# Biodegradation of Gallic Acid to Prepare Pyrogallol by *Enterobacter aerogenes* through Substrate Induction

Wenjun Li [a,b] and Chengzhang Wang [a,b,c]

Pyrogallol, as an important chemical raw material and reagent, has been prepared by the decarboxylation reaction of gallic acid hydrolyzing tannin acid extracted from Chinese gall, but the decarboxylation reaction is known to cause serious environmental pollution. To obtain efficient strains to degrade gallic acid, a screening study was carried out to explore different strains and optimal fermentation conditions of single impact factors, as well as using response surface methodology. The antioxidant bioactivity of products containing pyrogallol in the fermentation medium was also estimated. The results indicated that *Enterobacter aerogenes* could degrade gallic acid into pyrogallol with 77.86% average yield under the optimal fermentation conditions of an inoculum size of 5%, substrate concentration of 0.32%, incubation period of 60 h, fermentation temperature of 32 °C, content of phosphate buffer at 25%, and an initial pH of 6.0 in fermentation medium. The products contained 66.5% pyrogallol and were tested for their antioxidant capacity. They proved to have stronger antioxidant capacity compared with ABTS, BHT, and even Vc. In conclusion, the study provided a simple, highly efficient method, superior to complex genetic engineering technologies, to degrade gallic acid into pyrogallol, suggesting the possibility of large-scale production in the future.

*Keywords: Enterobacter aerogenes; Pyrogallol; Substrate induction; Response surface methodology; Hydrophilic antioxidant*

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## INTRODUCTION

Chinese gall is a kind of insect gall formed on *Anacardiaceae*, specifically *Rhus chinensis* Mill., *Rhus potanninii Maxim.*, and *Rhus punjabensis*, tree species unique to China, primarily distributed in Qinling, Ba Mountains, Wudang with unique climate and proper soil (Zhang 1991; Li *et al.* 2003). Tannic acid is the major ingredient of Chinese gall, as hydrolysable tannins (Li *et al.* 2008), so it is hydrolyzed easily to gallic acid (3,4,5-hydroxy benzoic acid), which is an important chemical raw material used widely in coatings, the chemical industry, leather, cosmetics, and minerals (Zhang *et al.* 2013). In addition, gallic acid can be used as a starting material in the synthesis of pyrogallol, lipid compounds of gallic acid, 3,4,5-hydroxy benzoic acid, 3,4,5-trimethoxybenzaldehyde (TMB), 3,4,5-methoxy benzyl pyrimidine (TMP) (Zhang *et al.* 2005), and other products.

At present, pyrogallol (1,2,3-trihydroxy benzene), a polyphenol, has been applied in a variety of industrial sectors as an important chemical raw material and reagent, especially in photography, used to make colloidal solutions of metals as a developer, used

for absorption of oxygen in gas analysis, used as a mordant in the dyeing of hair or staining leather, and used as an important intermediate in medicine, food, and cosmetics (Yoshida and Yamada 1985; Kumar *et al.* 1999). However, it is still prepared traditionally by chemical decarboxylation of gallic acid catalyzed by high temperature or pressure (Zeida 1998) in the presence of highly concentrated hydrochloric acid, resulting in the serious pollution of wastewater with a high concentration of salt. Gallic acid is obtained by enzymatically catalyzing tannic acid by tannase, and gallic acid is further catalyzed to pyrogallol by decarboxylation (Haslam *et al.* 1961; Brune and Schink 1992) (Fig. 1). Therefore, some studies have explored new eco-friendly and high-efficiency methods. Among them, an aquatic plant named *Myriophyllum spicatum* was found to produce pyrogallic acid (Satoshi Nakai *et al.* 2000), but few studies have been performed on it. In addition, the studies on biological conversion have shown the benefits of an absence of corrosion, no waste acid, less by-products, lower cost compared with the chemical-based method, and the most important point is that the target substrate can be converted into the target product if the conditions are suitable (Soni *et al.* 2012). However, the first step is to find a strain having the ability to degrade gallic acid to prepare pyrogallol.

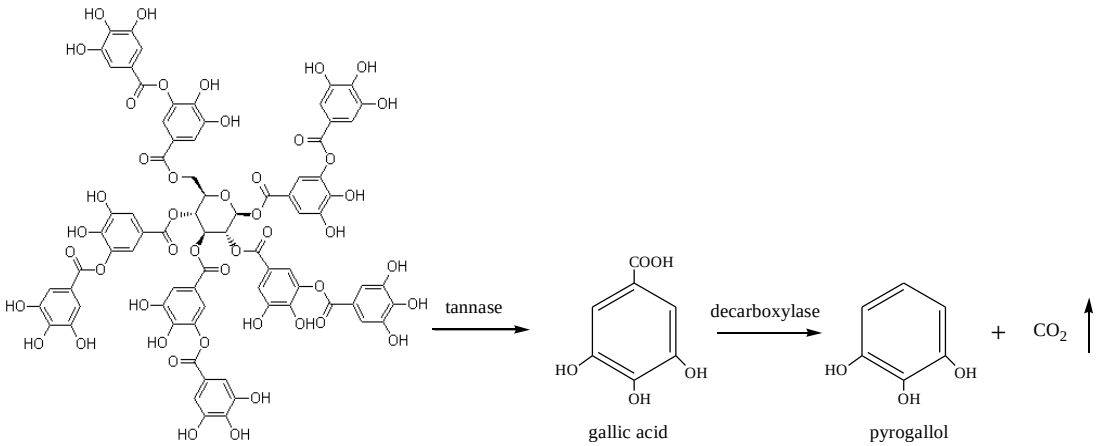


**Fig. 1.** The route of degradation of gallic acid to produce pyrogallol

Currently, some bacterial species have been found to degrade gallic acid, but most of them need to be cultivated under anaerobic conditions or immobilized to produce pyrogallol in a flow reactor. *Enterobacter* spp. can degrade gallic acid to pyrogallol with shake-flask cultivation, but the low yield of pyrogallol makes the method impractical for purposes of producing further products (Grant and Patel 1969; Yoshida *et al.* 1982; Yoshida and Yoshida 1985; Gupta *et al.* 1986; Samain *et al.* 1986; Krumholz *et al.* 1987; Kumar *et al.* 1992; Nakajima *et al.* 1992; Haddock and Ferry 1993). Additionally, some bacteria have been identified as having both tannase and gallic acid decarboxylase activity, such as *Pantoea agglomerans* (Zeida 1998), *Streptococcus gallolyticus* (Osawa *et al.* 1995a), *Lonepinella koalarum* (Osawa *et al.* 1995b), *Lactobacillus plantarum* (Kar *et al.* 2003), *Lactobacillus paraplantarum* (Kar *et al.* 2003), and *Lactobacillus pentosus* (Kar *et al.* 2003). Having a mixture of activities increases the difficulties in controlling the reaction to get the target product. Some advanced biotechnology methods have been applied to improve the situation; for example, scientists synthesized a new type of *Escherichia coli*, called *E. coli* KL7/pSK6.161, by genetic engineering (Spiros *et al.*

