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Short communication

Establishment of CTAB Turbidimetric method to determine hyaluronic acid content in fermentation broth

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ABSTRACT

The characters of visible spectra on the formation of turbidity between hyaluronic acid (HA) and cetyltrimethylammonium bromide (CTAB) were investigated. The effects of reaction time and temperature on the formation of turbidity were also explored. Consequently, CTAB Turbidimetric method (CTM), a quick and safe method to determine HA content in large batch fermentation broth samples, was established. CTM and Bitter–Muir method (BMM) were respectively applied to measure HA content and were thoroughly compared. The results indicate that CTM was superior to BMM in terms of accuracy, precision and sensitivity. CTM is a promising alternative to BMM to determine HA content in fermentation broth.

1. Introduction

Hyaluronic acid (HA), a linear, unbranched acid mucopolysaccharide consisting of alternating *N*-acetyl-D-glucosamine and D-glucuronic acid, is a valuable biopolymer in the medical and cosmetic market (Fong Chong & Nielsen, 2003). In recent years, instead of extracting HA from rooster combs and umbilical cord, preparing HA through microbial fermentation is being used more. Determination of HA content in fermentation broth is not only one of the necessary steps when screening HA high-yielding bacterial strains but also a routine measurement during the fermentation process.

Currently, the conventional method to determine HA content in fermentation broth is BMM (Bitter–Muir method) (Huang, Chen, & Chen, 2006; Jeon et al., 2007; Liu, Du, Chen, Wang, & Sun, 2008; Segura et al., 2005). It gives excellent results when applied to the analysis of polysaccharides which contain only glucuronic acid (Roden, Baker, Cifonelli, & Mathews, 1972). However, the analysis of results will show significant error if solution samples contain impurities (Zhou, Guo, Li, & Hu, 2001). Additionally, the operation of BMM has certain risks since concentrated sulphuric acid is used to treat samples and pretreatment is quite complex (Bitter & Muir, 1962). Although other biochemical methods such as specific hyaluronidase digestion and HPLC methods have certain advantages on accuracy and precision, they are rarely applied to determine large batch fermentation broth samples (Gu & Yan, 2003; Prieto et al., 2005).

The turbidimetric method here described is based on the formation of turbidity between HA and CTAB, a cation surface active agent. The amount of turbidity developed when CTAB is added to a HA solution is proportional to the amount of HA in the system (Nicola, 1955). However, the stability of turbidity is easily affected by a variety of factors (Scott, 1960). This study explored the characters of visible spectra and the effects of reaction conditions on the formation of turbidity. Consequently, CTM (CTAB Turbidimetric method) was established and compared with the conventional BMM.

2. Materials and methods

2.1. Materials

HA standards were obtained from Sigma Corporation; Glucuronic acid was an analytical reagent; CTAB was purchased from Sinopharm Corporation in China; Preparation of CTAB reagent was as follows: 2.5 g CTAB was dissolved in 100 ml of 0.2 mol L^{-1} NaCl solution (Nicola, 1955).

2.2. Preparation of HA sample solutions

The fermentation broth was centrifuged and the supernatant mixed with 2.5 volumes of absolute ethanol, and then rested at 4 °C for 1 h (Roden et al., 1972). After centrifuging again, the sediment was collected and dissolved in 5 volumes of deionized water.

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2.3. Procedure of CTM

Ultraviolet and visible spectrophotometer (UV-1201, Rili Corporation in China) was used for determinating the absorbance. 1 ml of HA standards was introduced into each test tube while ionized water was used as a blank. When precise 2 ml of CTAB reagent was added into each test tube, the timer was set in order to accurately control reaction time and the solutions were gently shaken to ensure fully mixed. Subsequently, the solutions were left until the 9th min and then transferred to a cuvette. Absorbance was read against the blank at the 10th min and the readings of UV were taken at 400 nm. Absorbance was plotted against HA standard concentrations to constitute calibration lines. Sample solutions were determined as previously described. The HA concentration of samples could be calculated based on a calibration line and the HA content of samples worked out.

3. Results and discussion

3.1. Determination of the suitable absorption wavelength

HA standards with different concentrations mixed with CTAB reagents and accurately reacted for 8 min, spectral curves within 340–800 nm were scanned. As shown in Fig. 1(a), the difference in absorbance at lower wavelengths was larger than that at higher ones. This implies that within lower wave bands absorbance can more accurately reflect HA concentration. Fig. 1(b) indicates that the absorbance had better linear dependence on HA concentration at any wavelength and the correlation coefficients were 0.999, 0.999, 0.996, 0.996, 0.995 and 0.998, respectively. With the decrease of wavelength the slopes of standard curves gradually increased. Since a higher slope could more accurately and sensitively reflect HA concentration, this study selected 400 nm as the wavelength for further research.

