Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(5):27-33

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Impact of de-aeration methods on the culture of *Clostridium butyricum*

Hua-li Jian[1], Guang-jun Song[2] and Mei-de Liao[3*]

[1]*College of Food Science, South China Agricultural University, Guangdong, PR China*
[2]*Guangzhou Yuan Da Biological Technology Development Co. Ltd., Guangdong, PR China*
[3]*College of Natural Resources & Environment, South China Agricultural University, Guangdong, PR China*

ABSTRACT

Clostridium butyricum is a kind of anaerobic bacteria, so de-aeration is extremely important during culture. In this paper, adding sodium thiosulfate and cysteine as well as mixed culture with Saccharomyces cerevisiae were adopted in de-aeration during Clostridium butyricum culture. The experimental results showed that the optimal dosage of sodium thiosulfate was 0.004g/mL, where the biomass of Clostridium butyricum reached 3.53×10^8 cells/mL; the optimal dosage of cysteine was 0.004g/mL, where the biomass of Clostridium butyricum reached 4.46×10^8 cells/mL; during mixed culture with yeast, when the inoculum biomass ratio between Clostridium butyricum and Saccharomyces cerevisiae was 0.521, the biomass of Clostridium butyricum reached the maximum volume 5.428×10^7 cells/mL with the yeast biomass of 1.87×10^6 cells/mL. When inoculum biomass ratio between the Clostridium butyricum and Saccharomyces cerevisiae was 1.217, the biomass of yeast reached the maximum volume 2.4×10^6 cells/mL with the Clostridium butyricum biomass of 5.175×10^7 cells/mL.

Keywords: *Clostridium butyricum*, reducing oxygen agent, *Saccharomyces cerevisiae*, mixed culture, biomass

---

INTRODUCTION

Clostridium butyricum is a typical microorganism which produced butyric acid can be separated from the soil and intestines of healthy animals. As the main metabolites of *Clostridium butyricum*, butyric acid is a kind of short chain fatty acids with many significant physiological functions [1-3]. Since 1944 when Japan officially put the *Clostridium butyricum* formulation into clinical application as the Bacillus preparation, the *Clostridium butyricum* has been cultured in large amount [4, 5]. However, *Clostridium butyricum* is a kind of strict anaerobic bacteria which is very sensitive to oxygen. So for a long time, the anaerobic bioreactor was relied on or adding reducing agent such as glutathione salt reduction method for culturing *Clostridium butyricum*. In the study, combining with the reality of *Clostridium butyricum* actual production in the livestock breeding industry [6, 7], the biological de-aeration culturing method was adopted as an innovation. The yeast of high nutritional value with rich protein which had a long history of application in the livestock husbandry was mixed with *Clostridium butyricum* for culture. The products of mixed culture would not only provide nutrition for breeding animals, but also for prevention and treatment of intestinal damage.

EXPERIMENTAL SECTION

Strains

Clostridium butyricum and *Saccharomyces cerevisiae* were used in the present study. These strains were supplied by Guangdong Provincial Institute of Microbial Culture Collection Center and maintained on slants of sulfur ethylation agar and YPG agar at 4℃ respectively.

Culture medium

Liquid sulfur ethylation culture media, tryptone 15g; L-Cystine 0.5g; Glucose 5g; Yeast extract 5g; sodium chloride

2.5g; Sodium thinobycliate 0.5g; Resazurin 0.001g; Agar 0.75g; methylene blue 0.05g; distilled water 1000mL; pH7.1.

YPG (Yeast Extract Peptone Dextrose): Peptone 2g; Yeast extract 1g; Glucose 2g; methylene blue 0.05g; distilled water 100mL; pH 7.0.

