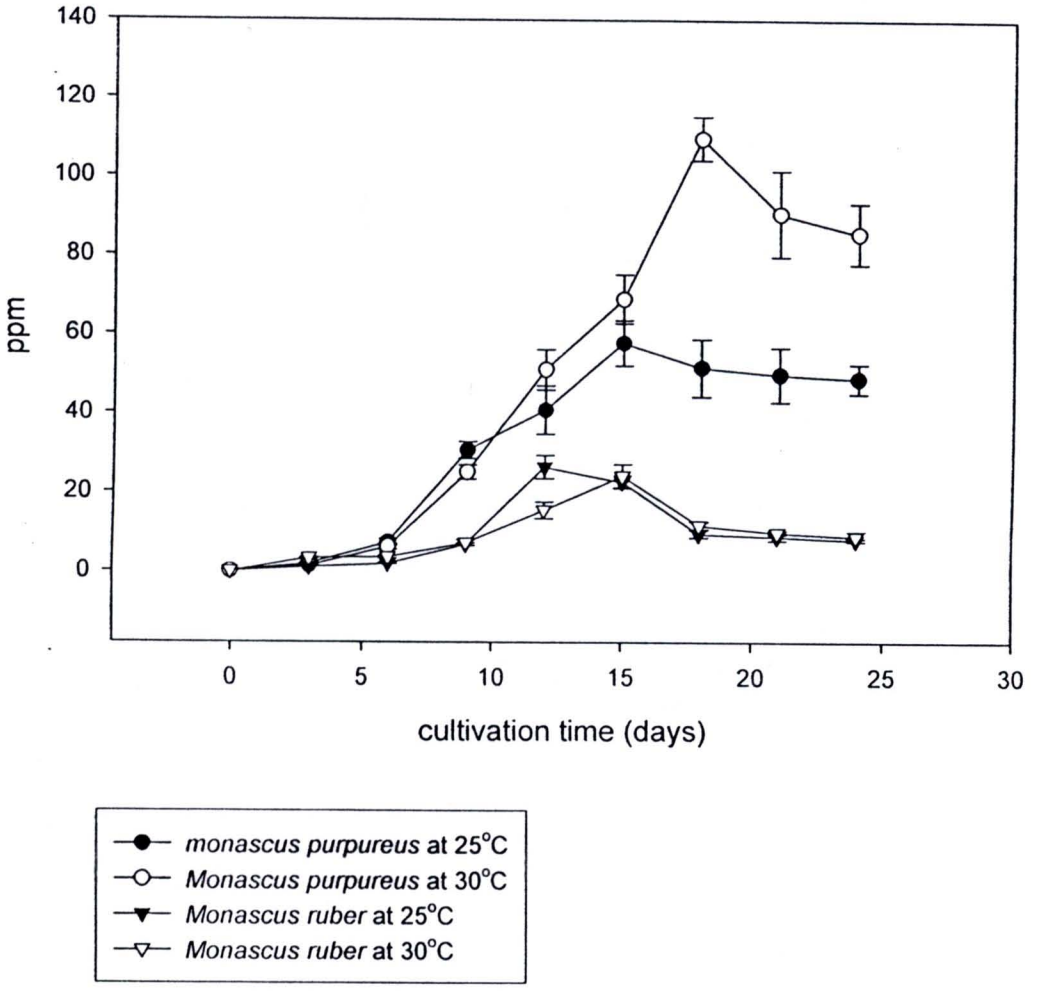


Fig 4.3 Effects of Monascus strains and cultivation temperatures on monacolin K production

According to the results above, *Monascus purpureus* BCC 6131 significantly produced monacolin K more than *Monascus ruber* TISTR 3006. The opimum temperature for *Monascus purpureus* BCC 6131 to produce monacolin K was 30°C as had been reported with *Monascus purpureus* CCRC 31615 by Su et al., 2003. At 30°C,

Monascus purpureus BCC 6131 produced 109.862 ±5.4930 ppm of monacolin K. Therefore *Monascus purpureus* BCC 6131 was selected as a potential strain, which can be used to produce red fermented rice with high concentration of monacolin K.

