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Vitreoscilla hemoglobin promotes Salecan production by *Agrobacterium* sp. ZX09*

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Abstract: Salecan is a novel exopolysaccharide produced by the strain *Agrobacterium* sp. ZX09, and it is composed of only glucose monomers. The unique chemical composition and excellent physicochemical properties make Salecan a promising material for applications in coagulation, lubrication, protection against acute liver injury, and alleviating constipation. In this study, we cloned the *Vitreoscilla* hemoglobin gene into a broad-host-range plasmid pCM158. Without antibiotic selection, there was negligible loss of the plasmid in the host *Agrobacterium* sp. ZX09 after one passage of cultivation. The expression of *Vitreoscilla* hemoglobin was demonstrated by carbon monoxide (CO) difference spectrum. The engineered strain *Agrobacterium* sp. ZX09 increased Salecan yield by 30%. The other physiological changes included its elevated respiration rate and cellular invertase activity.

Key words: Salecan, *Vitreoscilla* hemoglobin, *Agrobacterium* sp. ZX09, Invertase, Respiration rate
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1 Introduction

Among the polysaccharides produced by bacteria, β-glucans consisting of a backbone of β-1,3-D-glucose units with various lengths of β-1,6 side chains have the notable physiological effect of immunological activation (Miura *et al*., 1996). Immunologists have found the β-glucan receptors, dectin-1 and complement receptor 3, on the surface of innate immune cells (Brown and Gordon, 2001; Vetvicka *et al*., 2007). There are many application studies associated with β-glucan immune activation, e.g., tumoricidal activity, prevention of infection, and damage reduction of radiation exposure (Thompson *et al*., 2010). β-Glucans are also found to decrease the levels of serum cholesterol and liver low-density lipoproteins, leading to lowering of atherosclerosis and cardiovascular disease hazards (Barsanti *et al*., 2011). Salecan, produced by the strain *Agrobacterium* sp. ZX09, is a novel water-soluble β-glucan-like polysaccharide. It consists of the following repeating units: →3)-β-D-Glcp-(1→3)-[β-D-Glcp-(1→3)-β-D-Glcp-(1→3)]$_3$-α-D-Glcp-(1→3)-α-D-Glcp-(1→ (Xiu *et al*., 2010). This special structure of Salecan is water-soluble and has potential biological activities owing to its β-1,3-glucosides. It reduces the acute hepatic injury in mice induced by alcohol (Chen *et al*., 2011) and carbon tetrachloride (Chen *et al*., 2012). It has a potential to become a hydrophilic laxative to cure constipation (Zhou *et al*., 2013). It can also possibly be used in the food industry as a thickening agent due to its excellent rheological properties (Xiu *et al*., 2011). Therefore, increasing the yield of Salecan has a vital significance for its industrial application.

It is well known in fermentation by aerobic organisms that the supply of O_2 usually becomes a

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serious limitation because of its low solubility, especially during high-cell-density fermentation or when fermentation media are highly viscous. *Vitreoscilla*, an obligate aerobic bacterium (Pringsheim, 1951), survives and grows in an O_2-poor environment owing to its synthesis of hemoglobin (VHb). VHb binds O_2 at low extracellular concentrations and delivers it to the terminal respiratory oxidase, thus enhancing respiration (Park *et al*., 2002). The *vgb* gene from *Vitreoscilla* has been successfully engineered into various organisms to improve cell density, protein synthesis, and metabolic productivity (Khosla and Bailey, 1988; Frey and Kallio, 2006; Su *et al.*, 2010; Frey *et al*., 2011). The *vgb* gene is generally integrated into the genome of the host organisms to form stable inheritance, but this technique is usually limited to the genome-sequenced organisms.

In this study, a broad-host-range vector pCM158 (Marx and Lidstrom, 2002), hosting *vgb* gene, was demonstrated to persist in Salecan-producing *Agrobacterium* sp. ZX09 without antibiotic selection pressure and to improve the respiration rate, invertase activity, and Salecan production.