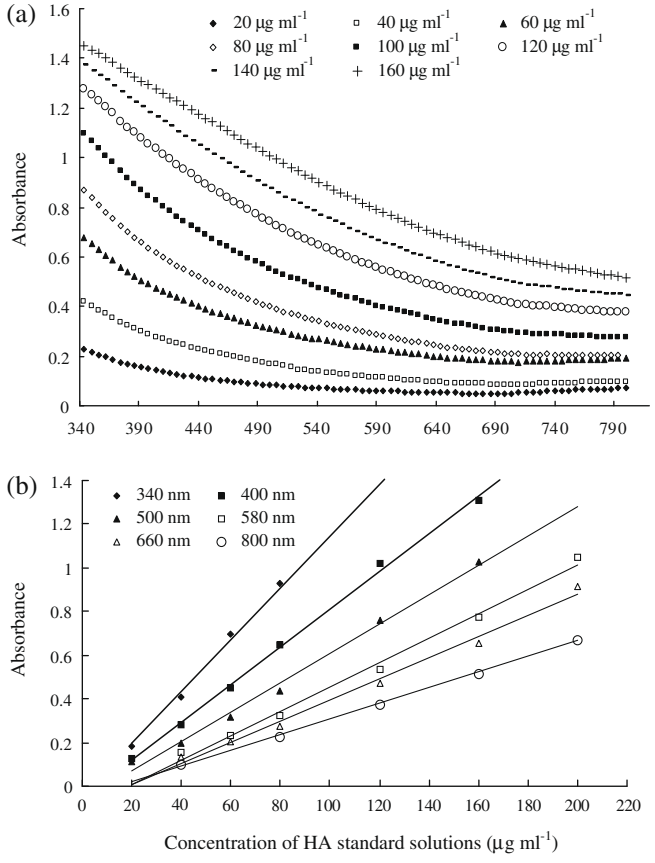

Fig. 1. Determination of the suitable wavelength. Absorption spectra of reaction systems in 340–800 nm (a) were carried out. The standard curves based on different wavelengths were shown in (b).

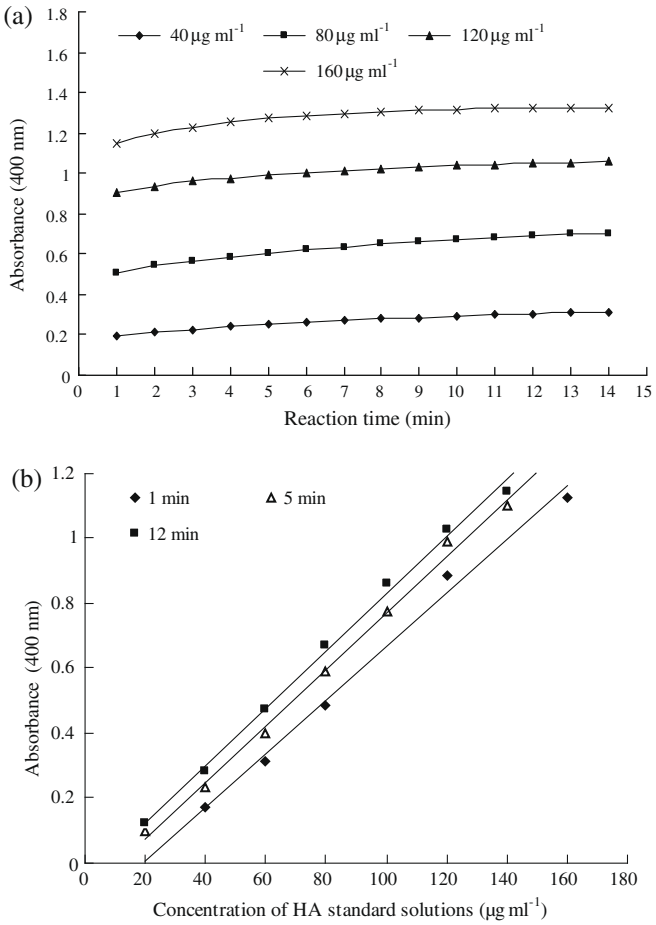

Fig. 2. Effect of reaction time. The absorbance histories of reaction systems following reaction time (a) were showed and the standard curves based on different creation time (b) were presented.