2000). That approach was able to produce pyrogallol from glucose with a high yield, but the method was too complicated to operate and popularize. Previous investigations on the enzymatic production of pyrogallol were mostly carried out using gallotannin or taratannin because tannins are readily available in nature (O'Donovan and Brooker 2001; Odenyo *et al.* 2001), but the catalyzed reaction was extremely slow, and only 25.8% or 28.5% of the substrate was degraded (Yoshida and Yamada 1985).

To explore a better way to degrade gallic acid by microorganisms, it was decided to use a much more simple and effective method: substrate induction to screen a bacteria to degrade gallic acid effectively, from the typical strains belongs to *Citrobacter* spp. and *Enterobacter* spp. Substrate induction means that it is possible to make a strain to degrade a specific substrate into another target product by controlling the substrate in the culture. Therefore, in order to find the target strain and determine the optimum conditions, firstly, the strategy of changing the gallic acid as the sole substrate with the other ingredients or outside conditions of culture medium has been applied for a long time in many different fields (Sayre *et al.* 1956; Petit *et al.* 1978; Sun *et al.* 2007). The yield was still lower than those prepared by chemical methods because of the presence of other by-products in the process. Possibly this result was just a consequence of the normal metabolism of the bacteria from the peak of by-products detected in the HPLC. Therefore, in this work it was attempted to optimize and improve the fermentation process. Pyrogallol, as a type of polyphenol, was shown to be better than BHT and *Vitamin C*(*Vc*) hydrophilic antioxidant (Halliwell 1996), so to test the antioxidant capabilities of products containing pyrogallol in the fermentation medium, assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and (3-ethyl-benzothiazoline-6-sulfonic acid) ABTS were estimated (Thaipong *et al.* 2006) because they have been frequently used to detect antioxidant capabilities in plants, foods, and other products (Brand-Williams *et al.* 1995; Miller and Rice-Evans 1997; Gil *et al.* 2002; Leong and Shui 2002).

## EXPERIMENTAL

All chemicals, including standard compounds of gallic acid and pyrogallol, unless specified otherwise, were obtained from Sigma Chemical Co. and were of certified reagent grade.

*Microorganism and growth conditions*

The Gram-negative bacteria, which were identified as *Enterobacter* spp. and *Citrobacter* spp. on the basis of morphological, biochemical, and 16S ribosomal RNA gene sequence features, were preserved in the Chinese common microbe bacterial preservation administration center. The *Enterobacter* spp. culture was maintained on a fluid medium containing 0.06% $MgSO_4 \cdot 7\ H_2O$, 0.4% $(NH_4)_2SO_4$, 0.2% gallic acid, and 30 mM phosphate buffer solution (with a pH value of 6.6) (Yoshida *et al.* 1982); the *Citrobacter* spp. culture was maintained on a fluid medium containing 2.0% gallic acid, 0.2% $(NH_4)_2HPO_4$, 0.1% $KH_2PO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% yeast extract (Yoshida and Yamada 1985).

*Screening the bacteria producing high yields of pyrogallol*

The two types of bacteria to be screened were cultivated in various culture conditions for various amounts of time, after which their fermentation broth was collected to determine whether the pyrogallol was produced or not, and culture media for *Enterobacter* spp. and *Citrobacter* spp. were prepared separately, as shown in the previous paragraph. Two inoculating loops of strains were taken from a prepared agar slant preservation medium to inoculate a 250-mL flask (liquid loading quantity: 100 mL). Shake cultivation was conducted at 30 °C and 180 rpm, and samples were taken and tested for the production of pyrogallol every 12 h, continuing for a week based on their growth curve.

*Optimization of culture conditions for pyrogallol production with biochemical assay*

The strains screened were cultivated on an agar slant culture medium for 5 h; then, five inoculating loops of strains were taken with sterile operation to a 250-mL flask (seed culture medium: 100 mL), cultivated with standing for 5 h, at 37 °C. Next, 5 mL of seed liquid was added to the fermentation medium at 30 °C and 180 rpm for 60 h in a table concentrator. The study explored some factors that affected the yield of pyrogallol, such as the inoculum size (1 mL to 10 mL), the substrate concentration (0.1% to 3.0%), incubation period (0 to 120 h), fermentation temperature (20 to 50 °C), and fermentation medium initial pH value (5.0 to 8.0). According to the results of single factor experiments, Box-Behnken was applied to design response surface experiments of 3 factors and 3 levels.

*Extraction of pyrogallol from fermentation broth*

The gallic acid was degraded by *Enterobacter aerogenes* in the fermentation broth for approximately 60 h. Then, approximately 20 mL of the fermentation liquor was extracted with 60 mL of diethyl ether, concentrated, filtered with a 0.45-μL filter membrane, and dissolved with 5 mL of mobile phase ($CH_3COOH$ (concentration of 0.5% ) :$CH_3OH$=0.37:0.63 (vol/vol)) and prepared to be detected.

*Analysis*

Gallic acid and pyrogallol were analyzed with an HPLC equipped with a Thermo C18 column (packing material size: 5 μm; the standard of chromatographic column: 4.6×250 mm); the eluent was a mixed solution of 37% $CH_3COOH$ (concentration of 0.5% ) and 63% $CH_3OH$ (vol/vol), and monitored using a PDA e detector at 263 nm with a flow rate of 1.0 mL/min. Authentic gallic acid and pyrogallol were used as reference materials. The retention times of gallic acid and pyrogallol were 3.077 and 3.443 min, respectively. The column was maintained at room temperature, and the samples were kept at 15°C and then analyzed with the Empower pro Software. The standard yield curve of pyrogallol was $y$ = 1318523.5$x$+6474.1, correlation coefficient $R$ = 0.99951; the standard curve of gallic acid was $y$ = 13379425.0$x$+60043.8, correlation coefficient $R$ = 0.99951.