2 Materials and methods

2.1 Strains, plasmids, media, and culture conditions

Escherichia coli strain DH5α was used as the host for bacterial transformation and plasmid construction. The plasmid pCM158 was used as *vgb* expression vector, and the *vgb* gene was derived from pUC19-vgb. Salecan-producing bacterium, *Agrobacterium* sp. ZX09, was isolated from a soil sample from the ocean coast of Shandong, China (Xiu *et al*., 2010). The culture medium for screening transformation and producing Salecan was Htm composed of NaH_2PO_4 1 g, KNO_3 3 g, $CaCl_2$ 0.07 g, $MgCl_2$ 0.2 g, $FeSO_4{\cdot}7H_2O$ 0.0125 g, $MnSO_4$ 0.003 g, $ZnCl_2$ 0.0075 g, sucrose 30 g, and H_2O 1000 ml, pH 7.2 (Xiu *et al*., 2010). The strain hosting pCM158-vgb plasmid was maintained on Htm agar medium by adding 50 μg/ml kanamycin. A single colony on an agar plate was first inoculated in seed medium (Htm) and aerobically incubated at 30 °C for 24 h with shaking at 250 r/min. A 1% inoculum was added to a 250-ml flask containing 50 ml of medium. The flask culture was incubated at 30 °C for 72 h at 250 r/min and the yield of Salecan was measured after cultivation for 12, 24, 36, 48, and 72 h.

2.2 Construction of pCM158-vgb plasmid and transformation

The VHb coding gene *vgb* was first cloned from the plasmid pUC19-vgb (Khosla and Bailey, 1988) by polymerase chain reaction (PCR) with the two primers of vgb-F (AAGCTTACAGGACGCTGGGGT) and vgb-R (CAATATTTGTCCCAAGTTT). The PCR products were then cloned into pMD18-T vector to form pMD18-T-vgb, and sequenced to be correct without mutations. Last, the *vgb* gene fragment was cut down from the pMD18-T-vgb by restriction endonucleases *Sph*I and *Eco*RI, and cloned into the corresponding sites of pCM158 plasmid to form pCM158-vgb. Electrocompetent cells were prepared in super optimal broth (SOB) medium. Once the optical density at 600 nm (OD_{600}) of the culture reached about 0.5 to 0.6, the cells were placed on ice and washed three times with ice-cold 10% glycerol. About 100 ng of pCM158-vgb and pCM158 DNA were added to 100 μl of the competent cells and the resulting mixtures were electroporated in 0.1-cm-gap cuvettes at 2.0 kV, 25 μF, 200 Ω in a Gene Pulser electroporation apparatus (BioRad). Cells were resuspended in 1 ml SOB medium. The cells were allowed to recover at 30 °C for 2 h, and then plated on Htm agar plates with 50 μg/ml kanamycin. Strains of *Agrobacterium* sp. ZX09 hosting the plasmids of pCM158-vgb and pCM158 were respectively designated VHb^+ and VHb^-. Plasmid stability was studied by inoculation of the strain into Htm medium without antibiotics and incubating at 28 °C with shaking (Guo *et al*., 2010). After 48 h of cultivation, the same amounts of cells were sprayed onto six solid medium plates: half of the plates lacked antibiotics and half contained antibiotics. Plasmid stability was indicated by the ratio of the colony-forming units (CFUs) formed on the plates with antibiotics to the ones formed on the plates without antibiotics.

2.3 CO-difference spectral analysis of VHb

The *vgb* gene expression was determined by carbon monoxide (CO) difference spectra of VHb (Liu and Webster, 1974). Cells of VHb^+ and VHb^- were harvested from 100 ml Htm medium after cultivation, then washed with potassium phosphate

buffer (KH_2PO_4 5.44 g/L, $Na_2HPO_4 \cdot 12H_2O$ 43 g/L; pH 7.2), disrupted by grinding in liquid nitrogen, and resuspended in 5 ml of potassium phosphate buffer. The cell debris was separated by centrifugation at 15000×*g* for 5 min. The liquid samples were exposed to CO for 10 min and then used for CO-difference spectral analysis by PowerWave HT Microplate Spectrophotometer (BioTek Instruments, Inc.).

2.4 Analytical methods

The viscosity of Salecan was determined by a NDJ-1 rotational viscometer (Shanghai Changji Instruments Co., Ltd., China) to indicate the relative yield of the sugar. The productivity of exopolysaccharide was expressed in terms of the weight after ethanol precipitation, collected by centrifugation, and dried under reduced pressure (Xiu *et al*., 2010).