4.2.2 Effect of moisture content on monacolin K production of M. purpureus BCC 6131

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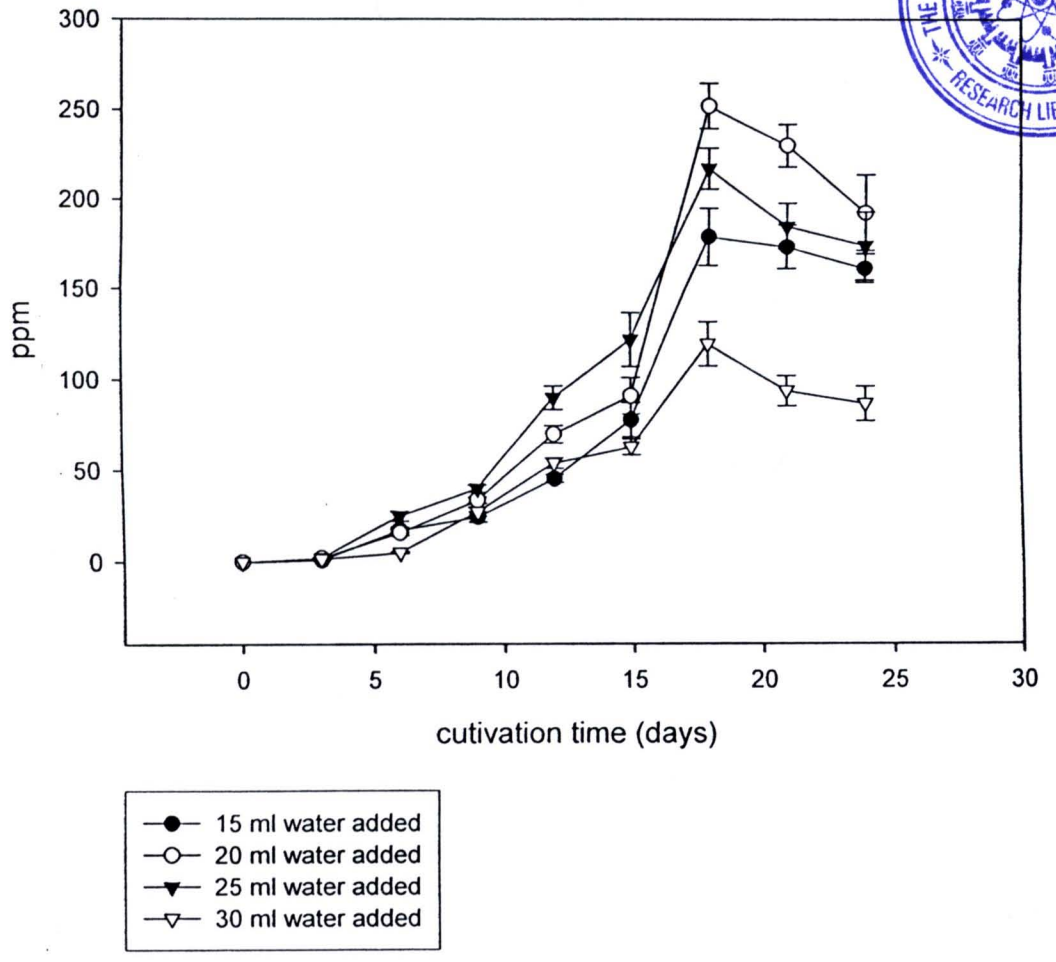

Fig 4.4 Effect of moisture content on monacolin K production of M. purpureus BCC 6131

Moisture content is among factors that are important for the growth of *Monascus* species. A substrate which is excessively dry or wet is not suitable for the growth of *Monascus* species and production of secondary metabolite. The results obtained from this study revealed that the high amount of water at 50 w/w did not lead to the high amount of monacolin K production. On the contrary, the optimal water amount was 20 ml of water with 30 g of rice. At this level, the highest amount of monacolin K produced by *M. purpureus* BCC 6131 was 252.0728±12.4036 ppm. According to this result, this amount of water added could be used for the further experiments in order to produce high amount of monacolin K.

4.2.3 Effect of rice substrate on monacolin K production of M. purpureus BCC 6131

Since unpolished rice is a healthy food, it was selected to be one of the substrates to produce monacolin K. Although, unpolished rice did not satisfactory produce high amount of monacolin K. As we can see in Fig. 2.6 in appendix 2, the *Monacus* culture grown on unpolished rice had longer mycelia and they did not produce much pigment. The reason why unpolished rice did not serve as a suitable substrate for producing *Monascus* fermented rice might come from the nature of unpolished rice that it did not allow *Monascus* mycelia to penetrate and utilize nutrient from the grains or some constituents that might inhibit the production of monacolin K. Therefore, It is worth conducting furthers studies to investigate the cause that suppressed monacolin K production in unpolished rice in this study.

Polished rice and broken rice did not provide a significant difference in monacolin K production (220.0195±13.9542 ppm for polished rice and

202.9862±14.3414 ppm for broken rice). Therefore the shape or size of rice grains did not affect the production of monacolin K by *M. purpureus* BCC 6131.