Determination of the growth curve of *Clostridium butyricum*

The prepared test tubes containing 9mL liquid sulfur ethylation culture media were randomly divided into experimental and control groups. 1%(v/v) of inocula were picked in the experimental group, meanwhile, the equal volume of sterile water was added in the control group. They were incubated at 37℃and the OD_{600} value of experimental group and control group were measured respectively in every 8 hours (fully shaked before measurement); OD_{600} value in the experimental group was subtracted by OD_{600} value in the control group and OD_{600} value of bacteria was obtained, then, the growth curve of *Clostridium butyricum* could be drawn [8].

To establish the relationship curve between OD_{600} value of *Clostridium butyricum* and *Saccharomyces cerevisiae*（yeast） and biomass of them in mixed culture

In order to facilitate the determination of the biomass of yeast and *Clostridium butyricum* in mixed culture, combination of Hemocytometer counting with OD_{600} value determination was adopted in this paper to establish a calculation method. *Clostridium butyricum* and yeast suspension of different concentrations were prepared. The hemocytometer was used to calculate the cell concentration and the spectrophotometer was adopted to measure the OD_{600} value. Then mix bacteria with different known concentrations, measure the OD_{600} value and establish a fitted curve.

De-aeration capacity measurement of inorganic reduction matter (methylene blue indicator method)

0.000g/mL, 0.002g/mL, 0.004g/mL, 0.006g/mL, 0.008g/mL, and 0.010g/mL inorganic compounds of sodium thiosulfate reduction were added to the test tube containing 9 mL liquid sulfur ethylation culture medium. After high-temperature sterilization, *Clostridium butyricum* was incubated with 1%(v/v) inocula at 37℃. The height of blue liquid was observed and recorded in every 12h.

The impact of inorganic and organic reduction matter on *Clostridium butyricum* culture

The certain amount of inorganic reducing compound sodium thiosulfate(or organic reducing compound cysteine) was added into sulfur-ethylation culture medium so the final concentration would respectively be 0.001g/mL，0.002g/mL，0.003g/mL，0.004g/mL，0.005g/mL，and 0.006g/m. After high-temperature sterilization, each concentration randomly was divided into control group and experimental group. 1%(v/v) of inocula were picked in the experimental group, meanwhile, the equal volume of sterile water was added in the control group. They were incubated at 37℃ for 48h. The OD_{600} value of experiment group and control group were measured respectively and the bacterial biomass was calculated.

The impact of biological de-aeration on *Clostridium butyricum* culture

The prepared test tubes containing 9mL YPG culture medium were randomly divided into experiment group and control group. 1%(v/v) of inocula were picked in the experimental group according to the volume ratio of *Clostridium butyricum* and yeast in the Table 1 respectively, meanwhile, the equal volume of sterile water was added in the control group. Then the OD_{600} value of inoculum liquid was determined and the biomass ratio of *Clostridium butyricum* and yeast was calculated. They were incubated at 30℃ for 48h. The OD_{600} value of experiment group and control group were measured respectively. At the same time, the hemocytometer was used to calculate the yeast number [9, 10].

Table 1 The inoculation ratio of *Clostridium butyricum* and *Saccharomyces cerevisiae* in mixed culture

Group		1	2	3	4	5	6	7	8
Inoculum volume (*Clostridium butyricum*: *Saccharomyces cerevisiae*)	Volume ratio	1∶9	2∶8	3∶7	4∶6	5∶5	6∶4	7∶3	8∶2
	Biomass ratio	0.134	0.302	0.521	0.804	1.217	1.810	2.820	4.830

RESULTS AND DISCUSSION

The growth curve of *Clostridium butyricum*

The growth curve of *Clostridium butyrate* on the sulfur-ethylation culture medium was shown in Fig.1, and data in the Fig.1 was the determination value of culture liquid after diluted for 10 times (same as below). From the *Clostridium butyrate* growth curve the log phase of *Clostridium butyrate* growth at about 2h-10h, which reached to the late logarithmic phase and the equilibration period after 16h. The biomass could reach to the maximum volume

at about 40h.