The respiratory rate was determined by inoculating 1 ml of the seed medium into a 1-L flask bottle containing 250 ml Htm medium. The bottle was sealed by a cap, and incubated at 30 °C on a rotary shaker at 150 r/min. The decrease in dissolved O_2 was recorded online by an optical O_2 sensor VisiFerm DO Arc 120 (Hamilton Company) inserting through the cap.

Invertase activities were measured by DNS colorimetry (Miller, 1959). Cells of VHb^+ and VHb^- were separated from 100 ml culture broth by centrifugation, grinded in liquid nitrogen, and resuspended with 2 ml sodium phosphate buffer (0.2 mol/L NaH_2PO_4 93.5 ml, 0.2 mol/L Na_2HPO_4 6.5 ml; pH 5.7). A 500-μl sample of the cell suspension was mixed with 500 μl of 0.1 g/ml sucrose solution. After 10 min, the hydrolysis was stopped by immersion of the reaction mixture in a boiling water bath for 5 min. Glucose generated from sucrose hydrolysis by invertase was measured by DNS colorimetry. Invertase activities were expressed as milligrams of glucose per minute per milligram of protein (mg/(min·mg)).

3 Results

3.1 Maintenance of pCM158-vgb and its expression in *Agrobacterium* sp. ZX09 without antibiotic selection

The *vgb* gene was cloned into the broad-host-range plasmid pCM158 to form pCM158-vgb (Fig. 1), and then pCM158-vgb was successfully introduced into *Agrobacterium* sp. ZX09 by electroporation. The plasmid persisted well in *Agrobacterium* sp. ZX09. Under no antibiotic selection, there was a negligible loss of the plasmid for one passage of two days cultivation (Fig. 2), but after five passages of cultivation the loss of the plasmid was apparent. Plasmid loss is not easy as commonly thought. Velappan *et al*. (2007) have shown that even some incompatible plasmids could persist together in host for several passages without antibiotic selection. Persistence of plasmids in host without antibiotic selection was crucial for their practical usage in fermentation; the use of antibiotics would dramatically increase the cost of fermentation, and the waste containing the antibiotics discharged into the environment would pose serious health problems. Foreign genes were usually integrated into the genome of the host for stable propagation. However, it needs double crossover and clear genetic background of the host. The process is time-consuming, especially for non-model organisms. Another shortcoming is that the genomic-integrated foreign gene is single-copied, thus limiting the gene product. On the contrary, plasmids can overcome these shortcomings. They are multi-copied and easy to handle without the genetic background of the host. In this experiment, we showed that the broad-host-range plasmid pCM158 can persist well in *Agrobacterium* sp. ZX09 without antibiotic selection at the end of cultivation and should have practical usage for the increase of product yield.

The expression of VHb was demonstrated by CO-difference spectrum owing to its binding to CO. The absorbance spectrum of the solution is subtracted from the spectrum after saturation with CO. Bubbling CO shifted the absorption maxima to 419 nm, which are characteristic of the VHb-CO complex. There was an absorption peak at 419 nm in *Agrobacterium* sp. ZX09 hosting pCM158-vgb (VHb^+), but it was absent in the strain hosting the empty vector pCM158 (VHb^-) after bubbling CO (Fig. 3). Cells of VHb^+ and VHb^- were collected for analysis after 2-d culturing in Htm medium without adding antibiotics. These results showed that VHb was stably expressed and further indicated that the plasmid could persist in *Agrobacterium* sp. ZX09 without antibiotic selection.

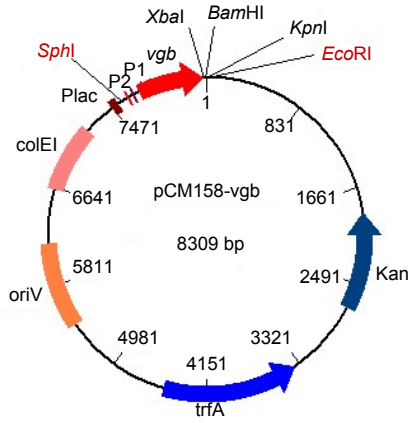

Fig. 1 Schematic map of plasmid pCM158-vgb

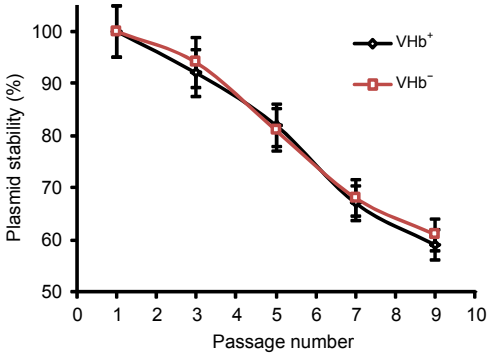

Fig. 2 pCM158 and pCM158-vgb maintenance in *Agrobacterium* sp. ZX09 without antibiotic selection

