

# **An Effective Cloning, Heterologous Expression and Physiological Activity in *Lactococcus lactis* NZ9000 of Catalase Gene from *Escherichia coli* DH5 $\alpha$**

## **Abstract:**

In order to clone active Catalase gene from bacteria, we introduced a method of shotgun integrating specific screen from *Escherichia coli* DH 5 $\alpha$ . Genome DNA was extracted from *E.coli* DH5 and partially digested with Sau3AI. Then some fragments more than 2.26kb were collected and ligated with T4 DNA ligase into the BamHI-cleaved plasmid pUC18 and transformed competent *E. coli* TG1 cells. The transformants were incubated anaerobically on brain heart infusion (BHI) containing tannic acid. The method could detect catalase activity and screen the catalase-positive clones. The catalase protein can be identified by SDS-PAGE. The results showed that the recombinant plasmid pUC18-kat was constructed successfully by PCR identification and restriction enzyme digestion. Opening the reading frame from DNA Sequence DH5a katE. The sequence long is 2262nt and coding protein are 753 Amino Acids, Molecular Weight 84198.72 Daltons. This method is simple, and possess of popularization and application value. It would lay a foundation for cloning an active catalase later. Further, in this study, the fragment of 2,262 bp catalase gene katE was cloned into the expression vector pQE30 and transformed into *Escherichia coli* M15, and KatE protein was expressed after the induction with Isopropylthio- $\beta$ -D-galactoside. The KatE protein was separated on SDS-PAGE and recovered using a His-tag affinity. New Zealand white rabbits were immunized with the purified protein to harvest polyclonal antibodies. Since *L. lactis* has no catalase, katE was inserted into *Escherichia coli* - *L. lactis* shuttle vector pMG36e and electrotransformed into *L. lactis* NZ9000. The expression of the KatE protein was confirmed by SDS-PAGE analysis and Western blot. Further experiment demonstrated that the expression of the KatE gene in *L. lactis* NZ9000 is able to produce active catalase that can provide efficient antioxidant activity. Additionally, to understand the import of catalase katE gene of *Lactococcus lactis* on the body's physiological changes of immune function in mice, Enzyme-linked immunoassay (ELISA) was used on the blood of mice, to measure IgG, IgE, CD4 and CD8 levels, and find out whether there is a difference between Mice Groups eating the recombinant *L.lactis* NZ9000 and other groups i.e. (recombinant *E.coli* DH5 $\alpha$  and *L.lactis* NZ9000; *E.coli* DH5 $\alpha$  and saline) The experiments showed that recombinant *L.lactis* NZ9000 was significantly higher than the other on IgG concentrations; IgE, CD4 or CD8 levels are not significant . Mice with increased IgG levels of IgG after the intake of recombinant *L. lactis* NZ9000 helps to explain the recombinant *L. lactis* NZ9000 regulatory role in humoral immunity of mice; IgE level did not change, CD4 and CD8 levels were also not changed, thus suggesting that there is no significant effect of cellular immunity on the body in a short time.

**Key words:** method of shotgun, scening, catalase-positive, transformants , NZ9000, .

## **Introduction:**

*Lactococcus lactis* is commonly used in fermented industry, especially in fermented dairy products. In addition, *Lactococcus lactis* is one type of host bacteria which could induce expression of common, important research and application value. And *Lactococcus lactis* is relatively simple on rapid growth and metabolism, with separated decomposition synthesis metabolism. As the small genome beneficial genetic engineering such as separation and purification of extracellular proteins, these advantages make *Lactococcus lactis* has major research value in the regulation of metabolism (Rochat et al., 2012; Van de Guchte et al., 1989). At present, *Lactococcus lactis* in milk products have been widely used as a leavening agent for sour cream, yogurt, yogurt, soy milk beverage and dairy production.

Catalases are antioxidant metalloenzymes that divide H<sub>2</sub>O<sub>2</sub> into water (H<sub>2</sub>O) and O<sub>2</sub> (Abriouel et al., 2004). Therefore, in microbiology, the catalase test is also used to differentiate between bacterial species in the lab (Percy, 1984). Catalase is a very highly conserved enzyme that is ubiquitous in bacteria, fungi, plants and animals. Bacterial catalases are widespread in aerobes such as *Escherichia coli* (*E.coli*). And Catalases are classified into three major families including Mn-catalases, catalase-peroxidases and monofunctional catalases (Arockiaraj et al., 2012).