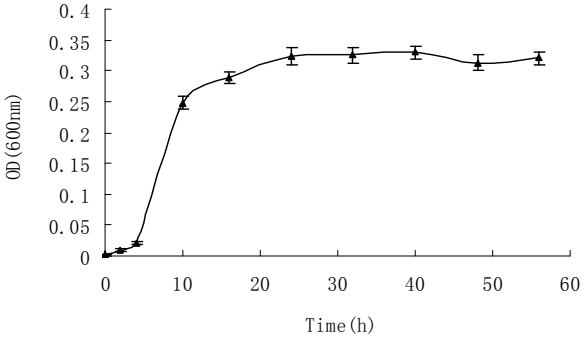

Fig.1 The growth curve of *Clostridium butyricum*

The relationship between OD_{600} value and biomass (cell concentration) of yeast

The analysis of corresponding relation between OD_{600} value of yeast suspension and biomass was shown in Fig.2, where regression equation (1) was obtained by regression analysis.

$$Y = 0.680X - 0.02 \quad (1)$$

$$R^2 = 0.9700$$

Where Y is the yeast biomass($\times 10^7$ cells/mL), X is the OD_{600} value.

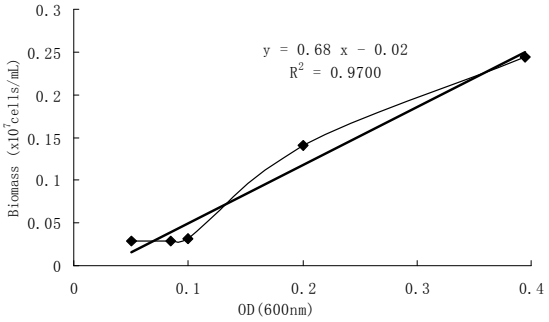

Fig.2 The relationship between OD_{600} value and biomass of yeast

The relationship between OD_{600} value and biomass (cell concentration) of *Clostridium butyricum*

The analysis of corresponding relation between OD_{600} value of *Clostridium butyricum* suspension and biomass was shown in Fig.3, where regression equation (2) was obtained by regression analysis.

$$Y = 14.43 X - 0.93 \quad (2)$$

$$R2 = 0.9800$$

Where Y is the *Clostridium butyricum* biomass, ($\times 10^7$cells/mL), X is the OD_{600} value of *Clostridium butyricum* suspension.

The relationship between biomass of *Clostridium butyricum*, yeast and mixed suspension OD_{600} value

The *Clostridium butyricum* and yeast with different concentration was mixed and the OD_{600} value of the mixed suspension was determined. The result was shown in Fig.4 and equation (3) was obtained from the figure.

$$\text{Mixed Suspension } OD_{600} = \frac{\text{yeast}OD_{600} + \textit{butyricum}OD_{600}}{2} \quad (3)$$

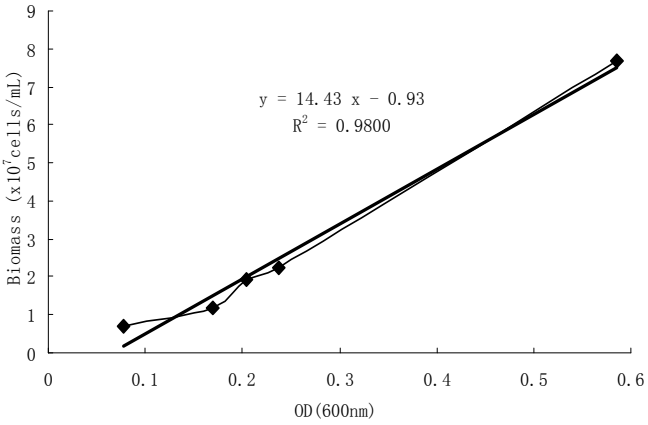

Fig.3 The relationship between OD_{600} value and biomass of *Clostridium butyricum*