These calculations were performed using the following equations. The yield of pyrogallol was given by,

$$y=(5\times c / v_1)\times v_0 / m_0\times 100\% \quad (1)$$

where $v_1$ is the extraction volume; $v_0$ is the actual volume of the fermentation broth; $m_0$ is the theoretical production of pyrogallol; $c$ is the concentration of pyrogallol, calculated by its standard curve; and the number 5 represents the volume (in mL) of the mobile phase used to dissolve the extract.

The degradation rate of gallic acid is given by,

$$y = (1 - 5 \times c' / c_0' \times v_1) \times 100\% \quad (2)$$

where $c'$ is the concentration of gallic acid, calculated by its standard curve; and $c_0'$ is the initial concentration of gallic acid.

*Oxidation resistance and bacteriostatic testing*

To evaluate the oxidation of the extract containing pyrogallol, the two most common radical scavenging assays were used. 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (Miller and Rice-Evans 1997; Leong and Shui 2002) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Brand *et al.* 1995; Gil *et al.* 2002) have been frequently used to estimate antioxidant capacities (Floegel *et al.* 2011). The DPPH assay was done according to the method of Brand-Williams (Brand *et al.* 1995), with some modifications. Briefly, a solution of 1 mM DPPH.in 95%(v/v) methanol was stirred for 30 min, then the absorbance of solution was adjusted to 0.650±0.020 at 517 nm using fresh 95% (v/v) methanol. After that, 0.1 mL of standard or sample were mixed with 3 mL of DPPH solution and incubated for 30 min in the dark. Then the specimens were monitored for their absorbance. Controls consisted either of 0.1 mL acidified distilled deionized water in 3 mL of DPPH solution for vitamin C standard or 0.1 mL of 50%(v/v) methanol in 3 mL of DPPH solution for samples. For the ABTS assay, the procedure followed the method of Floegel (Floegel *et al.* 2011) with some modifications (Brand *et al.* 1995; Thaipong *et al.* 2006). In this procedure 2.5 mM of ABTS was mixed with 1 mM of 2,2-azobis(2-amidinopropande) dihydrochloride in 10 mM phosphate buffered saline(PBS) solution, pH 7.4. Then, the mixture was heated in a water bath at 60 °C for 30 min, after which the blue-green $ABTS^+$ solution was cooled to room temperature, filtered through nylon syringe filters, and diluted with fresh PBS buffer until absorbance of 0.650±0.020 at 734 nm. After that, 0.02 mL of vitamin C standard or sample were mixed with 0.980 mL of $ABTS^+$ solution and incubated for 10 min in 30 °C water bath; then their absorbance was monitored at 734 nm after 10 min. A control consisted either of 0.02 mL acidified distilled deionized water in 0.980 mL of radical solution for vitamin C standard or 50%(v/v) methanol in 0.980 mL of radical solution for samples.

## RESULTS AND DISCUSSION

### The Strains for Producing Pyrogallol

The strains for producing pyrogallol were screened from *Enterobacter* spp. and *Citrobacter* spp. cultivated on the above cultures modified at the same initial pH. In the study of Zeida *et al.* (1998), high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) methods were applied to detect the appearance of a pyrogallol peak to confirm the strain researched. However, the present results showed that only *Enterobacter aerogenes* No. CICC23008 could degrade gallic acid to form pyrogallol after cultivating for 48 h in modified culture. Although the strain is a

facultative anaerobe, it could not have grown on the shaking culture medium under aerobic conditions, and the following figures (Figs. 3 through 8) demonstrate this point.

HPLC (Fig. 2a,b,c) provides evidence of the existence of pyrogallol in the process; Fig. 2(a) shows the mixed standards of gallic acid and pyrogallol, Fig. 2(b) shows the presence of pyrogallol for the first time after cultivating for 48 h, and Fig. 2(c) shows the maximum content after cultivating for 60 h.

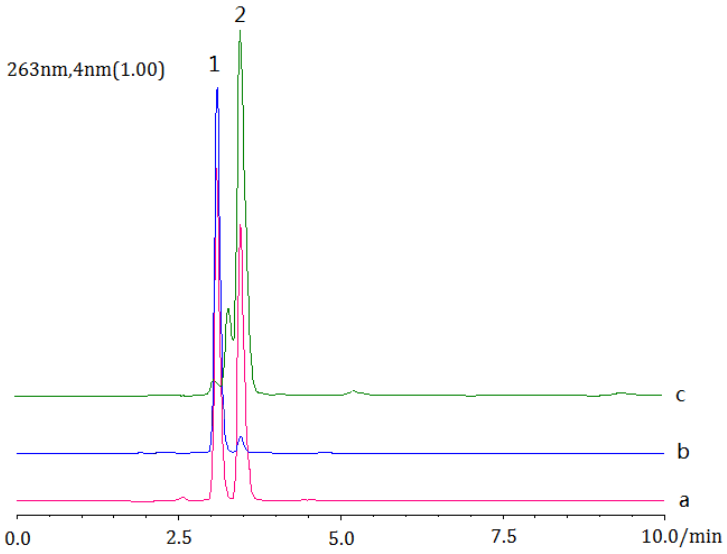


**Fig. 2.** Detection of pyrogallol from fermentation liquor by HPLC: (a) mixed standards of gallic acid and pyrogallol; (b) after 48 h of fermentation; (c) after 60 h of fermentation

## Optimization of Culture Conditions for Pyrogallol Production by *E. aerogenes* with Biochemical Assays

To optimize the culture medium to obtain the maximum yield of pyrogallol, various single factors were tested. Among them, the effects of various important factors on the yield of pyrogallol and the degradation rate of gallic acid were determined. The trend line of the yield of pyrogallol was shown to describe the overall situation. Then, on the basis of these results, response surface methodology was applied to determine the combined effect of three more significant factors at three levels.

## Effects of the Various Factors

*Inoculum size*

An inoculum size ranging from 1 to 10 mL was added to inoculate the culture medium containing 100 mL of fermentation broth with shaking. From the following figure, the yield of pyrogallol increased with inoculum size before 5 mL, but then tended to be stable from that point on (Fig. 3).