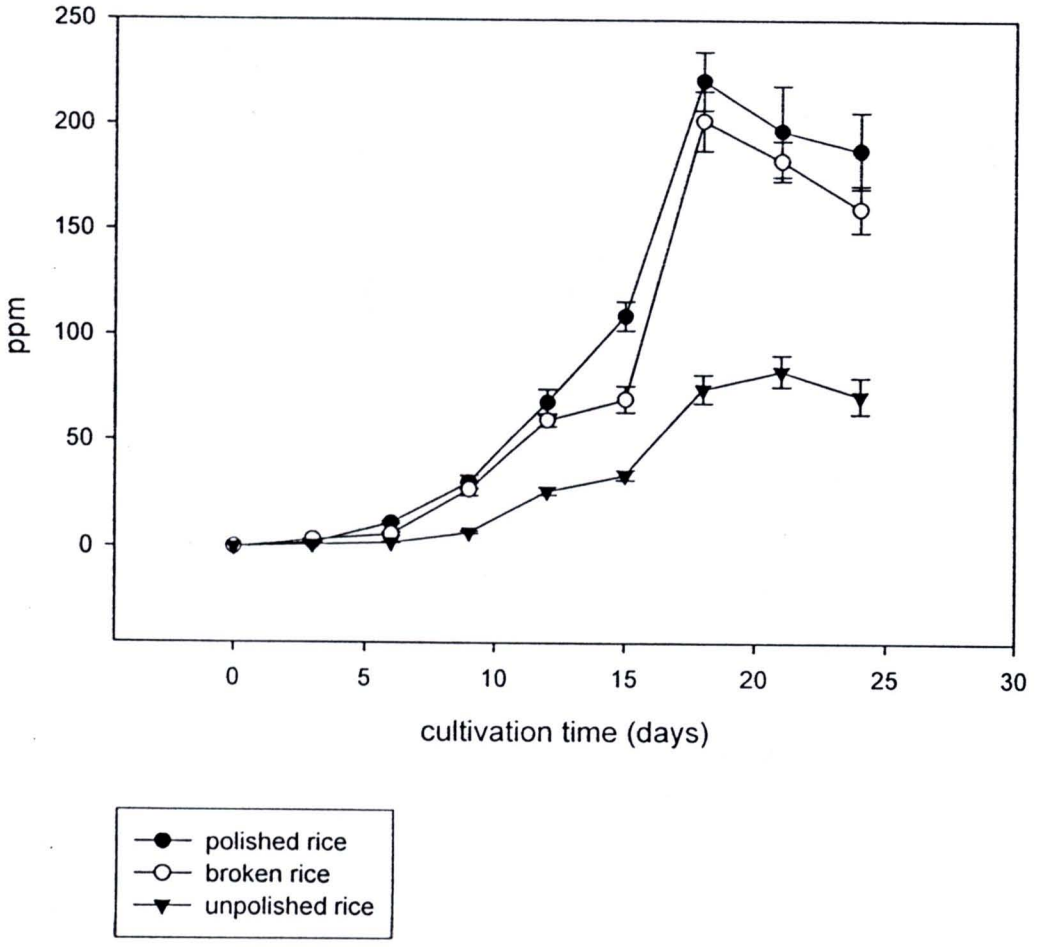

Fig 4.5 Effect of rice substrate on monacolin K production of M. purpureus BCC 6131

4.2.4 Effect of inoculum size on monacolin K production of M. purpureus BCC 6131

According to the results obtained, inoculum size had significant effect on monacolin production of *M. purpureus* BCC 6131 which supported the study of *M. purpureus* CCRC 31615 (Su *et al.*, 2003). The amount of monacolin K production increased gradually when the inoculum size was increased from 1cm^2 to 4 cm^2

(352.0936±20.1156). Although, the increased monacolin K level when the inoculum size was increased might have come from the PDA itself. Therefore, to avoid the effect from additional PDA, the amount of PDA inoculated on rice should be controlled in every inoculation.

It can be concluded that the optimal condition for monacolin K production from *M. purpureus* BCC 6131 is 30 g polished rice with 20 ml water added and the inoculum size of $4cm^2$.