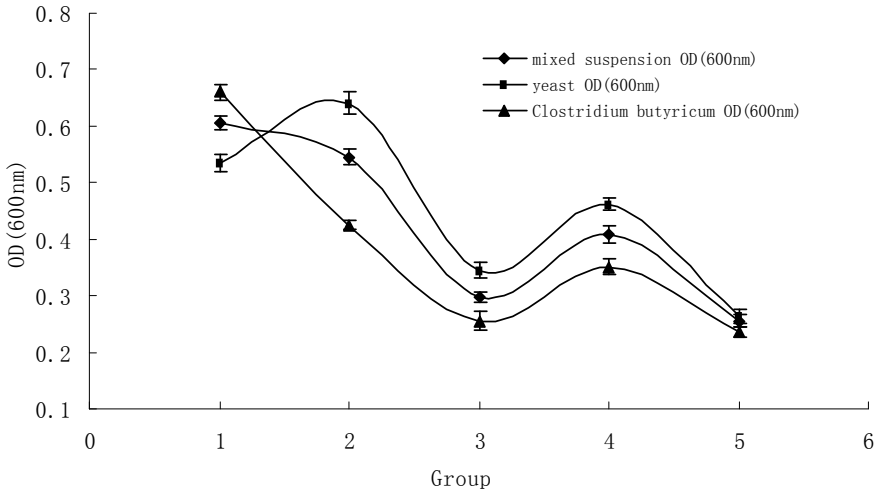

Fig.4 The relationship between biomass of *Clostridium butyricum*, yeast and mixed suspension OD_{600} value

Analysis of de-aeration effect (methylene blue indicator method)

The analysis result of methylene blue de-aeration effect was shown in Fig.5, which indicated that during initial culture, the additive volume of sodium thiosulfate was positively correlated with the de-aeration effect. The height of blue layer was smaller, which indicated that oxygen removal effect was better, the more *Clostridium butyrate* to grow. With the culture time extending and the oxygen entering continuously, the height of blue layer increased gradually to 50h. During the late culture, the height of blue layer was almost identical to 8cm when the additive volume of sodium thiosulfate was 0-6mg/mL, while the height of blue layer were 5cm and 6cm respectively when the additive volume of sodium thiosulfate were 8mg/mL and 10mg/mL. It showed that the additive volume of sodium thiosulfate(0-6mg/mL) would not influence the de-aeration effect during the late culture, and the high concentration of sodium thiosulfate(>6mg/mL) was effective for oxygen removal.

The impact of inorganic and organic reduction matter on *Clostridium butyricum* culture

The determination of impact on the *Clostridium butyricum* growth when using inorganic reducing sodium thiosulfate and organic reducing cysteine (reduced) as oxygen scavenger were shown in Fig.6 and Fig.7 respectively. The results showed that with the concentration of sodium thiosulfate and cysteine increasing, the de-aeration efficiency was improved as well. when the concentration of sodium thiosulfate and cysteine were 0.004g/mL, the OD_{600} of *Clostridium butyricum* reached to the maximum 3.53×10^8 cells/mL and 4.46×10^8 cells/mL respectively. When the additive concentration exceeded 4mg/L, it would show toxicity during the *Clostridium butyricum* growth; on the other hand, with the oxygen in the air continued to dissolve, the added oxygen scavenger continued to be oxidized and consumed, so the growth of *Clostridium butyricum* would be limited during late culture.

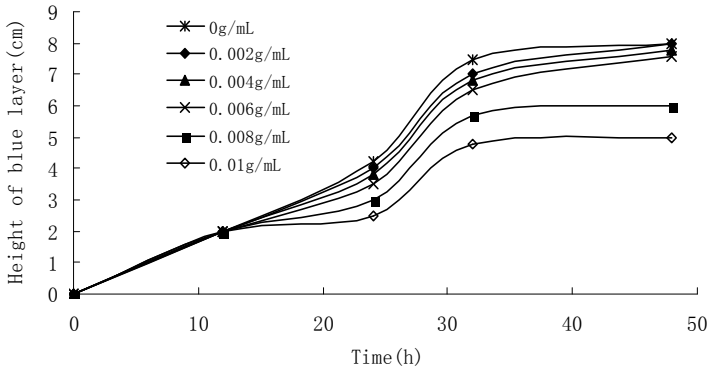

Fig.5 The oxygen removal effect of sodium thiosulfate with different concentration indicated by methylene blue indicator

