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## Study on the Methods of $\beta$ - Carotene Extraction of *Spirulina platensis*

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### Research Article

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#### ABSTRACT

In order to select the best methods of  $\beta$ - carotene extraction of *Spirulina platensis*, we use *Spirulina platensis* 1 and 2 as materials, freezing and thawing and HPLC as extraction methods, compare a variety of polar or non-polar organic solvents and different solvents to extract the amount of  $\beta$ -carotene. The result shows that in strain 1 and strain 2, with the solid-liquid ratio of 1: 6, the best extraction solvent respectively are: chloroform: methanol=2: 1 and chloroform: methanol=1: 2. Moreover, strain 2 are more suitable for industrially production of  $\beta$ - carotene.

### INTRODUCTION

*Spirulina* is a prokaryote belonging to Cyanophyta, Oscillatoria which is rich in all elements and nutrients required for the human body. The elements and nutrients derived from *Spirulina* help to achieve balanced amino acids, proteins, carotenoids, vitamins, minerals and polysaccharides components for human body<sup>[1-2]</sup>. *Spirulina* offers numerous advantages, as it is easier to culture, low-cost, showing stronger adaptability, hence has a huge economic and social benefits. *Spirulina* is the most excellent variety in worldwide which has been continuously developed and utilized in recent years<sup>[3]</sup>.

So far more than 600 carotenoids have been identified while only 40 could be absorbed and used by human<sup>[4]</sup>.  $\beta$ - carotene is one of the carotenoid, which not only can be used as a precursor of vitamin A, but also a potent antioxidant, which can effectively remove the body of free radicals and oxidants, improve the immune capacity, prevent atherosclerosis, cancer, alcoholic-liver disease, at the same time,  $\beta$ -carotene as a coloring agent and food additives are used in food and medical industries<sup>[5-8]</sup>.

It is reported that the content of  $\beta$ - carotene in 6 g *Spirulina* corresponds to the carotenoid content of 20 eggs. Further,  $\beta$ - carotene content in *Spirulina* is much higher than some animals or plants; therefore extracting  $\beta$ - carotene from *Spirulina* has become the focus of current research<sup>[9]</sup>. YueHui Zhu in an analysis of  $\beta$ - carotene content of *Dunaliella* showed the optimum extraction conditions is: 2 ml pure acetone as extraction solvent, solvent and culture fluid=2: 5, 5 min under ultrasonic. XueYan Li, HaiQiang Chen in the analysis content of the carotenoid of leeks obtained the following optimum conditions: petroleum ether: acetone =1:2 as the extraction agent, feed solvent =1:4 (g/mL), and extraction 1 time with 90 min at 30°C<sup>[4,10]</sup>. HongMei Lu, YiZeng Liang measured carotenoids in foods by high performance liquid chromatography<sup>[11]</sup>.

In order to find the best solvent extraction of  $\beta$ - carotene system, HPLC method was used to this study. Focusing on two strains of *Spirulina platensis* 1 and 2, we use five different extraction solvent to extract  $\beta$ - carotene with repeated freezing and thawing extraction according to the same steps. Finally to compare the content of  $\beta$ - carotene and identify suitable *Spirulina platensis* for expansion production of  $\beta$ - carotene industrially.

### MATERIALS AND METHODS

#### Material

Two strains of *Spirulina platensis* 1 and 2, coming from Cell Engineering Laboratory of the College of Life Science in Shanxi Normal University.

## Formulation

**Table 1.** Broth ingredients, pH = 7.5-8.0

Broth Ingredients							
NaHCO <sub>3</sub>	NaNO <sub>3</sub>	NaCl	K <sub>2</sub> SO <sub>4</sub>	KH <sub>2</sub> PO <sub>4</sub>	MgSO <sub>4</sub>	CaCl <sub>2</sub>	FeSO <sub>4</sub>
16.8 %	2.7%	1.0%	1.0%	0.4%	0.1%	0.04%	0.005%