Catalase as the other enzymes, was made of amino acids, four polypeptide chains, each containing 500 amino acids, the optimum pH is 7. almost all the aerobic microorganisms contain the enzyme, do some anaerobic microorganisms(Boon EM, et al. 2007). in addition, catalase can oxidize some cytotoxic substances, such as formaldehyde, formic acid, phenol and ethanol. These oxidation processes need to be accomplished by hydrogen peroxide through the following reactions.  $H_2O_2 + H_2R \rightarrow 2H_2O$ . It is changed with the change of pH value and temperature, the activity of hydrogen peroxide decomposing enzyme is also changed, and its activity was maximal at about pH7. The enzyme is widely used in food and milk products industry. (Yuzugullu Y et al. 2001, Boucher, I. et al. 2002, Martirosyan A.O., et al. 2004, Jacek S, and Loewen P C. 2002)..

Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long (Boon EM, et al. 2007). The optimum pH for human catalase is approximately 7, Almost all aerobic microorganisms use catalase. It is also present in some anaerobic microorganisms, They are widely used in food either alone in milk pasteurization and cheese production or its combination with glucose oxidase in the production of dietetic beverages (Yuzugullu Y et al. 2001, Boucher, I. et al. 2002, Martirosyan A.O., et al. 2004, Jacek S, and Loewen P C. 2002). [M1]

It was shown in a study that gallate decarboxylase activity was elevated in the presence of tannic acid in *S. gallolyticus* (Chung K T et al. 1998). This shows that some enzyme activity could make the organism have a selective advantage over *S. bovis* when grown in the presence of tannins (Engesser, D. M et al 1994).

The safety of biotechnology products has always been the focus of attention, in which host bacteria is a crucial factor. Because *Escherichia coli* produces endotoxin, if the traditional *Escherichia coli* cloning and expression of exogenous gene, its expression products need to undergo complex separation and purification to reach the food and drug standards. The establishment of food grade gene expression system provides the

conditions for the extensive application of foreign gene expression products beneficial to human health in the fields of functional foods, health care products and cosmetics. There are a lot of extrachromosomal factor in *Lactococcus lactis* (such as plasmid and phage) could provide an excellent material for the development of molecular biology and gene delivery system(Rochat *et al.*, 2012; van de Guchte *et al.*, 2002).

At present, many methods are used to clone genes. Such as those obtained from genome bank, PCR, shotgun, RT-PCR and so on (Sambrook, J.*et al.* 1989). To clone the active catalase from bacteria, an effective approach was assayed (Chung K T *et al.*1998). In this study, we cloned the active catalase of *E.coli* DH5a. We used methods including cloning, vector constructed, sequencing, and expression. We also screened the selected activity of catalase by the presence of tannic acid in recombinant strains in vitro. Additionally, to understand the import of catalase katE gene of *Lactococcus lactis* on the body's physiological changes of immune function in mice, the levels of IgG, IgE antibody and CD4 and CD8 cells in the blood of experimental animals were examined by enzyme-linked immunoassay (ELISA) for laying the foundation of further study of recombinant *L lactis*.

## Materials and methods

### A. DNA manipulations and plasmid construction

Table 1.The bacterial strains and plasmids used in this study

Bacterial strain or plasmid and kit	Description	Source or reference
1.Bacterial strains	<i>E. coli</i> Top10,TG1,DH5a Plasmidless <i>Lactococcus lactis</i> NZ9000	Invitrogen and this lab.
2.pMD18-T 3.Qiagen Midiprep plasmid kit 4.Examing kit	Cloning vector, Ap <sup>r</sup> , 2.692 kb, pBluescript SK+/ Midiprep plasmid purification kit Examing for T and B cells kit	New England Biolabs Qiagen Shanghai and Jianglai Biotechnology limited company)
5.pET-28a(+)	Expression vector, Ap <sup>r</sup> , 5.369 kb	Gibco BRL
6. pTkatE	MD18-T containing 2.5-kb <i>Bam</i> HI insert of <i>catalaseE</i> DH5a genomic fragment	This work

7. pVE3655 and pMG36e	PET28a(+) containing 2.262-bp <i>Xba</i> I- <i>Sph</i> I PCR product insert of catalase gene, plasmids pVE3655 and pSEC(donated by Dr.Gruss)	This work
-----------------------	--	-----------