Additionally, although 8 mL of inoculum supported the maximum production, 49.99%, the yield reached 48.79% with 5 mL, very close to the maximum. Therefore, 5 mL was the best choice in the following experiment and was considered economical; this value was the same as the another *Enterobacter* spp., in which 5% inoculum was the final result (Soni *et al.* 2012a,b). The degradation rate of gallic acid showed that most gallic

acid was used by this strain for growing and degrading into pyrogallol or other similar products found from Fig. 3.

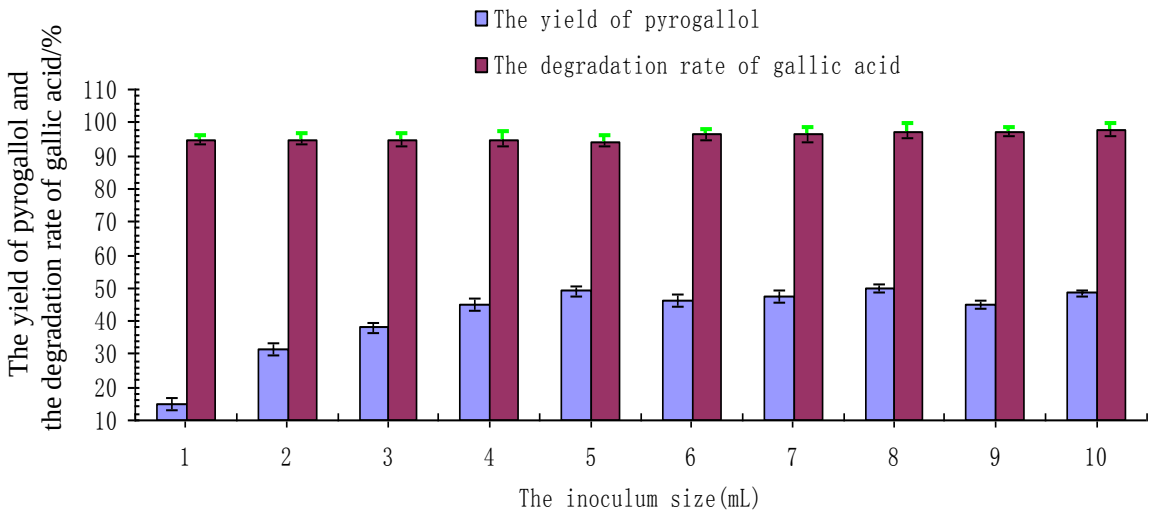


**Fig. 3.** Effect of inoculum size on pyrogallol production (0.2% gallic acid, 25% phosphate buffer, at 30°C, cultivated for 60 h at pH 6.2, and 180 r/min shaking)

*Substrate concentration*

Because the optimal substrate concentration was relative to the inoculum size, substrate concentrations ranging from 0.1% to 2.5% were tested and repeated several times, but the following strange results were acquired every time: 0.7% gallic acid in the fermentation broth showed the maximum pyrogallol production (Fig. 4).

Although the yields of pyrogallol with lower and higher substrate concentrations decreased, 0.4% gallic acid yielded more pyrogallol than did 0.5% and 0.6%, except for 0.7% gallic acid. Besides, there was no pyrogallol when the substrate concentration was more than 0.8%.

Although these results are surprising, repeated tests showed no change, and the results might have appeared because the addition of gallic acid changed the pH. This means that the gallic acid was a type of acid, so 0.7% gallic acid changed the pH to 5.5 in the fermentation system. Moreover, high concentration of gallic acid would inhibit the action and decrease the yield of pyrogallol. Therefore, 0.7% gallic acid was the result of the balance of pH and the concentration of gallic acid, and 0.4% gallic acid was used for further testing, and the speculations will be proved in the next experiments.

In many similar studies, 0.2% gallic acid was chosen (Yoshida and Yamada 1985; Zeida *et al.* 1998; Soni *et al.* 2012a,b), whereas this study demonstrated that the substrate concentration and inoculum sizes were related with each other. In other words, higher substrate concentration matched larger inoculums size, so that the balance of them was 0.4% of gallic acid to match 5% of inoculum sizes. In addition, the degradation rate of gallic acid showed a slight fluctuation when the concentration of gallic acid was higher than 0.8%, and no pyrogallol was detected. It was therefore reasonable that the much higher concentration of gallic acid inhibited the process of degradation into pyrogallol but produced other compounds from the high degradation rate above 0.8% gallic acid.

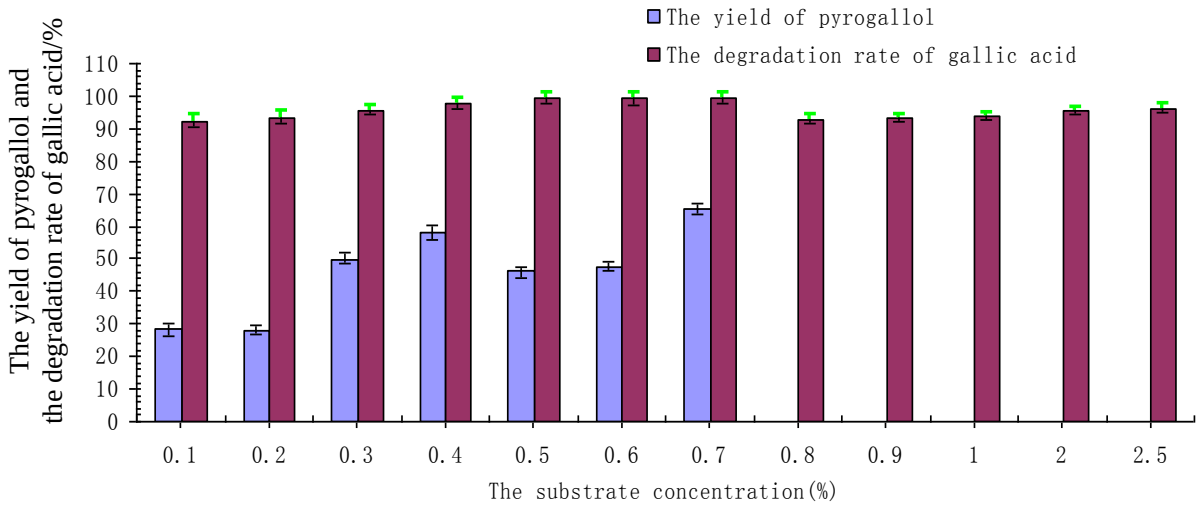


**Fig. 4.** Effects of substrate concentration on pyrogallol production (5% inoculum size, 25% phosphate buffer at 30 °C, cultivation for 60 h at pH 6.2, and 180 r/min shaking)