One passage was defined as 48-h cultivation in Htm medium without antibiotics. Plasmid stability was indicated by the ratio of the CFUs formed on the plates with antibiotics to those formed on the plates without antibiotics. Results were averages (±standard deviation) of triplicate experiments

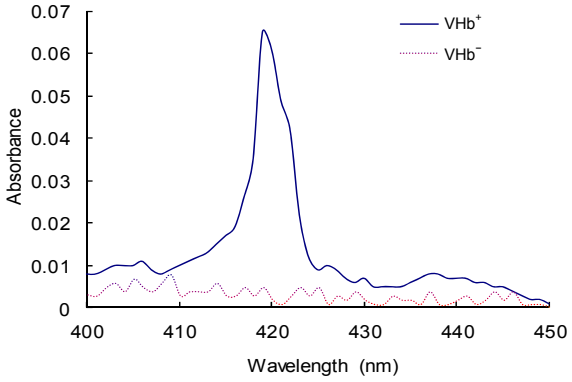

Fig. 3 CO-difference spectral analysis of VHb$^+$ and VHb$^-$

Cells of VHb$^+$ and VHb$^-$ were collected for analysis after 2-d culturing in Htm medium without adding antibiotics

3.2 VHb increased Salecan production

The viscosity yields of the two strains were monitored at 12 h intervals for 72 h (Fig. 4). The viscosity of VHb$^+$ was apparently higher than that of VHb$^-$, indicating the higher Salecan production by VHb$^+$. At the end of fermentation, viscosity yield of VHb$^+$ was about 33% higher than that of VHb$^-$. The obvious viscosity difference was manifested even at the first 12 h of the fermentation process. The Salecan production of VHb$^+$ was about 30% higher than that of VHb$^-$ (Fig. 5). Polymer synthesis is a high-energy and O_2 demanding process, whereas polymer in fermentation liquid inhibits O_2 transfer on account of its high viscosity. Therefore, polymer fermentation should be very sensitive to O_2 supply.

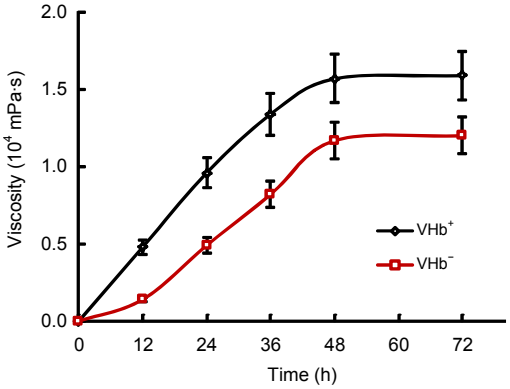

Fig. 4 Viscosity yields of VHb$^+$ and VHb$^-$

The two strains were monitored at 12 h intervals for 72 h on a rotary shaker at 250 r/min. Results were averages (±standard deviation) of triplicate experiments

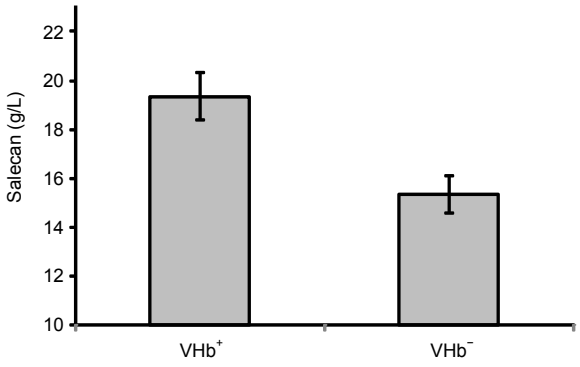

Fig. 5 Exopolysaccharide productivities of VHb$^+$ and VHb$^-$

The fermentation was performed on a rotary shaker at 250 r/min for 48 h. Results are averages (±standard deviation) of triplicate experiments