## Cloning and transformation.

In order to clone the catalase gene, total genomic DNA was isolated and purified with DNA purifying kit (Qiagen). Then it was partially digested with *Sau*3AI, and the DNA fragments of 1.5 to 2.5, 2.5 to 3.5, 3.5 to 5, and 5 to 7 kb were purified from the agarose gel using the QIAEX II kit (Qiagen). DNA fragments ligated with T4 DNA ligase (Qiagen) into the *Bam*HI-cleaved plasmid pBluescript SK+/- were used to transform electrocompetent *E. coli* Top10 cells (Sambrook, J *et al.* 1989). The gene library was constructed to about 1,500 clones. The transformants were screened for catalase activity on brain heart infusion (BHI) containing tannic acid (BHI-tannic acid medium) and ampicillin (150 µg/ml), which is the same medium used for screening for tannase activity (Chung K T, *et al.*1998). This medium was used as a plate-screening assay which could detect catalase activity when cloning into catalase-positive bacteria such as *E. coli*. First, the plates were incubated anaerobically for 2 days to allow the colonies to grow and to prevent darkening of the medium; the plates were then kept aerobically for a further 24 h at 37°C to induce the catalase gene (Hikmate A *et al.*2002). A zone of clearing surrounding the colonies which developed during aerobic incubation indicated tannic acid degradation as a result of catalase activity. Plasmid DNA (pHA01) of one clone was isolated using the Qiagen Midiprep plasmid purification kit (Qiagen3 in table 1), [M2] and the insert was sequenced bidirectionally at GATC Biotech (Konstanz, Germany) using M13 universal oligonucleotide primers and custom primers. Plasmid DNA (pMD18-T-kat) exhibiting presumptive catalase activity on BHI-tannic acid medium was shown to harbor a 2.26kb DNA fragment insert. The expression vector (pET28a(+)) was constructed as followed.

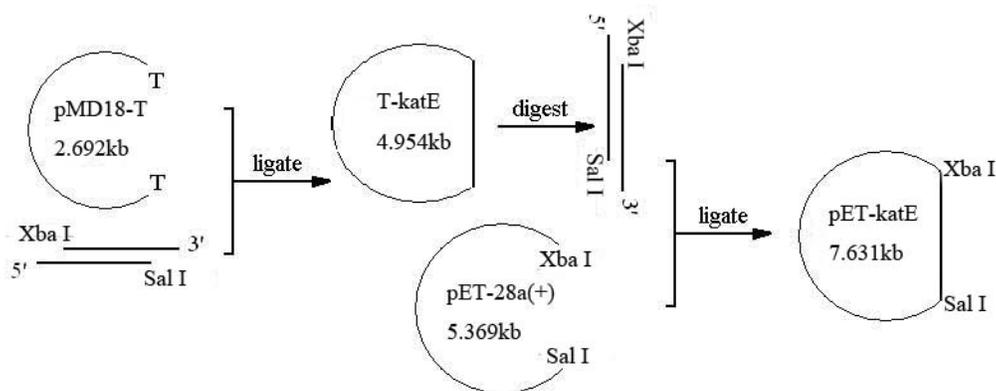


Fig. 1:Construction of recombinant plasmid pET-katE

*E. coli* was transformed by the method of electroporation (Powell IB *et al.*1988) which was pre-digested with Sall and XbaI restriction enzymes. After ligation, the reaction mixture was employed to transform DH5 $\alpha$  competent cells, and the recombinant plasmid having a size larger than pET28a was then screened out. It was confirmed that the cloned DNA fragment of 2.2 kb was the *katE* gene and the construction of expression plasmid pET 20b/*katE* had thus been accomplished.

#### **Amplification by PCR and oligonucleotide synthesis:**

PCRs were performed with a Perkin-Elmer Cetus (Norwalk, Conn.) apparatus using Taq DNA polymerase (Promega) as recommended by the manufacturer. Oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems, San Jose, Calif.). A 2262-bp fragment was PCR amplified from the pUCkat matrix.

The oligonucleotides used were XbaI-K-UP :  
5'-TCTAGAATGTCGCAAKATAACGAAAAGAACCCAKATCAGC-3' (oligo 1) for  
the coding strand and Sall-K-Down :  
5'-GTCGACTTACGCCGGGATTTTGTCAATCTTAGGAATGCGTG-3' (oligo 2) for  
the complementary strand.