*Incubation period*

Because *E. aerogenes* follows a growth pattern, the time course of pyrogallol production by *E. aerogenes* was monitored and detected every 12 h for 132 h. *Enterobacter aerogenes* showed a maximum yield of pyrogallol of 63.41% when grown in fermentation broth for 60 h (Fig. 5). This is a relatively long time compared to other strains; for example, *Citrobacter* spp. 64-1 degraded 0.2% gallic acid to produce the maximum yield of pyrogallol after cultivating for 48 h (Yoshida and Yamada 1985), and another *Enterobacter* spp. reached the maximum gallic acid decarboxylase activity after just 20 h (Soni *et al.* 2012b).

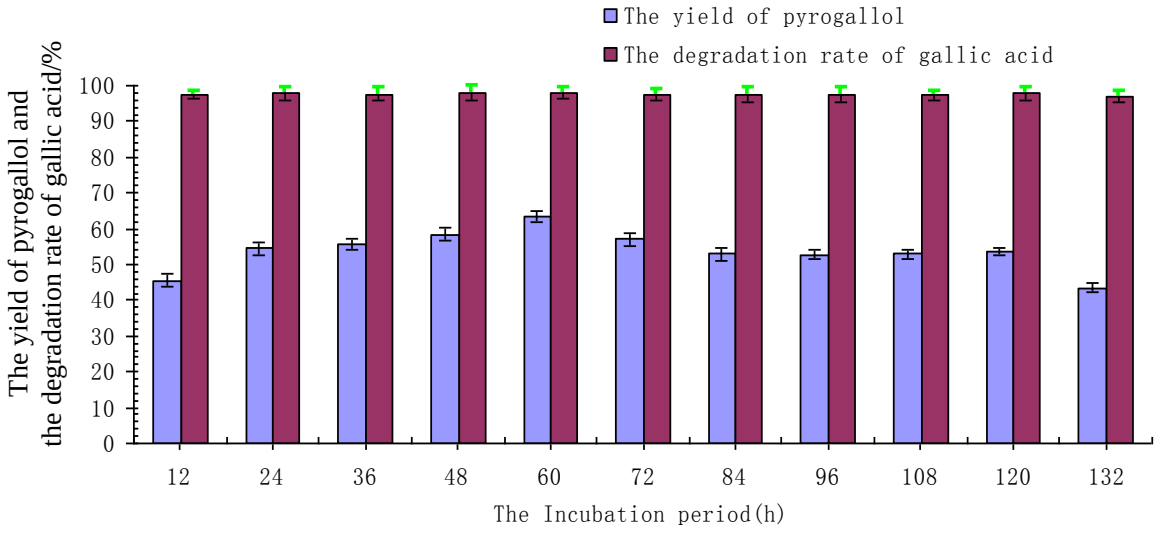


**Fig. 5.** Effects of incubation period on pyrogallol production (5% inoculum size , 0.4% gallic acid, 25% phosphate buffer, temperature of 30 °C, 6.2 pH value, and 180 r/min for shaking)

Because of the properties of the strain and the culture medium provided, the strain needs an acclimation process to fit the new culture medium completely to degrade the target substrate. To explore this reason, changes in the pH of the fermentation broth were recorded every 12 h, and the results showed the pH was 6.14 in the beginning 12 h, and then began to decrease until it reached 5.40 in the final 132 h. The pH was monitored using a pH meter throughout the process (Table 1). The results showed that the final pH was lower than the initial pH, and there was little change in the degradation rate of gallic acid. The gallic acid degraded quickly at first and maintained a high degradation rate, but considering the final results, maybe both pyrogallol and gallic acid have been used for growing by the strains or forming other compounds like weak acid, so the yield of pyrogallol was lower after cultivating for 132 h.

**Table 1.** Fermentation Broth pH

| Incubation period (h) | 12 | 24 | 23 | 48 | 60 | 72 | 84 | 96 | 108 | 120 | 132 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| pH | 6.14± 0.01 | 5.90± 0.02 | 5.65± 0.02 | 5.56± 0.01 | 5.53± 0.02 | 5.45± 0.01 | 5.47± 0.01 | 5.43± 0.01 | 5.40± 0.01 | 5.37± 0.01 | 5.40± 0.01 |

*Fermentation temperature*

Most bacteria are sensitive to temperature, so the degradation of gallic acid to pyrogallol using *E. aerogenes* was performed at various fermentation temperatures. As shown in Fig. 6, the yield of pyrogallol was essentially unchanged below 40 °C, reached the maximum yield of 63.56% at 35 °C, then decreased markedly; no pyrogallol was produced when the temperature was higher than 45 °C. The degradation rate of gallic acid showed a trend similar to that of the substrate concentration, so the explanation for the results may be the same. Two other *E. aerogenes* strains had optimal temperatures of 30 and 35 °C, similar to the present results (Soni *et al.* 2012a,b).