## Method

We prepare the algae mud with centrifuge at 8000 rpm, 10 min at 4°C and five different ratios of organic solvents according to the (Table 2)<sup>[8]</sup>. Take prepared algae mud 1 ml into five 10 ml centrifuge tubes respectively and freeze them 30 min, then put under at 37°C water bath to melt 10 min. Repeated freezing and thawing as described above can be 4-5 times<sup>[12]</sup>. Then we add to the above-configured organic solvents of each 6 ml, label, mix them up fully, and then put in a refrigerator at 4°C extracting 1 day at dark. One day later, put the above mixtures into centrifugation at 10000 rpm, 4°C, 10 min. Following centrifugation, the upper layer containing β- carotene is transferred 1.5 ml into a centrifuge tube and dry under a gentle stream of nitrogen gas, and then dissolves in ethyl acetate and then filter them with micro porous membrane for use. At the same time, we take 2 mg standard solution of β- carotene dissolved in 200 ul of chloroform, and then dissolve it in ethyl acetate and the capacity to 20 ml. After that, dilute with ethyl acetate to 10 ml at a concentration of 1 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml respectively.

**Table 2.** Organic solvent components and grouping table (unit: ml)

	A	B	C	D	E
A	Chloroform	Chloroform	Petroleum	Petroleum	Acetone
	4	2	ether3	ether2	
B	Methanol2	Methanol4	Acetone3	Acetone4	

Take prepared β-carotene of standard and sample solutions to filter with pinhole type membrane to vial of about 1.5 ml respectively. Mobile phase linear Isocratic elution: 50% mobile phase A (90% acetonitrile: water: triethylamine=9: 1: 0.01, 10% ethyl acetate) and 50% mobile phase B (20% acetonitrile: water: triethylamine=9: 1: 0.01, 80% ethyl acetate) are employed over 30 min at a rate of 1.0 ml/min under wavelength of 450 nm and 25°C with injection volume of 10 ul in the Symmetry C18 column (4.6 × 250 mm, 5 µm particle size)<sup>[13]</sup>.

## RESULTS AND ANALYSIS

### Standard Curve

Relation between β- carotene concentration (ug/ml) of standard solution and the peak area (Figure 1).

β- Carotene Analysis of Standard Solution of Strain 1, Strain 2

HPLC-chromatogram of β- carotene from standard products (Figure 2).

Peak time of β- carotene of Standard solution is about 26 min to 27 min analyzed by HPLC-chromatography.

(Figures 3-12) and (Tables 3 and 4).

So the comparison of total β- carotene content extracted out of 1 ml algae mud of strain 1 and strain 2 with solid-liquid ratio of 1: 6 shows below: (Figure 13).

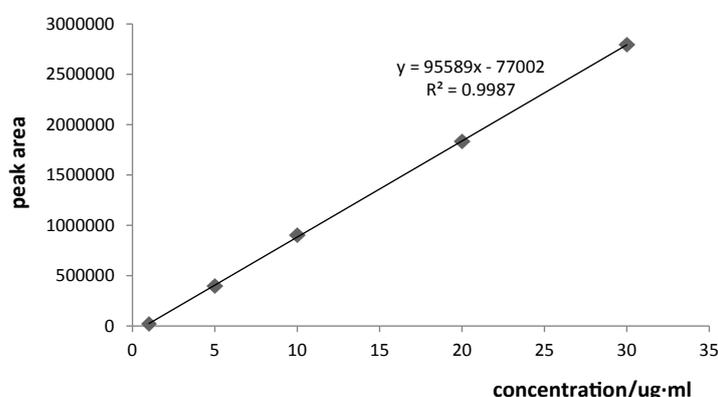
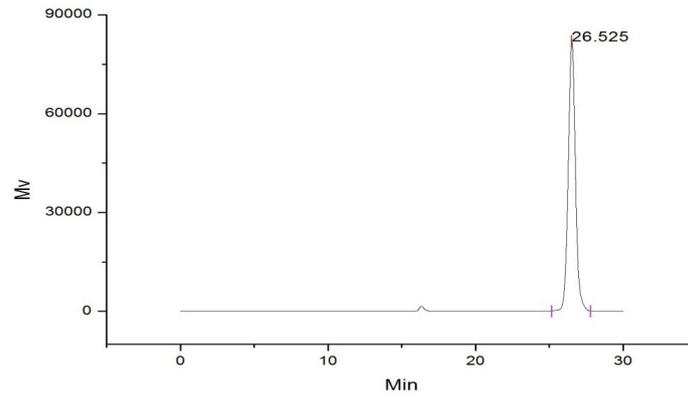
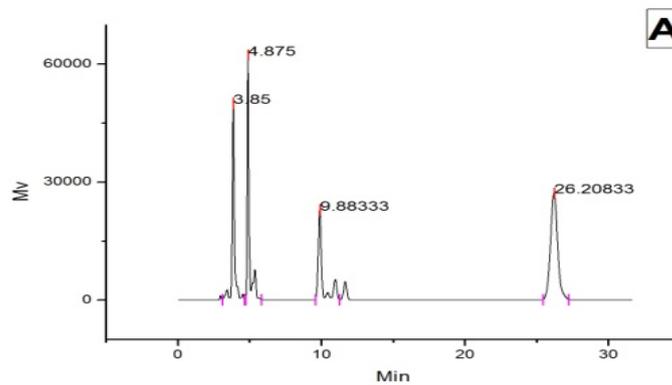