The reaction conditions 94 degree Celsius for 3 min, then 30 cycles of 94 degree Celsius for 0.5 min, 50 degree Celsius for 50 sec, 72 degree Celsius for 1 min, followed by a final extension of 5 min at 72 degree Celsius. The amplified fragment was purified using the QIA quick PCR purification Kit (QIAGEN Inc), and digested with EcoR I and Not I. The resulting fragment was respectively ligated into the contained T7 promotor plasmid pET28 digested with EcoR I and Not I. This DNA fragment was then cloned on pET28 vector in *E. coli* TG1, resulting in pET28 *kat*. Then *katE* was also inserted into *Escherichia coli* - *L. lactis* shuttle vector pMG36e and electrotransformed into *L. lactis* NZ9000.

#### **Protein detection of catalase:**

In order to assay the production as well as the expression of catalase induced by IPTG in *E. coli* BL21 (DE3) cells, the cell extracts by ultrasonic crashing and supernatants of the different *E. coli* recombinant clones were examined by SDS-PAGE (Hikmate Abriouel,*et al.*, 2004, Bernhard T *et al.*, 2004). Preparation of cellular and supernatant protein fractions of *L. lactis* was confirmed by SDS-PAGE analysis and Western blot. For fractionation between cell and supernatant fractions, 2 ml samples of nisin-induced *L. lactis* cultures were centrifuged for 5 min at 6000 g at 4 degree Celsius. Protein extracts were then prepared as previously described (Le Loir *et al.*, 1998). Further experiment demonstrated that the expression of the *KatE* gene in *L. lactis* NZ9000 is able to produce active catalase that can provide efficient antioxidant

activity.

### **Recombinant protein expression**

The recombinant catalase plasmid was put into *Lactococcus lactis* NZ9000 competent cells by electrophore transformation, 30 degrees Celsius after cultured for 24 hours, then a single colony sister was picked out in finally fresh GM17 medium for incubation. When A600 is up to 0.5, the inducer nicin was added to a final concentration of 40ug/L, with no induction strains as control, after incubation 6h, the bacteria were detected by SDS-PAGE protein.

### **Bacterial cultivation and the sample prepared**

Microbial strains of recombinant *Lactococcus lactis* and normal NZ 9000 were obtained from the lab of Dr. Li (Central of biopharmaceutical engineering in Zhaoqing university, China). They were cultured in 100 mL of GM17 broth [M3] overnight at 30 degree Celsius. In order to induce recombinant *Lactococcus lactis* producing exoprotein a compound nisin was added [M4] to stimulate 5 h. The bacteria count was done by plate count method under a microscope. The bacterial cells were centrifuged for 10 min at 3,000×g and washed twice with the sterilized PBS (pH 7.4). Finally, bacteria were resuspended in GM17 medium (pH 7.4) under gentle vortex mixing. This preparation was made for oral consumption of the experimental mice [M5].

### **Sequence alignment and analysis of the kat gene.**

The cloned catalase gene was sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Sequence alignment and analysis of the *kat* gene were performed by DNA star software. A number of *kat* homologs have been aligned and analyzed with DNA star package. Sequence similarities were analyzed with the BLAST and FASTA programs.

### **The mice fed with the preparations**

Female BALB/c and C57BL/50 mice (7–8 weeks old) were gifted by Center of experimental animals in Sunyat University (China) and were divided into 5 groups with 10 mice in each group. Non-induced cultures and *L. lactis* NZ strains harbouring the control plasmid pVE3655 (Table 1) were used as negative controls. Groups consisting of at least 10 mice each were inoculated intragastrically with 0.5 ml of sampling fluid of recombinant *L.lactis* and recombinant *E.coli* (test groups) and of normal *L.lactis* and *E.coli* (control groups). The animals were kept in separated cages and had free access to an autoclaved pelleted diet and sterile water during the experimental period. Four weeks after inoculation, the animals were killed under ether anesthesia and blood of the experimental mice were obtained for microbiological and immunological studies. The spleen was gently disrupted through a nylon mesh and the cells analyzed for in vitro cellular proliferative response and T and B lymphocyte subset profile in response to *Helicobacter pylori* (*H. pylori*) antigens.