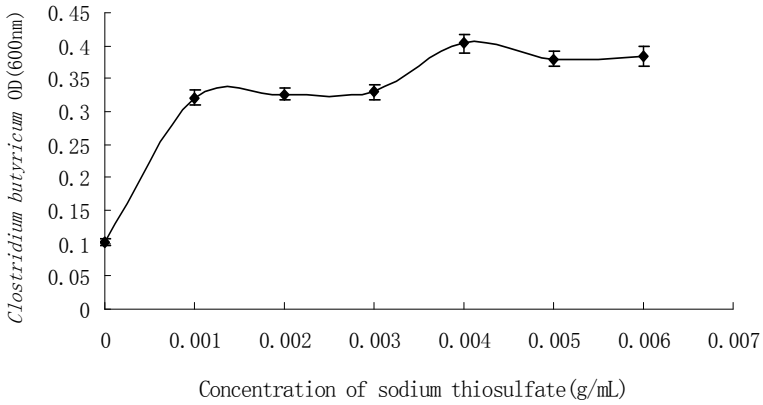

Fig.6 The impact of sodium thiosulfate as oxygen scavenger on *Clostridium butyricum* growth

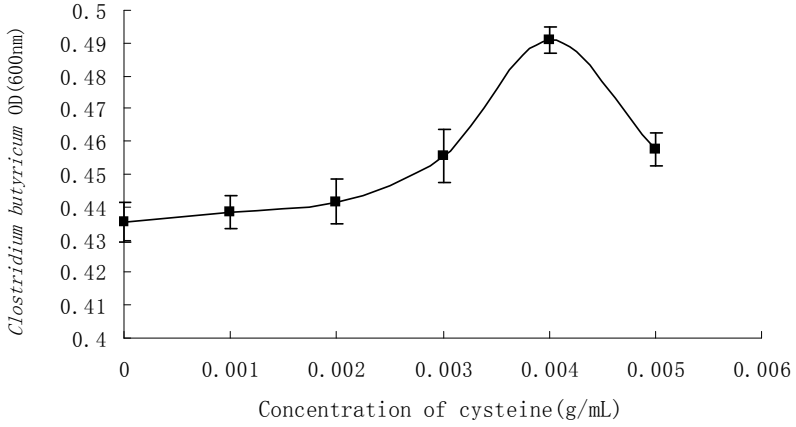

Fig.7 The impact of cysteine as oxygen scavenger on *Clostridium butyricum* growth

The impact of different inoculation ratio on growth of yeast and *Clostridium butyricum* in mixed culture

Combined with the actual situation of *Clostridium butyricum* and yeast in feed industry, facultative anaerobic growth and fast growth characteristics of yeast with high oxygen consumption rate were adopted in this paper to provide oxygen-free environment for the growth of *Clostridium butyricum* and achieve a total growth of both[11,12]. The mix-cultured yeast and *Clostridium butyricum* results (Fig.8, Fig.9) showed that during the all culture period, color blue did not appear in the culture liquid (or only blue appeared on the surface of culture liquid). It showed that a good anaerobic environment could be ensured by the mix-cultured system of yeast and *Clostridium butyricum*. At the same time, the initial inoculation ratio of the two bacteria has a significant impact on the growth of yeasts and *Clostridium butyricum*. When no yeast was picked in, *Clostridium butyricum* could not grow in the YPG medium. And when the inoculation ratio between *Clostridium butyricum* and *Saccharomyces cerevisiae* was 0.521, the

Clostridium butyricum biomass reached to the maximum value 5.428×10^7 cells/mL, and the yeast biomass now was 1.87×10^6 cells/mL. When the inoculation ratio was 1.217, the yeast biomass reached to the maximum volume 2×10^6 cells/mL, where the *Clostridium butyricum* biomass was 5.175×10^7 cells/mL. The results showed that the growth of *Clostridium butyricum* would be inhibited by the excessive inoculation of yeast, and the reason needed to be further studied.