Figure 1 shows: Within 1 ~ 30mg / L, β- carotene concentration (µg / L) and chromatographic peak area reflect a good linear relationship, the linear regression equation is  $Y = 95589X - 77002$ , and the coefficient of correlation is  $R^2 = 0.9987$ . Therefore this standard product can be used to identify the study.

**Figure 1.** Relation between β- carotene concentration (ug / ml) of standard solution and the peak area

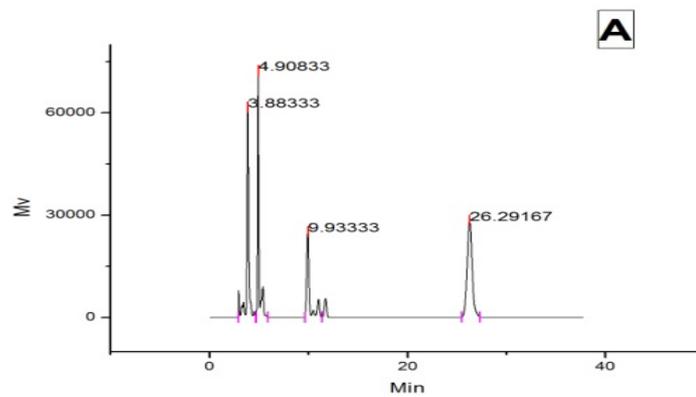


**Figure 2.** HPLC-chromatogram of  $\beta$ - carotene from standard products



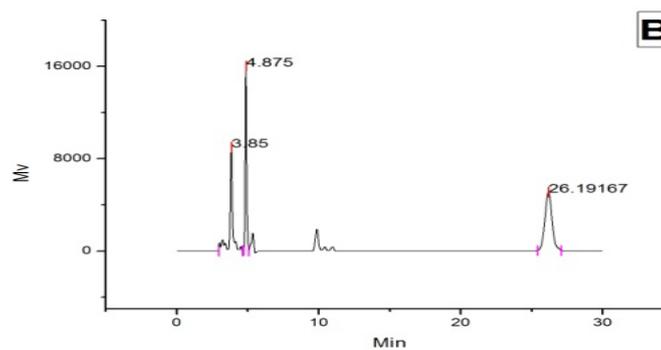
Chloroform: methanol = 2:

**Figure 3.** Strain1.



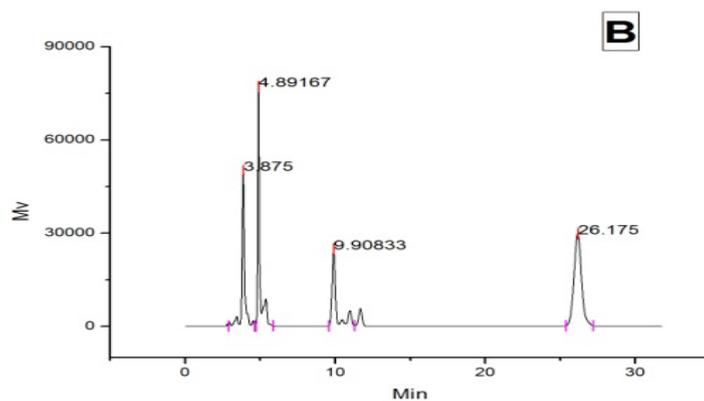
Chloroform: methanol = 2: 1

**Figure 4.** Strain 2.



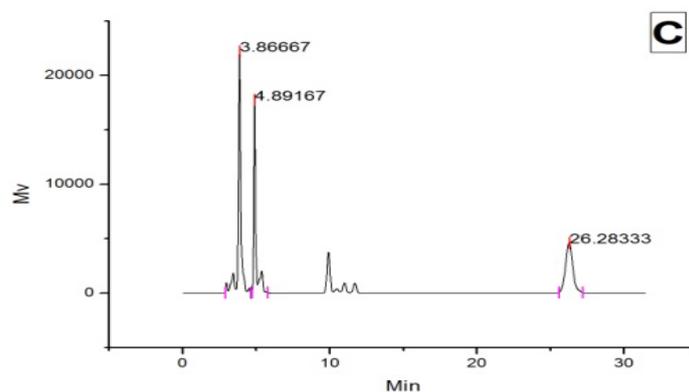
Chloroform: methanol = 1: 2

**Figure 5.** Strain 1



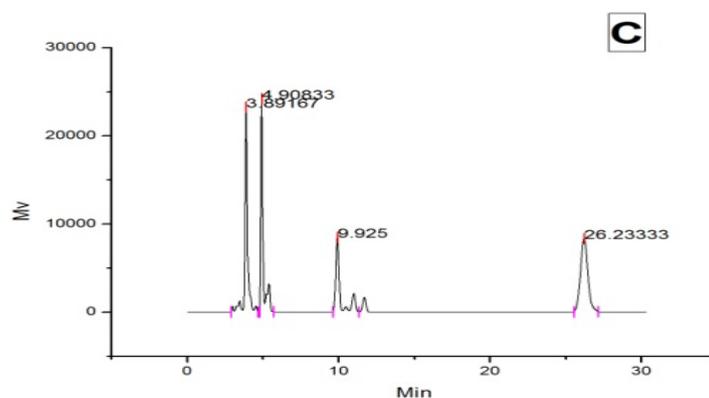
Chloroform: methanol = 1: 2

**Figure 6.** Strain 2



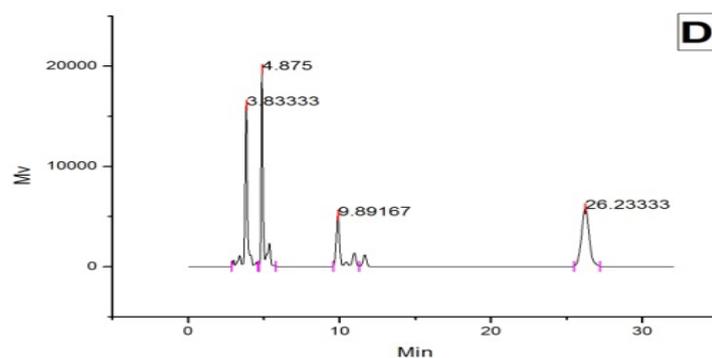
Petroleum ether: acetone = 1: 1

**Figure 7.** Strain 1



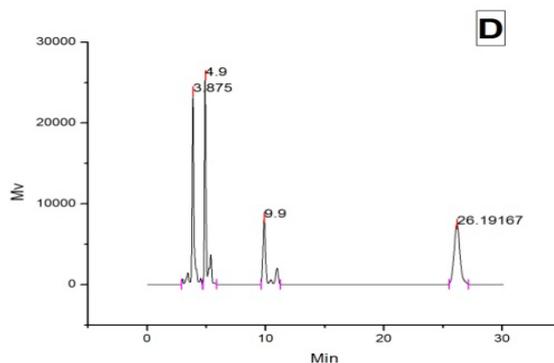
Petroleum ether: acetone = 1: 1

**Figure 8.** Strain 2



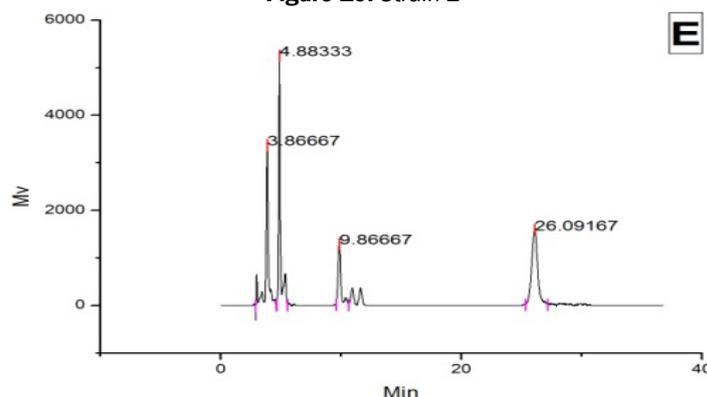
Petroleum ether: acetone = 1: 2

**Figure 9.** Strain 1



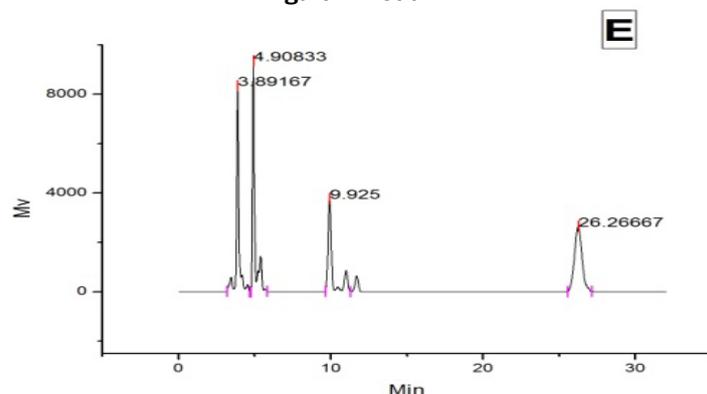
Petroleum ether: acetone = 1: 2

Figure 10. Strain 2



Acetone

Figure 11. Strain 1



Acetone

Figure 12. Strain 2

Table 3. All kinds of indicators in five per 1ml algae mud extraction methods of  $\beta$ - carotene from Strain 1

	A	B	C	D	E
peak time	26.20833	26.19167	26.28333	26.23333	26.09167
Y value (HPLC area value)	27338	25125	4581	5774	1540
1 ml organic solvent $\beta$ - carotene content	1.09155	1.06840	0.85348	0.86596	0.82166
6ml organic solvent $\beta$ - carotene content	6.54930	6.41040	5.12088	5.19576	4.92996

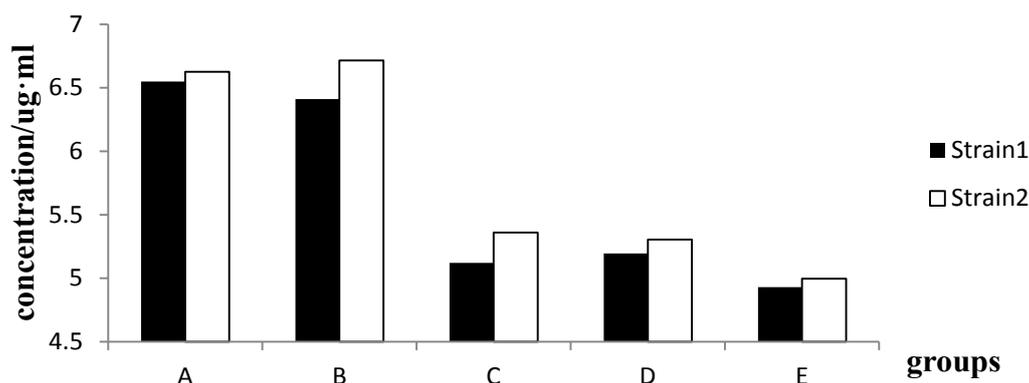
## DISCUSSION

Extracting  $\beta$ -carotene as the first step plays a vital role in quantitative analysis. The same material would yield variedly due to different methods of  $\beta$ -carotene extraction. The traditionally used spectrophotometry based method to extract  $\beta$ - carotene, could lead to light degradation, operational losses, and it is relatively complex while it is not a safe method for operators. Taking these into consideration, the present study employed -repeated freezing and thawing - extraction, so as to simplify the steps and to improve the accuracy of analytical methods. The principle here is that algal cells frozen in the dark at  $-20^{\circ}\text{C}$  and thawed at  $37^{\circ}\text{C}$

repeatedly keeping it away from sun, causes the formation of ice particles and increasing of salt concentration in cytoplasm [14]. This leads to the rupturing of the cell walls. Such methods are ideally in breaking walls in the late [14]. Due to repeatedly freezing and thawing in ratio of breaking walls could effect by different cell types, different organisms and different growth conditions, the extraction rate can be enhanced by increasing the times of freeze-thaw and extending extraction time. But repeated freeze-thaw extraction method is relatively time-consuming.