### **Immune function analysis of experimental mice.**

Spleen cells ( $1.0 \times 10^6$  cells in 900  $\mu$ l of RPMI 1640 medium containing 10% heat inactivated fetal calf serum, 200 mM L-glutamine and 50  $\mu$ g/ml gentamycin) from the experimental mice were cultured for 48 hr at 37 degree Celsius in 24 well microplates (Nunc). The cells were stimulated with 100ul of sonicated suspension of each *H. pylori* antigen preparation at a final concentration of 4ng/ml, stained with phycoerythrin-conjugated antibodies anti-IgG anti-CD4, anti-CD8 (Shanghai Jianglai Biotechnology limited company, China). The percentages of CD4 (helper), CD4/ CD8\_ (suppressor) T and B cells were determined by the examining kit[M6] (4.Shanghai Jianglai Biotechnology limited company in table 1). A total of 104 viable cells were analyzed after cell debris had been gated out. The results were expressed as percentage of blast cells (CD4, CD8\_ T and B cells) or lymphocytes (CD4, CD25\_ T cells). ELISA for katE-specific IgG and IgE antibodies was performed by porcine anti-rotavirus A IgG and IgE antibodies (Kit and Catalogue no.) as described in the manufacturer's instructions. but the secondary antibody was exchanged by peroxidase-labelled goat anti-porcine IgE (Thermo Fisher Scientific, Bonn, Germany) at a dilution of 1/10 000. The absorbance of each sample was measured at 450 nm.

### Salt stress

Salt stress experiments were performed in TYG broth. Early-exponential-phase cultures were harvested and resuspended in TYG broth containing 20% NaCl and incubated at 30 degree Celsius for 8h, plating serial dilutions at 2h intervals.

### Statistical Analysis:

The results were analyzed with statistical software SPSS 11.5 as median percentage values and analyzed by the variance analysis. The level of significance was set at  $P < 0.05$ .

## Results

### Genome DNA extraction

*E.coli* W3110 genome DNA was extracted and identified by 1percent agarose (see Fig

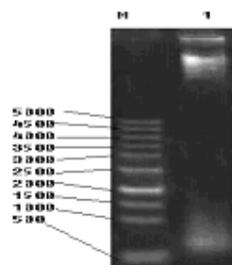


Fig 2 .DNA electrophoresis from *E. coli*  
M:Marker, 1 Genome DNA

2)

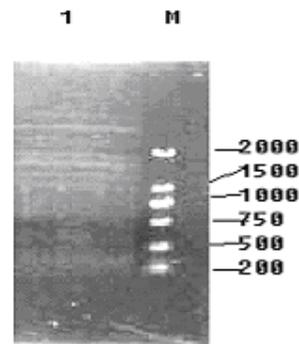


Fig 3 .Electrophoresis from *E. coli* total genomic DNA partially digested with Sau3AI  
M:Marker;1:genomic DNA partially digested with Sau3AI

### Positive clone selection

The positive clones more than 1500bp molecular weight with catalase activity on brain heart infusion (BHI) containing tannic acid and ampicillin for screening for tannase activity after transformation were selected ( see Fig 4,5).



Fig 4 a control plate without catalase activity



Fig 5 a plate-screening assay with catalase activity

### Kat gene identification

The *kat* gene was identified correctly by PCR and restrictive enzymes *Xba*I / *Sal* I (see Fig 6,7). The recombinant *L.lactis* containing *kat* gene was identified by PCR and restriction enzyme analysis. The recombinant plasmid was transformed into *E.coli* by electrophoration. The results from the Figure 7 and 8 showed that the *kat* gene isolated in the *E. coli* was correct.

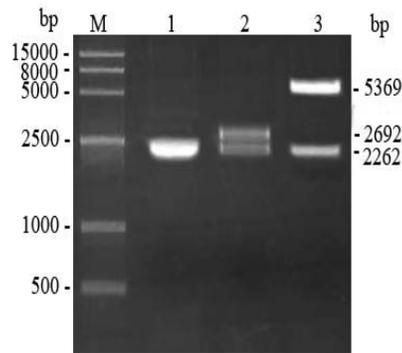


Fig.6 Identifikation of PCR product of *katE*, recombinant plasmid T-*katE* and pET-*katE*  
M: DNA marker; 1: PCR product; 2: T-*katE* digested with *Xba*I / *Sal* I; 3: pET- *katE* digested with *Xba*I / *Sal* I.