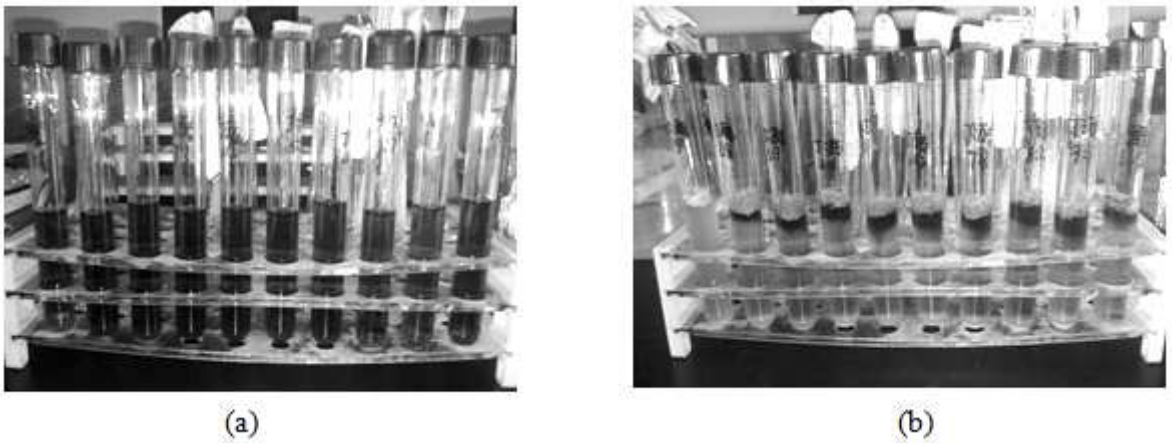

Fig.8 The oxygen removal effect of mixed culture indicated by methylene blue indicator
(a) YPG Medium before inoculation (blue)
(b) YPG Medium after mixed culture for 48h (no blue or only blue appeared on the surface of medium)

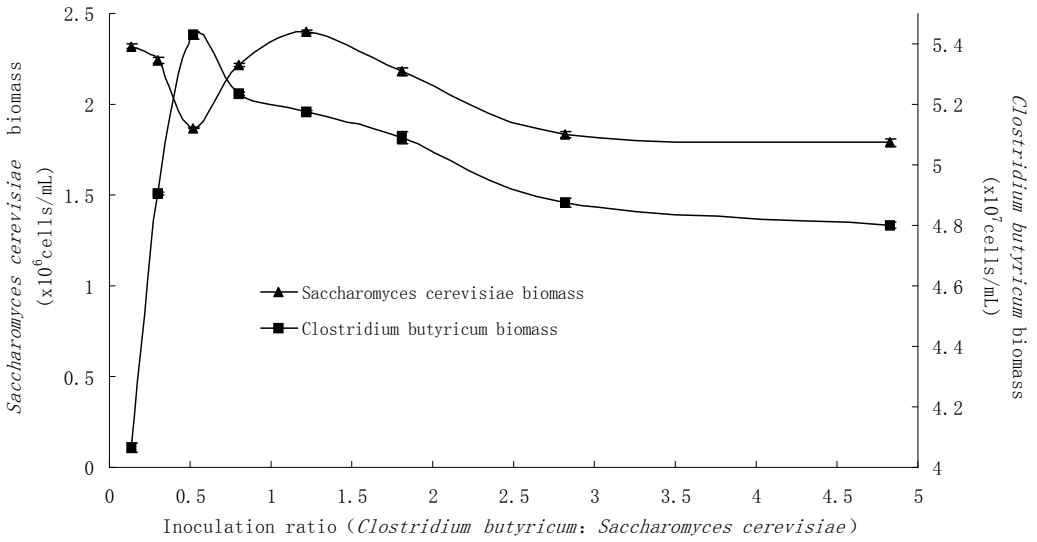

Fig.9 The impact of different inoculation ratio on growth of *Clostridium butyricum* and *Saccharomyces cerevisiae* in mixed culture