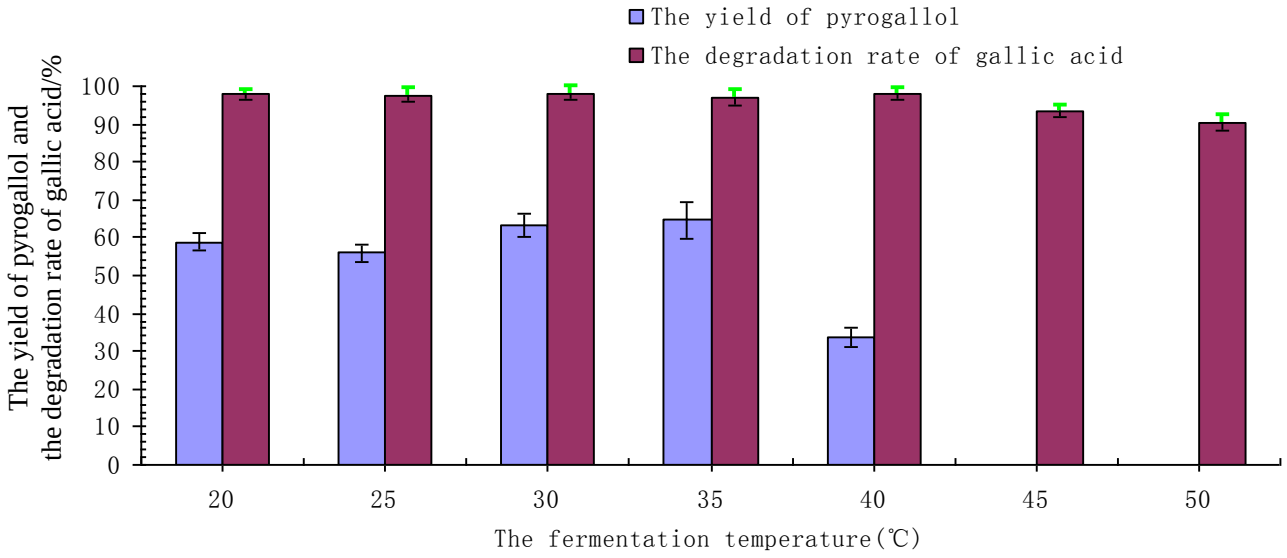


**Fig. 6.** Effect of fermentation temperature on pyrogallol production (5% inoculum size, 0.4% gallic acid, 25% phosphate buffer, cultivated for 60 h at pH 6.2, 180 r/min shaking)

*Phosphate buffer content*

To test the buffer system, different volumes of 0.2 M phosphate buffer were added to the fermentation broth, and the results are shown in Fig. 7. A 35% phosphate buffer provided a maximum pyrogallol yield of 56.39%, but too much phosphate buffer would bring too much inorganic salt to the system (Kleinman *et al.* 1979; Gómez *et al.* 2001), so 25% phosphate buffer, which had a similar yield, was the better choice.

Figure 7 also shows that the gallic acid was degraded before pyrogallol was present, which suggests that gallic acid was used for growing at the beginning phase. In addition, gallic acid was degraded into other compounds because of the high degradation rate of gallic acid without the matched high yield of pyrogallol, as shown in the figure.

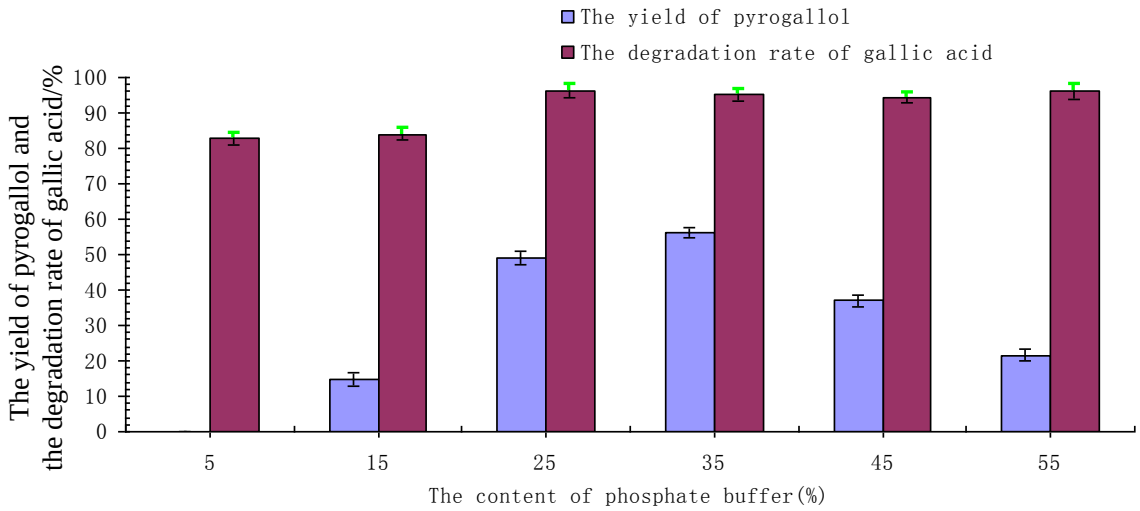


**Fig. 7.** Effect of phosphate buffer content on pyrogallol production (5% inoculum size, 0.4% gallic acid at 30°C, cultivated for 60 h at pH 6.2, and 180 r/min shaking)

*Initial pH value in fermentation medium*

The pH value noticeably influenced the extracellular protein content. To study its effect on the yield of pyrogallol and the degradation rate of gallic acid, seven different initial pH values were compared. The yield of pyrogallol reached the maximum when the pH value was 6.0.

The pH values that were too low or too high resulted in low yields (Fig. 8); this result is similar to that of *Citrobacter* spp. 64-1 with a pH value of 6.0 and *Enterobacter* spp. with a pH of 6.6 (Yoshida and Yamada 1985; Soni *et al.* 2012a). The pH value changed the path of the strain to produce pyrogallol, and it also affected the whole condition in the culture medium, which decided the production of pyrogallol. Gallic acid was mostly degraded into pyrogallol when the pH value was lower than 6.0 but was degraded into other compounds when the process was inhibited after 6.0 pH. From the above, the histogram of the degradation rate of gallic acid verified the explanation and suggested that speculations of the first paragraph of *Substrate concentration* were reasonable.