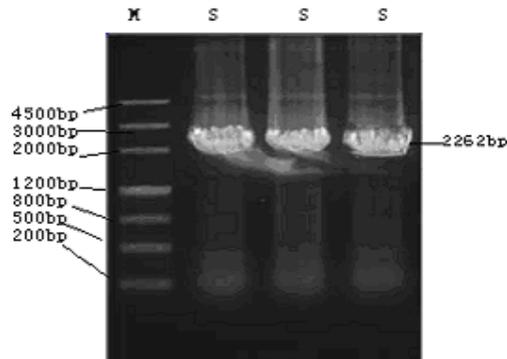


Fig 7. *kat* gene identifikationby PCR  
M: Marker, S: product by PCR

### Expression of *kat* gene in *E.coli*

The recombinant *E.coli* were incubated and induced by IPTG in LB medium for 6 hours. The KAT protein was identified by SDS-PAGE and Western-blot with the antibody prepared from the rabbits immuned by recombinant KAT protein (prepared in our laboratory). The results confirmed that KAT protein had been expressed obviously in *E.coli*. The molecular weight is 84.2kD. The plasmid containing *kat* gene was called pET-*katE*. And it was proved by Western-blot. The quantity of expression

was about 10 mg/ml (Figure8,9).

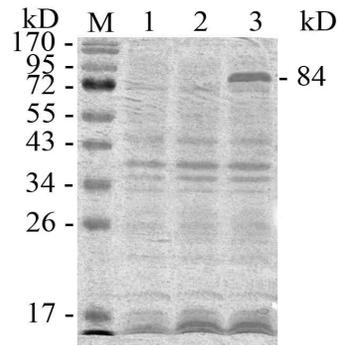


Fig. 8 SDS- PAGE analysis of KATE expressed in *E. coli* BL21 (DE3)  
M: protein marker; 1: BL21(DE3) with IPTG induction; 2: pET-katE clones transformed *E.coli* BL21(DE3) without IPTG induction; 3: pET-katE clones transformed *E.coli* BL21(DE3) with IPTG induction

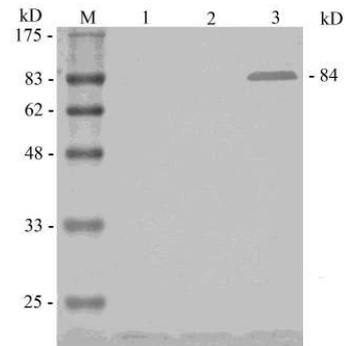


Fig. 9 Western blot analysis of the Kat protein expressed in *E.coli* BL21 (DE3) using Ni-NTA conjugate antibody  
M: prestained protein marker; 1: BL21(DE3) with IPTG induction; 2: pET-katE clones transformed *E.coli* BL21(DE3) without IPTG induction ; 3: pET-katE clones transformed *E.coli* BL21(DE3) with IPTG induction

The catalase activity of *L.lactis* was also dependent on the concentration of salt. Salt stress experiments showed that after 8 h incubation, 5.1% of NZ9000 were viable. However, only the recombinant *L.lactis* in TYG broth containing 20% NaCl and incubated at 30 degree Celsius for 8h exhibited greater salt sensitivity as compared to the parent culture NZ9000 strains surviving.[M7] From this result it would appear that this recombinant strain may be involved in responding to salt or osmotic stress. The recombinant *L.lactis* revealed growth difference in different physiological environment. It survived and revived surpassing normal *L.lactis* NZ9000 at low temperature (4-0 degree Celsius) . The result suggests that recombinant *L.lactis* has ability against environment salt stress.

### Sequence analysis

The open reading frame of pET-katE containing kat gene is 2262nt long, and translating protein is 753 amino acids, and its' isoelectric point is 5.67. The nucleotides and amino acids are as followed (see Fig 10 in appendix). The protein sequence was translated as shown in fig 11(See appendix).

It is known that the kat gene is katE because the sequence is homology to katE genes from *Escherichia coli* O157:H7 str and *Escherichia coli* str. K-12 substr, the homology is 98, 99.8 percent, respectively. Analysis of the amino acid sequence of katE of *E.coli* (cloned) revealed that it contains all the typical features of the KAT

proteins (Table 2).

Phylogenetic analysis. In order to root a tree of *katE* from 8 bacteria sequenced to date, a DNA polymerase phylogeny was inferred using the *kat* genes from those bacteria representing different Phyla which had a high score and the lowest e-values in tblastx searches against bacteria *kat* gene ( Fig.12).