CONCLUSION

The results of using inorganic reducing sodium thiosulfate and organic reducing cysteine (reduced) as oxygen scavenger showed that when the additive volume of sodium thiosulfate and cysteine were 0.004g/mL, the OD_{600} of *Clostridium butyricum* reached to the maximum 3.53×10^8 cells/mL and 4.46×10^8 cells/mL respectively.

Using YPG as the culture medium, test results of different inoculation ratios demonstrated that certain conspiracy relations existed between *Clostridium butyricum* and yeast; when the inoculation ratio between *Clostridium butyricum* biomass and yeast biomass was 0.521, the *Clostridium butyricum* biomass reaches to the maximum value 5.428×10^7 cells/mL, where the yeast biomass was 1.87×10^6 cells/mL. When the inoculation ratio was 1.217, the yeast biomass reached to the maximum value 2.4×10^6 cells/mL, where the *Clostridium butyricum* biomass reached to 5.175×10^7 cells/mL. But with excessive yeast inoculation, the growth of *Clostridium butyricum* would be reduced.

The preliminary *Saccharomyces cerevisiae* and *Clostridium butyricum* co-culture experiment demonstrated that the

Clostridium butyricum and *Saccharomyces cerevisiae* could be mixed cultured together with good growing conditions. By optimizing culture conditions the maximum biomass of *Clostridium butyricum* and yeast could be further improved. Moreover, during the mixed culture process, medium discoloration caused by oxygen dissolved permeability did not occur, which was significantly better than directly adding inorganic or organic reducing oxygen scavenger in the culture medium. Therefore, it had provided a new idea for production of *Clostridium butyricum* and yeast so as to offer feed additives of good quality.

Acknowledgements
This research was financially supported by the Programs to Foster the University Young Elitists of Guangdong Province (No. 2012LYM_0029). We thank the Guangzhou BiaoQi Electronics Technology Co., Ltd. for providing the detecting instrument.

REFERENCES

[1] TDH Woo; K Oka; M Takahashi, et al. *J. Med. Microbiol.*, **2011**, 60(11), 1617-1625.
[2] QX Gao; LL Qi; TX Wu, et al. *Vet. Microbiol.*, **2012**,160(3-4), 395-402.
[3] GT Cao; YP Xiao; CM Yang, et al. *J. Anim. Vet. Adv.*, **2012**, 11(15), 2665-2671.
[4] Q Kong; L Chen; W Zhou. *Sci. Tech. Food Ind.*, **2009**, 12, 197-199.
[5] ML Shi; Y Dai; L Zhang. *J. Hangzhou Teach. Coll. (Nat. Sci. Ed.)*, **2001**, 8, 38-40
[6] W Wang; T Archbold; MS Kimber, et al. *J. Anim. Sci.*, **2012**, 90(4), 400 -402.
[7] H Yan; R Potu; H Lu, et al. *PLoS ONE*, **2013**, 8(4), e59581.
[8] SG Xie; Q Dai; SM Zhao. *Microbiol. Chin.*, **2007**, 34(6), 1047-1051.
[9] J Zhou; NQ Xiao. *J. Anhui Agric. Sci.*, **2011**, 39 (15), 8824-8825.
[10] YL He; ZH Guo; ZJ Tan, et al. *Mod. Agric. Sci. Tech.*, **2010**, 2, 29-31.
[11] SL Wang; HY Ling. *J. Wuhan Univ. (Nat. Sci. Ed.)*, **2009**, 55(5), 587-590.
[12] J Stadie; A Gulitz; MA Ehrmann, et al. *Food Microbiol.*, **2013**, 35(2), 92-98.