**Table 4.** All kinds of indicators in five per 1 ml algae mud extraction method of  $\beta$ - carotene from Strain 2

	A	B	C	D	E
peak time	26.29169	26.17500	26.23333	26.19167	26.26667
Y value (HPLC area value)	28555	29984	8390	7518	2605
1 ml organic solvent $\beta$ - carotene content	1.10428	1.11923	0.89332	0.88420	0.83281
6ml organic solvent $\beta$ - carotene content	6.62568	6.71538	5.35992	5.30520	4.99686



From Figure 13, we can see that In Strain 1, the best extraction solvent system of chloroform: methanol = 1: 2 to get the  $\beta$ -carotene content of 6.54930 ug / ml; in Strain 2, the optimum extraction solvent system is chloroform: methanol=2:1 to reach the  $\beta$ -carotene concept of 6.62568 ug / ml. Consequently, Strain 2 is more suitable for expansion of production  $\beta$ - carotene industrially in terms of the five solvent systems.

**Figure 13.** Total  $\beta$ - carotene content extracted out of 1 ml algae mud of strain 1 and strain 2 with solid-liquid ratio of 1: 6

Different extraction solvent systems to extract the  $\beta$ - carotene content of Spirulina could have different effect [15]. Mo Yu Nan, who extracted carotenoids from orange cabbage and found ethanol : acetone=1:1 is the best; Yue Hui Zhu regarded acetone as optimum extraction for carotenoids of Dunaliella; Pengfei Guo, Chang Ying Hu with 95% ethanol as carotenoids optimum extraction system from papaya [16]. XiaoMing Hu, Wan Ling Cai used petroleum ether as  $\beta$ - carotene optimum extraction from carrot juice [17]. The present study to investigate the best extraction solvent systems of Strain 1 and Strain 2, to choose which algae is more suitable for the expansion culture industrially, has been provided five organic solvent systems to compare the content of  $\beta$ - carotene under the same conditions.

With the rapid use of HPLC, provide reliable and stable data's for its sensitivity and accuracy. Meanwhile, the isomerization and degradation of carotenoids could down to a minimum coupling with its reasonable analysis time [11]. In this study, we have tried many times to confirm the options of mobile phase, setting of temperature, selection of wavelength, the flow rate through the process of whole HPLC, so as to obtain the best analysis method to  $\beta$ - carotene from Spirulina. The results showed that the optimum parameter to extract  $\beta$ - carotene from Spirulina by HPLC system is to 50% A: 90% (acetonitrile: water: triethylamine=9: 1: 0.01), 10% ethyl acetate and 50% B: 20% (acetonitrile : water: triethylamine=9: 1: 0.01), 80% ethyl acetate as the mobile phase at the flow rate of 1.0 ml/min under the column temperature 25°C and wavelength 450 nm with the injection volume 10  $\mu$ l [13].

## CONCLUSION

The HPLC assay system is 50% A: 90% (acetonitrile: water: triethylamine=9: 1: 0.01), 10% ethyl acetate and 50% B: 20% (acetonitrile: water: triethylamine=9: 1: 0.01), 80% ethyl acetate as the mobile phases at a rate of 1.0 ml/min under wavelength of 450 nm with the injection volume 10  $\mu$ l and column temperature 25°C [14]. In Strain 1, the best extraction solvent system is chloroform: methanol=1: 2 to get the  $\beta$ - carotene content of 6.54930  $\mu$ g/ml; in Strain 2, the best extraction solvent system is chloroform: methanol=2: 1 to reach the  $\beta$ - carotene concentration of 6.62568  $\mu$ g/ml. Obviously, it is Strain 2 that is more suitable for expansion production of  $\beta$ - carotene industrially for five solvent systems in this study.

## ACKNOWLEDGEMENTS

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