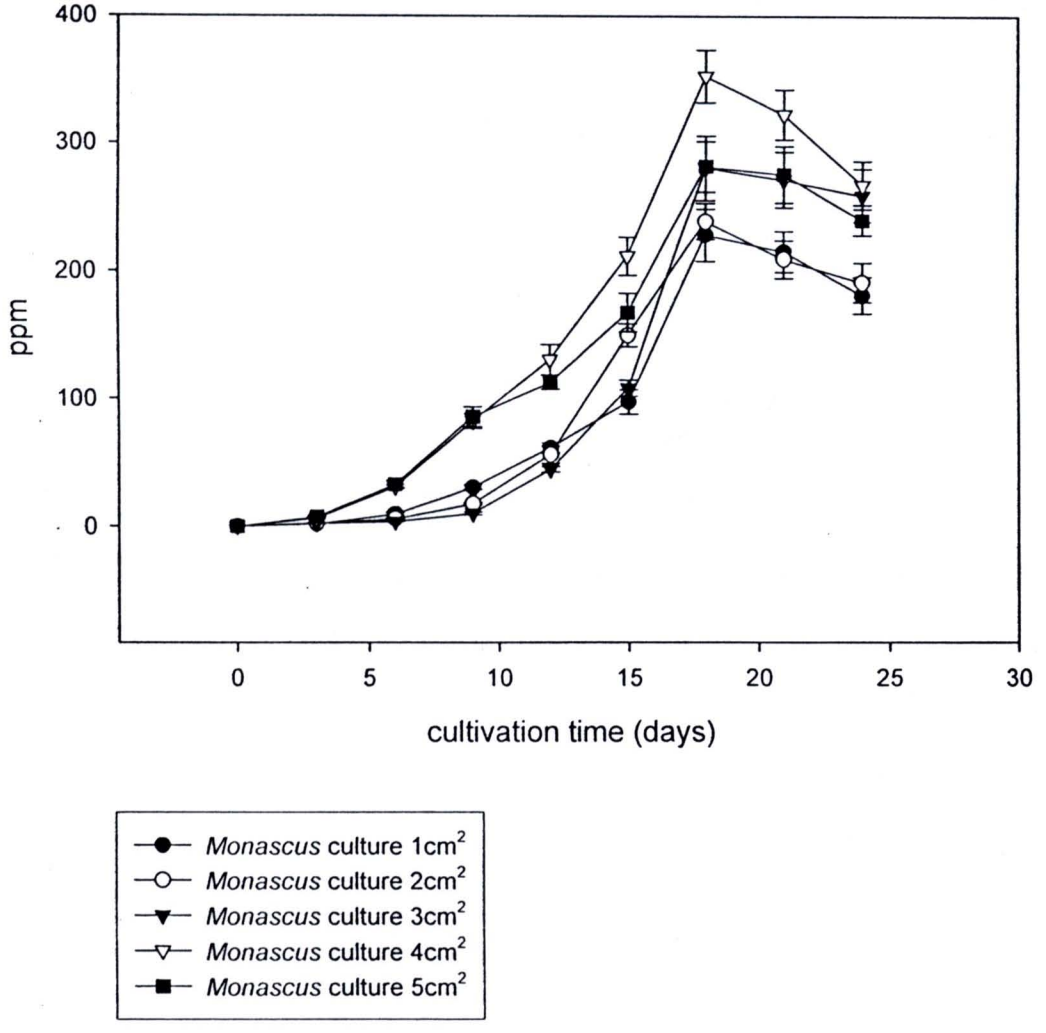

Fig 4.6 Effect of inoculum size on monacolin K production of *M. purpureus* BCC 6131

4.2.5 Effects of Monascus strains and cultivation temperatures on citrinin production

From the results shown, *Monascus ruber* TISTR 3006 produced citrinin approximately 5 times more than *M. purpureus* BCC 6131. That fact also led to the bigger ratio of monacolin K to citrinin of *M. purpureus* BCC 6131. Citrinin showed the similar trends as monacolin K- it dropped after having reached the maximum level. The decline in citrinin levels after it reached the maximum level is presumably related to the release of enzymes during cellular lysis (Damoglou *et al.*, 1984) or other of degradations.