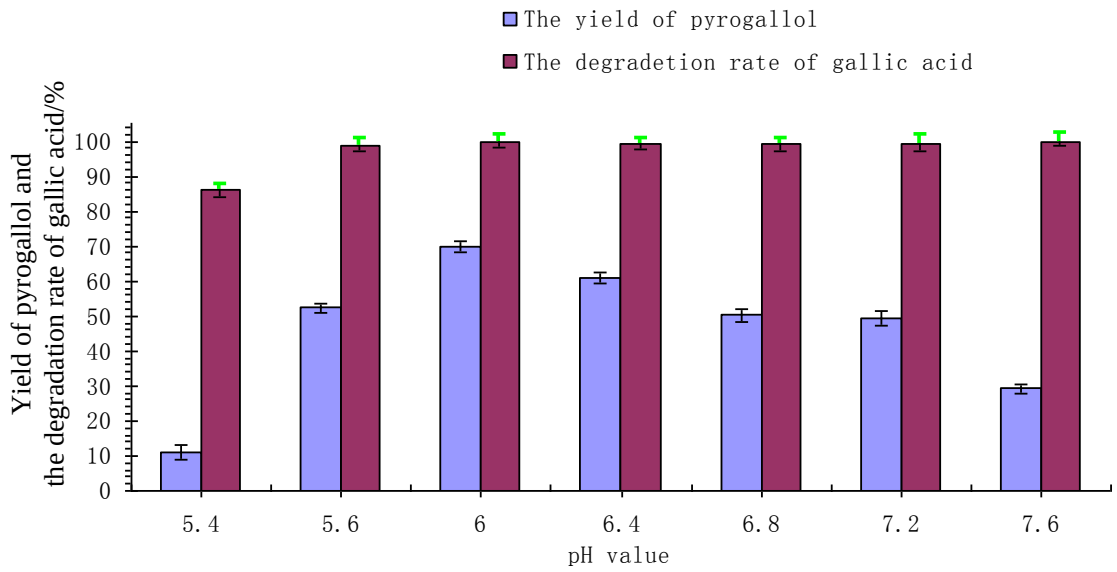


**Fig. 8.** Effect of pH value on pyrogallol production (5% inoculum size, 0.4% gallic acid at 30°C, cultivated for 60 h with 25% phosphate buffer, and 180 r/min shaking)

## Response Surface Methodology

Considering the yield of pyrogallol and the degradation rate of gallic acid, the influence of different single factors and their importance to the whole experiment, the substrate concentration (0.3% to 0.5%), fermentation temperature (25 to 35 °C), and fermentation medium initial pH value (5.6 to 6.4) were used as the main factors to search the optimization of culture conditions of the microbial degradation of gallic acid and verify final consequences. Their scopes are shown in Table 2, and the response was measured in terms of the yield of pyrogallol. The effect of the variables on the yield was calculated by Design Expert® 7' (Stat-Ease, Inc., Minneapolis, MN, USA), which was used to generate and analyze the experimental design of response surface methodology (Sun *et al.* 2007).

Table 3 shows the yields of pyrogallol using response surface methodology. Table 4 shows the analysis of variance for the response surface quadratic model; the lack of fit was 0.0108, less than 0.05, which indicated the fit of the equation was bad and an adjusted equation was needed. Then, the lack of fit was larger than 0.05 after considering the addition of the power of three to the equation (Zhang *et al.* 2011), shown in the following Table 5.

**Table 2.** Experimental Factor and Level of Response Surface Methodology

| Factors | Levels | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| $X_1$ Temperature (°C) | 25 | 30 | 35 |
| $X_2$ Initial pH value | 5.6 | 6.0 | 6.4 |
| $X_3$ Substrate concentration (%) | 0.3 | 0.4 | 0.5 |

**Table 3.** Program and Experimental Results of Response Surface Methodology

| No. | $X_1$ Temperature (°C) | $X_2$ Initial pH | $X_3$ Substrate concentration (%) | $Y$ Yield of pyrogallol (%) |
|---|---|---|---|---|
| 1 | -1 | -1 | 0 | 73.34 |
| 2 | 1 | -1 | 0 | 0 |
| 3 | -1 | 1 | 0 | 34.39 |
| 4 | 1 | 1 | 0 | 58.52 |
| 5 | -1 | 0 | -1 | 67.51 |
| 6 | 1 | 0 | -1 | 76.02 |
| 7 | -1 | 0 | 1 | 56.90 |
| 8 | 1 | 0 | 1 | 42.73 |
| 9 | 0 | -1 | -1 | 68.12 |
| 10 | 0 | 1 | -1 | 60.12 |
| 11 | 0 | -1 | 1 | 0 |
| 12 | 0 | 1 | 1 | 55.21 |
| 13 | 0 | 0 | 0 | 65.01 |
| 14 | 0 | 0 | 0 | 66.02 |
| 15 | 0 | 0 | 0 | 65.71 |
| 16 | 0 | 0 | 0 | 65.52 |
| 17 | 0 | 0 | 0 | 66.38 |

**Table 4.** Analysis of Variance for Response Surface Quadratic Model

| Source | Sum of squares | df | Mean square | F value | P value Prob>F |
|---|---|---|---|---|---|
| $X_1$ | 1248.51 | 1 | 1248.51 | 10.37 | 0.0146 |
| $X_2$ | 322.16 | 1 | 322.16 | 2.68 | 0.1458 |
| $X_3$ | 965.11 | 1 | 965.11 | 8.02 | 0.0253 |
| $X_1X_2$ | 1659.50 | 1 | 1659.50 | 13.79 | 0.0075 |
| $X_1X_3$ | 253.56 | 1 | 253.56 | 2.11 | 0.1900 |
| $X_2X_3$ | 998.68 | 1 | 998.68 | 8.30 | 0.0236 |
| $X_1^2$ | 181.00 | 1 | 181.00 | 1.50 | 0.2597 |
| $X_2^2$ | 2380.95 | 1 | 2380.95 | 19.78 | 0.0030 |
| $X_3^2$ | 12.80 | 1 | 12.80 | 0.11 | 0.7539 |
| Model | 8085.88 | 9 | 898.43 | 7.46 | 0.0074 |
| Residual | 842.54 | 7 | 120.36 | | |
| Lack of fit | 777.69 | 3 | 259.23 | 15.99 | 0.0108 |
| Pure error | 64.85 | 4 | 16.21 | | |
| Cor total | 8928.43 | 16 | | | |

**Table 5.** Analysis of Variance for Response Surface Quadratic Model (After Adding the Equation of the Third Power)

| Source | Sum of squares | df | Mean square | *F* value | *P* value Prob>*F* |
|---|---|---|---|---|---|
| $X_1$ | 301.52 | 1 | 301.52 | 19.42 | 0.0070# |
| $X_2$ | 557.11 | 1 | 557.11 | 35.87 | 0.0019* |
| $X_3$ | 1333.53 | 1 | 1333.53 | 85.87 | 0.0002* |
| $X_1X_2$ | 1659.50 | 1 | 1659.50 | 106.86 | 0.0001* |
| $X_1X_3$ | 253.56 | 1 | 253.56 | 16.33 | 0.0099# |
| $X_2X_3$ | 998.68 | 1 | 998.68 | 64.31 | 0.0005* |
| $X_1^2$ | 176.46 | 1 | 176.46 | 11.36 | 0.0199# |
| $X_2^2$ | 2369.18 | 1 | 2369.18 | 152.56 | < 0.0001* |
| $X_1^2X_2$ | 238.12 | 1 | 238.12 | 15.33 | 0.0112# |
| $X_1^2X_3$ | 423.43 | 1 | 423.43 | 27.27 | 0.0034* |
| $X_1X_2^2$ | 116.15 | 1 | 116.15 | 7.48 | 0.0410# |
| Model | 8850.78 | 9 | 804.62 | 51.81 | 0.0002* |
| Residual | 77.65 | 5 | 15.53 | | |
| Lack of fit | 12.80 | 1 | 12.80 | 0.79 | 0.4245 |
| Pure error | 64.85 | 4 | 16.21 | | |
| Cor total | 8928.43 | 16 | | | |