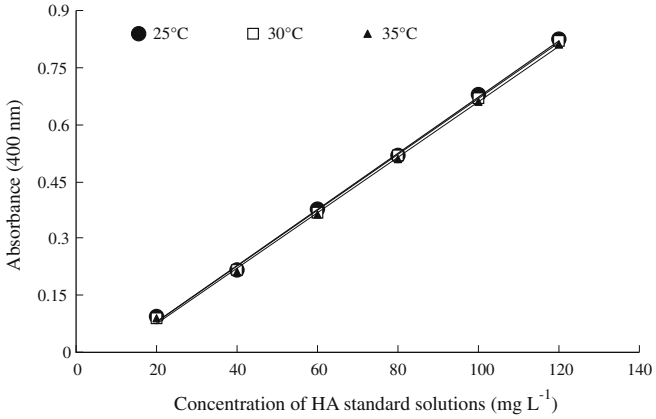

Fig. 3. Standard curves based on different creation temperatures.

Table 1
Comparation between recoveries of spiked samples.

Treatments	V_A[a]	CTM				BMM			
		V_1[b]	V_2	V_3	R[c] (%)	V_1	V_2	V_3	R (%)
1	20	19.59	20.95	18.11	97.75	18.28	19.72	15.90	89.83
2	40	39.59	40.14	40.14	99.89	33.07	33.71	32.43	82.66
3	60	58.92	62.57	58.92	100.23	40.02	49.13	48.02	80.65
4	80	86.89	87.97	87.43	109.29	66.14	68.21	73.14	86.45
5	100	104.87	104.32	104.87	104.67	88.56	86.18	88.72	87.82
Average					102.37				85.48

[a] V_A: Amount of standard sample which was added into fermentation broth before ethanol fractionation (μg ml^{-1}).
[b] V_1, V_2, V_3: Amounts of recovered standard at 3 repeats (μg ml^{-1}).
[c] R: Average recovery of 3 repeats (%).

Table 2
Comparation of precision between the two methods.

Samples	CTM							BMM						
	V_m[a]					V_a[b]	RSD[c] (%)	V_m					V_a	RSD (%)
	1	2	3	4	5			1	2	3	4	5		
1	196.67	195.97	198.68	198.68	209.49	199.90	2.59	163.35	157.79	166.53	160.17	149.04	159.34	3.93
2	204.01	203.34	204.01	204.50	203.90	203.95	0.19	155.88	159.06	161.45	162.02	160.08	159.70	1.43
3	295.91	297.26	303.34	296.60	297.78	298.18	0.94	253.67	232.20	219.48	226.78	228.32	232.09	5.25

[a] V_m: Measurement value at different times (μg ml^{-1}).
[b] V_a: Average value (μg ml^{-1}).
[c] RSD: The relative standard deviation (%).

3.2. Effect of reaction time

The absorbance at 400 nm was measured after the HA standards and the CTAB reagent reacted for different time. As shown in Fig. 2(a), with the reaction time the absorbance of every reaction system gradually increased. It indicates that the reaction systems are not very stable and the result is in good agreement with Scott (1960). The following study explored the difference between standard curves when reaction time was fixed. Fig. 2(b) shows that with the increase of the fixed reaction time the standard curves moved upwards and had a better linear relation between absorbance and HA concentration. The correlation coefficients were 0.996, 0.997 and 0.998, respectively. It is obvious that a standard curve with better linear relation can be obtained only if reaction time is accurately controlled and kept unanimous. The following study selected 10 min as reaction time.

3.3. Effect of reaction temperature

HA standard solutions and CTAB reagent were respectively incubated at different temperatures and reacted for accurate 10 min at corresponding temperature. Fig. 3 shows that the standard curves at different temperatures almost superimposed. This indicates that the effect of reaction temperature is negligible. However, because the Critical Solution Temperature of CTAB is 20 °C (Scott, 1960), solutions and reaction systems should be kept higher than 25 °C.

3.4. Comparation of accuracy, precision and sensitivity between CTM and BMM

As shown in Table 1, the average recovery of CTM was 102.37% while for BMM, was 85.48%. It indicates that CTM is much more accurate than BMM. A small quantity of remnant protein in sample solutions probably leads to the difference in accuracy. Table 2 shows that the average RSD of three samples which was determined by CTM was 1.24% while 3.54% was by BMM. It indicates that CTM is more precise than BMM, mainly because the procedure of CTM is so simple that less error will be caused. Table 2 also shows that for the same sample, the measurement results of CTM were obviously higher than those of BMM, these are mainly due to the difference in accuracy of the two methods. The limit of detection was calculated according to IUPAC (1987). The result shows that the limit of detection of CTM was 1.55 μg ml^{-1} while that of BMM was 4.14 μg ml^{-1}. So CTM is more sensitive than BMM.

4. Conclusion

CTM established in this paper is a quick method to determine HA in fermentation broth. CTM is superior to BMM in terms of accuracy, precision and sensitivity, and is easier to be handled. Since CTAB is a kind of cation surface active agent, the security of CTM is dramatically improved compared with BMM, which uses concentrated sulphuric acid as the main reagent. Therefore, CTM is a promising alternative to conventional BMM, especially when the measurement on large batch fermentation broth samples is performed.

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