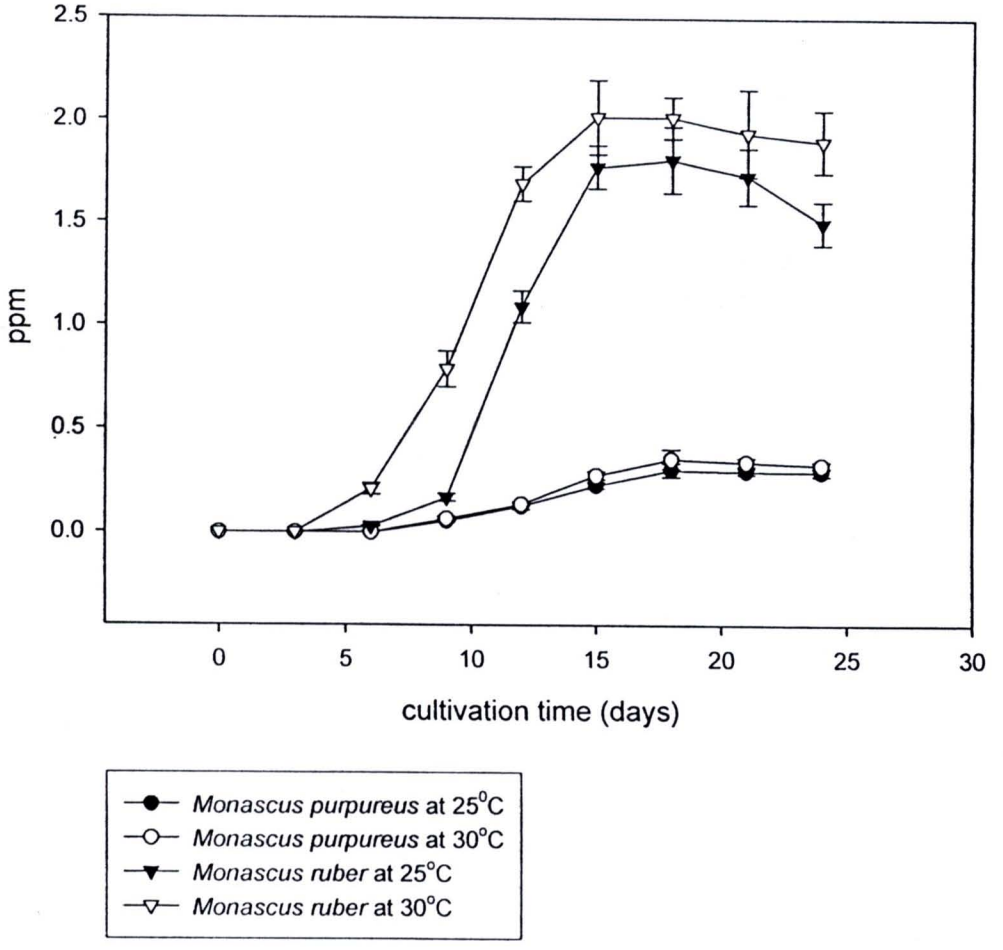

Fig 4.7 Effects of *Monascus* strains and cultivation temperatures on citrinin production

4.2.6 Effect of moisture content on citrinin production of M. purpureus BCC 6131

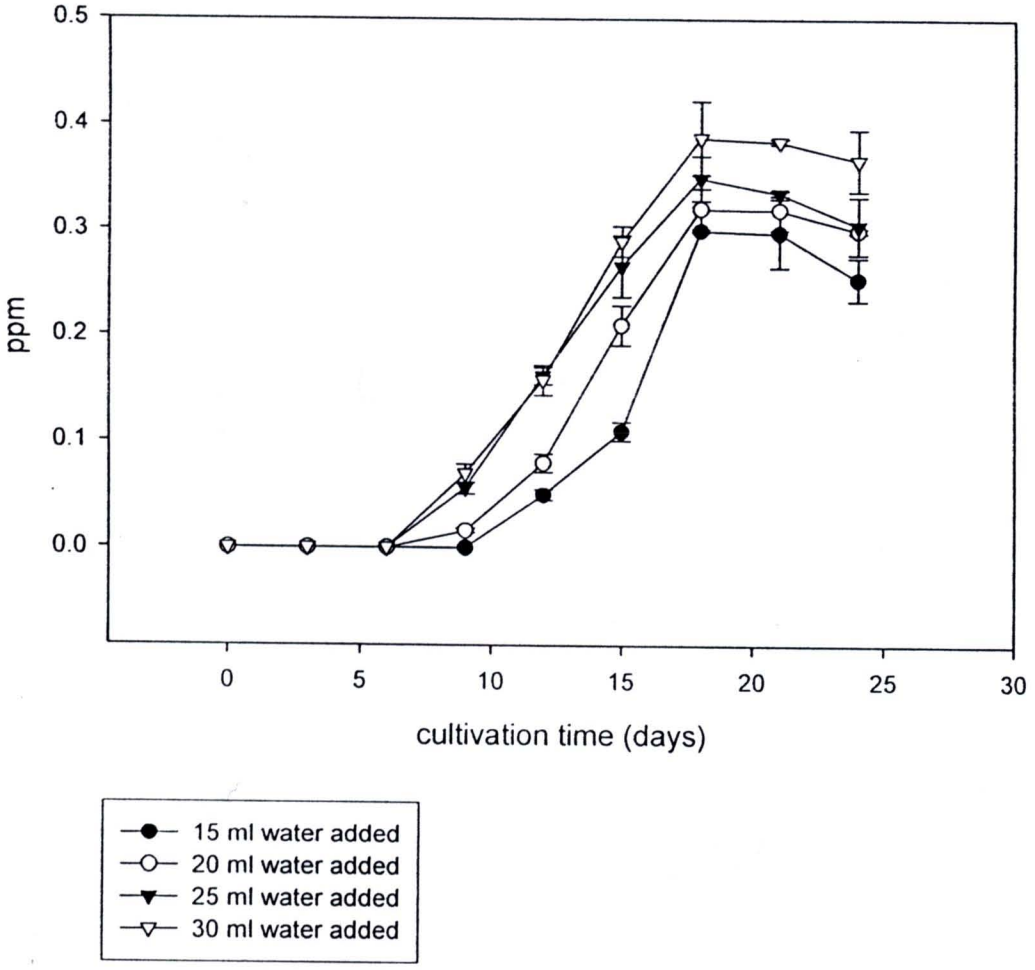

Fig 4.8 Effect of moisture content on citrinin production of *M. purpureus* BCC 6131

According to the results shown, the amount of citrinin production by *M. purpureus* BCC 6131 increased slightly when the amount of water added increased. This relationship had also been report in the study of *Penicillium citrinum* which showed that higher water activity was, the higher citrinin was detected. In addition citrinin was also detected earlier in cultivation time with water activity (Comerio et al., 1998). Taking these results into consideration, the amount of watered added could be kept low as long as the *Monascus* could germinate and produce monacolin K. Using

different level of water added presumably leads to the better ratio of monacolin K and citrinin production.