3.3 VHb increased respiration rate

The O_2 concentration in the liquid was monitored online in a sealed bottle. In order to set off the effect of cell concentration, the same amounts of VHb^+ and VHb^- were inoculated, and at the end of the experiment, the OD_{600} values of the cells were measured to be 0.048 and 0.046, respectively, for VHb^+ and VHb^-, a difference of less than 5%. In general, the two curves had a similar pattern of O_2 decreasing rate (Fig. 6a), but at different O_2 concentrations the consumption rates varied. The lower the O_2 concentration was, the VHb^+ consumed O_2 at a higher rate. At the interval of 20%–40%, 6%–10%, and 3%–6% air saturated O_2 concentration, the relative respiration rates of VHb^+ were 0.236, 0.082, and 0.065, respectively, while the values for VHb^- were 0.194, 0.042, and 0.022 (Figs. 6b–6d). Both VHb^+ and VHb^- dropped their respiration rates with the decrease of O_2 concentration, but with different dropping rates. VHb^+ consumed O_2 faster than VHb^-. At the three O_2 concentration intervals, the respiration rates of VHb^+ were 1.2, 1.9, and 3.1-fold that of VHb^-, respectively. While at the interval of 40%–100% air saturated O_2 concentration, the difference of respiration rate between VHb^+ and VHb^- was not obvious (data not shown). The growth limiting O_2 concentrations for bacteria are usually far below air saturated O_2 concentration. Above the growth limiting O_2 concentrations, the terminal oxidase is saturated by O_2 and the reaction will not increase with the rise of O_2 concentrations. This explains why at high O_2 concentration of 20%–40% the respiration rates of VHb^+ and VHb^- were similar; at very low O_2 concentration, VHb played a significant role in facilitating its host respiration. The localization of VHb was determined to be concentrated near the cell membrane (Ramandeep *et al*., 2001), and further study showed that VHb could bind the subunit I of cytochrome bo and provide O_2 directly to the terminal oxidase (Park *et al*., 2002), thus providing O_2 directly to the terminal oxidase.

3.4 VHb increased cellular invertase activity

The increase in respiration rate and product yield means more rapid carbon drainage and conversion. Since sucrose was the sole carbon source for *Agrobacterium* sp. ZX09, we measured the activity of the sucrose hydrolase enzyme invertase. The cellular