Pair Distances of Untitled ClustalW (Weighted), Percent Similarity in upper triangle in table 2

Percent Identity										
1	2	3	4	5	6	7	8	9		
■	98.0	99.8	24.9	26.2	26.5	25.5	25.0	25.1	1	E.coli <i>katE</i> (cloned).seq
	■	97.9	24.8	26.0	26.1	25.7	25.1	25.6	2	E.coli <i>katE</i> Seq #2.seq
		■	24.9	26.2	26.5	25.5	25.0	25.1	3	E.coliGK12 <i>katE</i> Seq #4.seq
			■	24.4	26.5	27.1	24.4	26.7	4	hay bacillus <i>cat</i> (ORF) Seq.seq
				■	26.4	25.9	58.3	27.5	5	Pseudomonas <i>katE</i> Seq #8.seq
					■	29.0	26.1	27.3	6	Salmonella <i>katE</i> Seq #6.seq
						■	24.6	27.0	7	Staphylococcus <i>carnosus</i> <i>cat</i> (ORF) Seq.seq
							■	26.5	8	yersinia <i>kat E</i> Seq #7.seq
								■	9	bacillus <i>subtilis</i> <i>kat E</i> Seq #5.seq

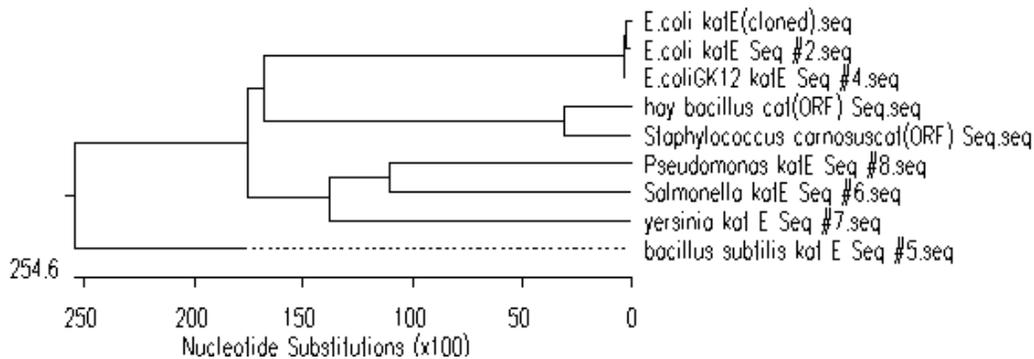


Fig 12. Phylogenetic tree analysis from *kat* genes of bacteria strains

### Detection of immunization in the blood of experimental animals

The results were expressed as percentage of blast cells (CD4, CD8, T and B cells). ELISA for *katE*-specific IgG and IgE antibodies was performed by porcine anti-rotavirus A IgG and IgE antibodies as described in the manufacturer's instructions. But the secondary antibody was exchanged by peroxidise-labelled goat anti-porcine IgE (Thermo Fisher Scientific, Bonn, Germany) at a dilution of 1/10 000. The absorbance of each sample was measured at 450 nm.

The concentration of *L.lactis* NZ9000 is  $8.4 \times 10^8$  cfu/ml, and that of *E.coli* DH5 $\alpha$  is  $7.2 \times 10^6$  cfu/ml.

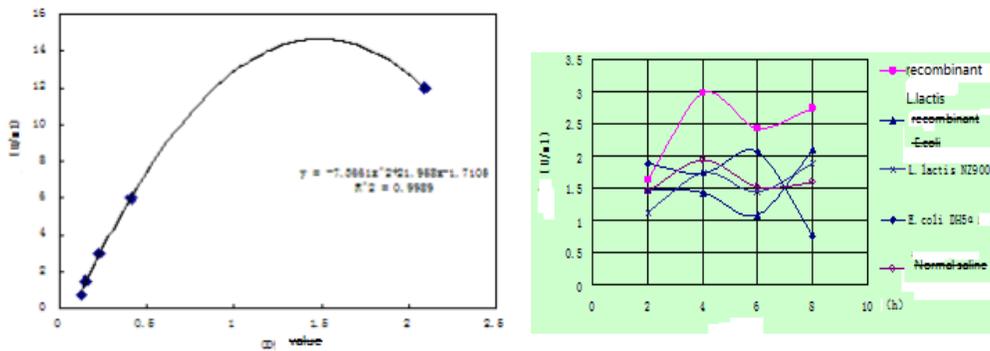


Fig 13. Standard curve of IgG Fig 14. Comparison of IgG among the different treatments