4.2.7 *Effect of rice substrate on citrinin production of M. purpureus BCC 6131*

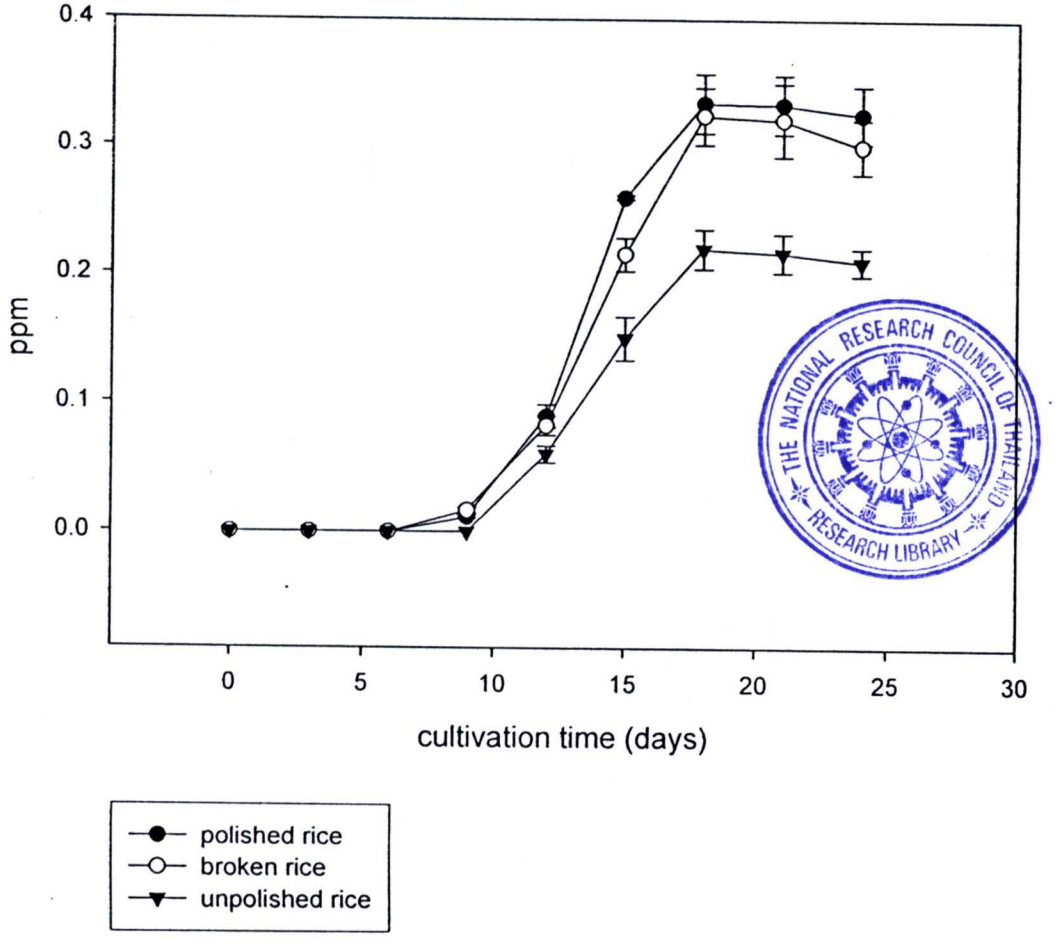

Fig 4.9 Effect of rice substrate on citrinin production of *M. purpureus* BCC 6131

Similarly to monacolin K production, using unpolished rice did not increase the production of citrinin. Even though citrinin level from unpolished rice was low (0.2201±0.0124ppm) compared with citrinin level from polished rice (220.0195±13.9542ppm), but the ratio of monacolin K to citrinin also follow that trend

- 340.5893±8.3847 for unpolished rice, 662.1431±11.3957 for polished rice. It means that unpolished rice is not a suitable substrate for monacolin K production from *M. purpureus* BCC 6131.