Note: #: $p < 0.05$ significance level; *: $p < 0.005$ significance level

$$Y=68.63-8.68\times X_1+11.80\times X_2-18.26\times X_3+20.37\times X_1\times X_2-7.96\times X_1\times X_3+15.80\times X_2\times X_3-6.46\times X_1^2-23.69\times X_2^2-10.91\times X_1^2\times X_2+14.55\times X_1^2\times X_3-7.62\times X_1\times X_2^2.$$

After adjustment, the results were found to fit the equation given above. The Model F-value of 51.81 implied that the model was significant. There was only a 0.02% chance that a "Model F-Value" this large could occur as a result of noise. Values of "Prob > *F*" less than 0.0500 indicated that the model terms were significant. In this case $X_1$, $X_2$, $X_3$, $X_1X_2$, $X_1X_3$, $X_2X_3$, $X_1^2$, $X_2^2$, $X_1^2X_2$, $X_1^2X_3$, and $X_1X_2^2$ were significant model terms. Values greater than 0.1000 indicated the model terms were not significant. If there were many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The "Lack of Fit F-value" of 0.79 implied the Lack of Fit was not significant relative to the pure error. There was a 42.45% chance that a "Lack of Fit F-value" this large could occur as a result of noise. Non-significant lack of fit was good, and the model could fit; therefore, this equation could be used to predict how *E. aerogenes* degrades gallic acid. Analysis of variance showed that all items in the equation of response values were significant, which indicated the influences of various specific experimental factors on the response value were not a simple linear relationship. Among them, the most significant factor was pH value; the effects of initial pH value of the fermented liquid were larger.

Using software for optimization, with the analysis of the response surface, the optimum conditions were predicted as follows: fermentation temperature was 31.58 °C, initial pH value was 6.07, and substrate concentration was 0.32%. Under these conditions, the predictive yield was 80.02%. To simplify the operation, the fermentation temperature was adjusted to be 32 °C, fermentation medium initial pH was adjusted to be

6.0, and the substrate concentration was adjusted to 0.32%. Then, three parallel experiments were simulated under the process conditions; the average yield of pyrogallol was 77.86% (RSD = 1.21%) and differed 2.70% compared with the predictive yield throughout the three parallel experiments. The model was definitively shown to be correct by these results.

## Oxidation Resistance and Bacteriostatic Testing

In this experiment, to achieve the exact hydrophilic antioxidants of products, three repeated experiments were carried out, and the final results are shown in Fig. 9. The capacity for scavenging free radicals with products containing 66.5% pyrogallol was much better than other reductants including ABTS and BHT, even better than *Vc* (Vitamin C), but products could not scavenge all free radicals like *Vc* because of the existence of impurities. It also exhibited strong antioxidant capacity of products compared with others.

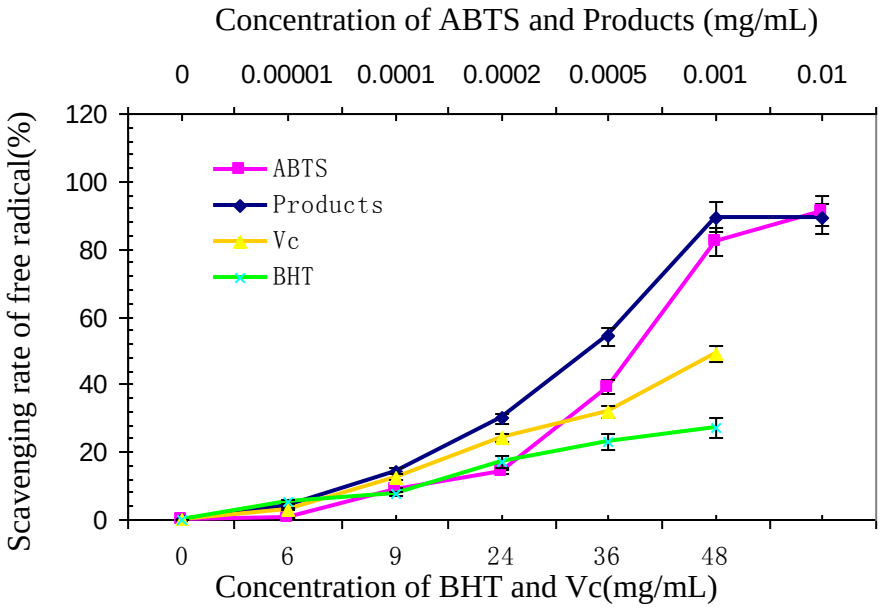


**Fig. 9.** Scavenging rate of free radicals. Note: the upper abscissa describes ABTS and products; the lower abscissa describes *Vc* and BHT.

Results from the study made it possible to explain the observed effects of single factors in a reasonable way. Moreover, since there are wide industrial applications of decarboxylase, the strain seems to be a prospective organism for further biotechnological exploitation without generating much pollution. Therefore, the research has important significance to direct further research on the large-scale production in the future.

## CONCLUSIONS

1. *Enterobacter aerogenes* was screened from two types of strains to degrade gallic acid to produce pyrogallol through substrate induction.

2. Single factors measured by the yield of pyrogallol and the degradation yield of gallic acid were researched, and the results were combined to design a response surface to produce pyrogallol with 77.86% average yield at the optimal fermentation conditions of inoculum size 5%, substrate concentration 0.32%, incubation period 60 h, fermentation temperature 32 °C, content of phosphate buffer 25%, and initial pH 6.0 in fermentation medium.
3. The antioxidant capacity of products containing 66.5% pyrogallol was explored, and the results showed the products were stronger than ABTS, BHT, and Vc.

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