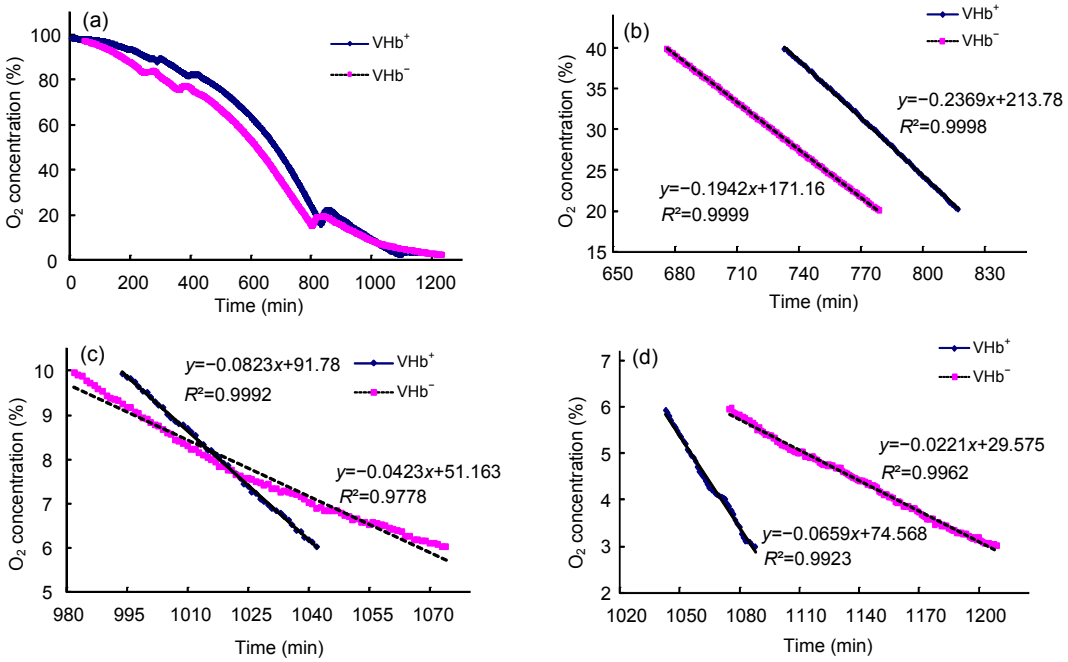

Fig. 6 Respiration rates of VHb^+ and VHb^-

(a) In general, VHb^+ and VHb^- had a similar pattern of O_2 decreasing rate. The O_2 concentration in the liquid was monitored online in a sealed bottle. In order to set off the effect of cell concentration, the same amounts of VHb^+ and VHb^- were inoculated. (b–d) Respiration rates at 20%–40% (b), 6%–10% (c), and 3%–6% (d) air saturated O_2 concentration interval. These formulae indicate the linear regression of the data. The coefficients indicate the change rate of O_2 concentration (y) with respect to time (x). R^2, the statistic parameter, measures the goodness of model fitting, with the value of 1 as perfect fitting

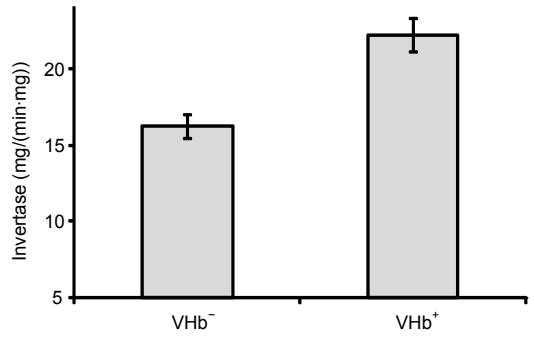

Fig. 7 Invertase activities of VHb^+ and VHb^-
Invertase activity was expressed as milligram of glucose per minute per milligram of protein (mg/(min·mg)). Results are averages (±standard deviation) of triplicate experiments

invertase activity in VHb^+ increased 37% compared with VHb^- strain (Fig. 7). It was reported that over-expression of invertase in plants increased oxygen consumption (Bologa *et al*., 2003). Therefore, the increase in respiration of VHb^+ was probably caused in part by the increase in invertase activity. The mechanism for the increase in invertase activity was unclear, though there were similar reports that the expression of invertase was suppressed under low O_2 concentration (Zeng *et al*., 1999; Bologa *et al*., 2003).

4 Conclusions

In order to overcome the O_2 limiting during Salecan fermentation, we cloned the *Vitreoscilla* hemoglobin gene, *vgb*, into a broad-host-range plasmid pCM158 to form pCM158-vgb. The plasmid could persist in the host *Agrobacterium* sp. ZX09 for at least one passage without antibiotic selection. The engineered strain *Agrobacterium* sp. ZX09 increased Salecan yield by 30% without antibiotic selection pressure. The benefit for the approach is its ease of handling and usefulness for field fermentation.

Acknowledgements

We would like to thank Mary E. LIDSTROM from the University of Washington, USA for providing the plasmid pCM158.

Compliance with ethics guidelines

Yun-mei CHEN, Hai-yang XU, Yang WANG, Jian-fa ZHANG, and Shi-ming WANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要：

本文题目：在土壤杆菌 ZX09 中透明颤菌血红蛋白的表达提高索拉胶的产量

***Vitreoscilla* hemoglobin promotes Salecan production by *Agrobacterium* sp. ZX09**

研究目的：提高胞外多糖索拉胶的产量。

创新要点：透明颤菌血红蛋白基因在质粒上表达，不加抗生素发酵生产索拉胶。

研究方法：将透明颤菌血红蛋白基因构建到广谱质粒 pCM158 上得到重组质粒 pCM158-vgb，重组质粒转入土壤杆菌 ZX09 中，使透明颤菌血红蛋白在土壤杆菌 ZX09 中表达。

重要结论：在土壤杆菌 ZX09 中颤菌血红蛋白的表达解决了发酵过程中氧受限的问题，提高了宿主菌的呼吸速率、蔗糖酶活性和黏度，将发酵产物索拉胶的产量提高了 30%。

关键词组：索拉胶；透明颤菌血红蛋白；土壤杆菌 ZX09；蔗糖酶；呼吸速率