4.2.8 Effect of inoculum size on citrinin production of M. purpureus BCC 6131

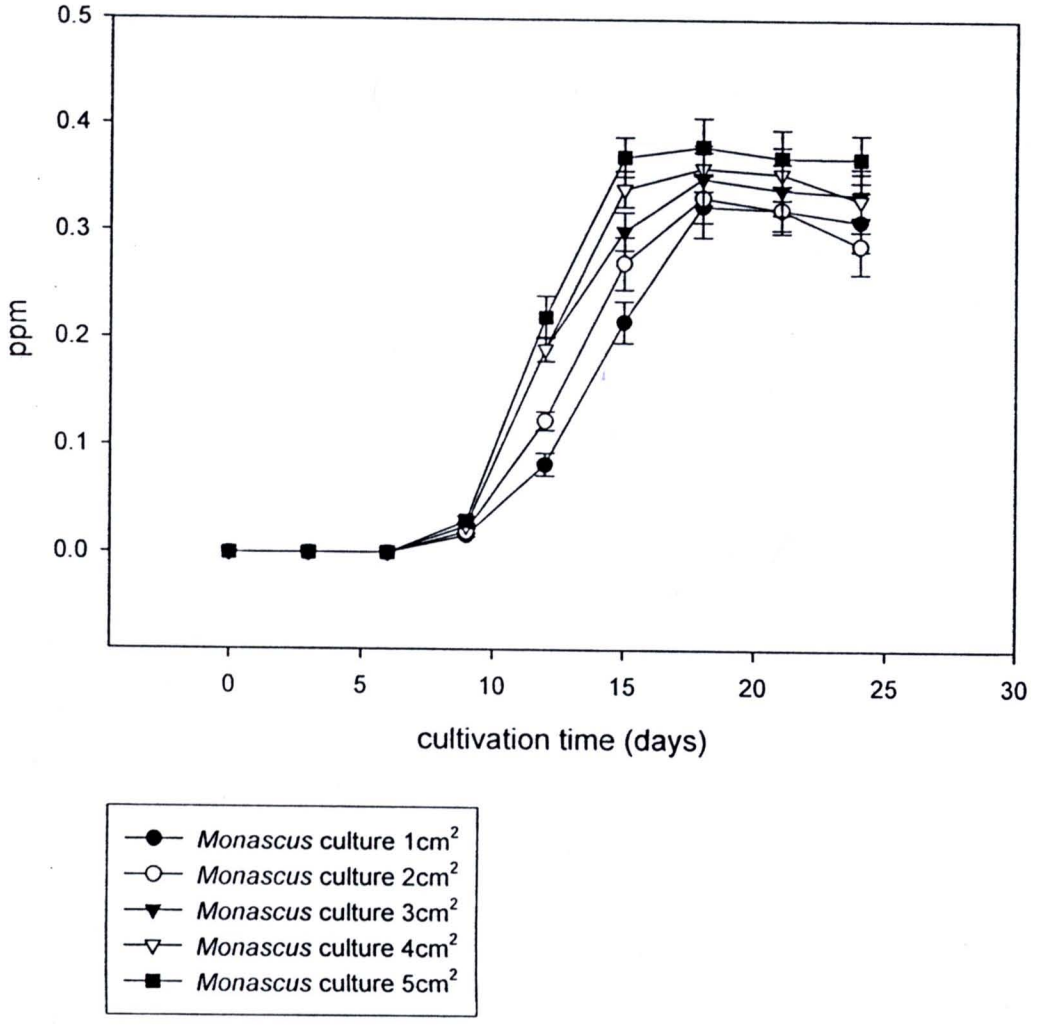

Fig 4.10 Effect of inoculum size on citrinin production of *M. purpureus* BCC 6131

According to the results, citrinin production was not affected from the inoculum size. The production slightly increased when the inoculum size increased, but not significant.

Since both monacolin K and citrinin are derived from polyketide pathway, the production of both monacolin K and citrinin may increase at the same time. Therefore any decision being made on optimum conditions should take into consideration of relationship between these two metabolites. Ratio of monacolin K to citrinin could be used as an indicator for the optimum condtions for producing red fermented rice as anti-hypercholesterolemic agent.

Table 4.2 through 4.5 concluded the production of monacolin K, citrinin along with ratio between them.

Table 4.2 monacolin K and citrinin content of red fermented rice from *M. purpureus* BCC 6131 and *Monascus ruber* TISTR 3006 on 18th day of cultivation

Metabolites	*M. purpureus* BCC 6131 at 25 °C	*M. purpureus* BCC 6131 at 30°C	*Monascus ruber* TISTR 3006 at 25°C	*Monascus ruber* TISTR 3006 at 30°C
Monacolin K (ppm)	51.70± 6.23	109.86 ±5.49	9.58±0.89	11.73±0.83
Citrinin (ppm)	0.30±0.031	0.35±0.04	1.81±0.13	2.01±0.10
Monacolin/citrinin	168.35±10.31	307.41±11.71	5.29±0.27	5.81±0.55

Table 4.3 monacolin K and citrinin content of red fermented rice from *M. purpureus* BCC 6131 at different moisture contents

Metabolites	15 ml water	20 ml water	25 ml water	30 ml water
Monacolin K (ppm)	178.93±16.0	252.07±12.40	217.00±10.28	119.65±11.46
Citrinin (ppm)	0.30±0.01	0.32±0.01	0.35±0.02	0.38±0.03
Monacolin/citrinin	596.29±9.47	785.82±7.70	620.19±5.30	308.20±4.19

Table 4.4 monacolin K and citrinin content of red fermented rice from *M. purpureus* BCC 6131 on different types of rice substrate

Metabolites	Polished rice	Broken rice	Unpolished rice
Monacolin K(ppm)	220.01±13.95	202.98±14.34	75.83±6.75
Citrinin (ppm)	0.32±0.02	0.33±0.02	0.2201±0.01
Monacolin/citrinin	662.14±11.39	623.64±9.36	340.58±8.38

Table 4.5 monacolin K and citrinin content of red fermented rice from *M. purpureus* BCC 6131 at different inoculum sizes

Metabolites	1cm^2	2 cm^2	3 cm^2	4 cm^2	5 cm^2
Monacolin K (ppm)	227.97 ±20.11	238.57 ±14.01	280.37 ±23.23	352.09 ±20.11	281.47 ±18.90
Citrinin (ppm)	0.32±0.02	0.33±0.02	0.35±0.02	0.36±0.02	0.38±0.02
Monacolin/citrinin	703.87 ±5.37	718.88 ±7.38	800.31 ±9.09	978.03 ±6.12	740.16 ±5.91

4.3 Stability test

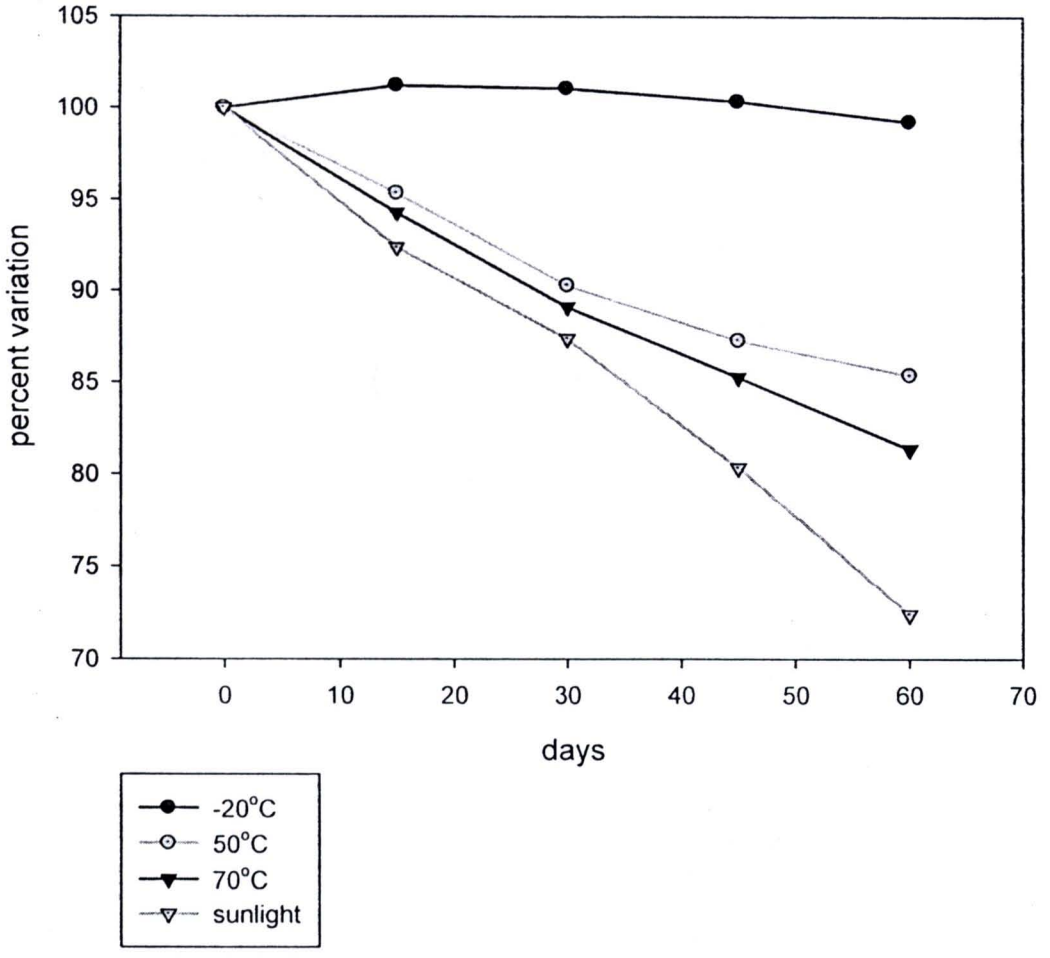

Fig 4.11 percent variation of monacolin K in red fermented rice after exposure to different storage conditions

Starting from the amount of monacolin K as 100%, the amount of monacolin K was declined considerably (72.3947±6.7290% in 60 days) in sunlight which indicated the light sensitivity of monacolin K content in red fermented rice. Also, at 50°C and 70°C monacolin K content was also decreased significantly (85.3827±7.9263% and 81.4947±8.0115 within 60 days respectively) showing heat-labile. Therefore, it is

recommended to store red fermented rice powder in a light-proof container in cool condition.