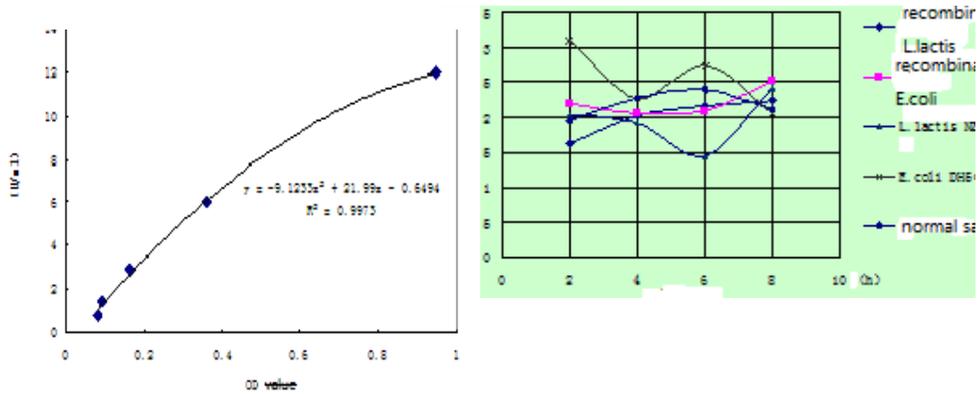


Fig 16. Comparison of IgE among the different treatments

### Comparison of statistical analysis

The level of IgG from the group with recombinant *L.lactis* NZ9000 treatment was higher than that of other treatments (see Tab 3.) There is a significant difference between them. Variation of IgG of the sera from mice with recombinant *L.lactis* NZ9000 was higher than that of other treatments, and the difference is significant ( $p < 0.05$ ; see Fig 13,14 and Tab3). However, the level of IgE was not difference between them ( $p > 0.05$ ; see Fig15, 16 and Table 4).

Table 3. Comparison of IgG among different treatment groups (u/ml)

groups	N				
		2h	4h	6h	8h
Recombinant <i>L. lactis</i> NZ9000	10	1.628±0.012	2.967±0.008	2.424±0.009	2.745±0.010
Recombinant <i>E. coli</i> DH5 α	10	1.483±0.021	1.431±0.011	1.079±0.013	2.010±0.009
<i>L. lactis</i> NZ9000	10	1.119±0.011	1.745±0.013	1.443±0.009	1.893±0.008
<i>E. coli</i> DH5 α	10	1.881±0.010	1.732±0.010	2.075±0.008	0.758±0.009
N.S	10	1.443±0.009	1.939±0.011	1.516±0.009	1.589±0.012

Table 4. Comparison of IgE among different treatment groups (u/ml)

Groups	n				
		2h	4h	6h	8h
Recombinant <i>L. lactis</i> NZ9000	10	1.619±0.006	2.30±0.013	2.178±0.0010	2.245±0.011
Recombinant <i>E. coli</i> DH5α	10	2.211±0.010	2.061±0.015	2.100±0.015	2.521±0.010
<i>L. lactis</i> NZ9000	10	2.049±0.011	1.917±0.011	1.444±0.009	2.425±0.011
<i>E. coli</i> DH5α	10	3.101±0.009	2.283±0.011	2.749±0.011	2.069±0.012
N.S	10	1.957±0.012	2.270±0.009	2.405±0.013	2.120±0.012

The results showed that the concentration of CD4 and CD8 from experimental animals with recombinant *L. lactis* NZ9000 was not higher than that of the other groups. There was no significant difference between them ( $P > 0.05$ , see Tab 5,6 and Fig 18,20).

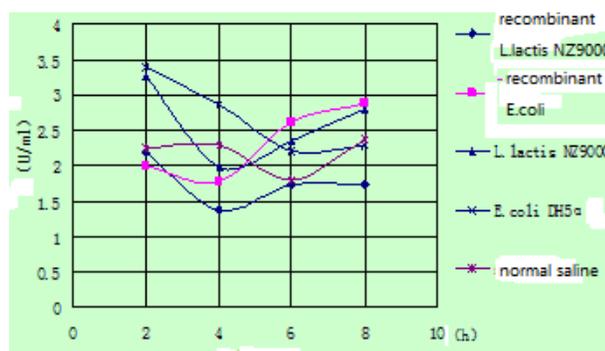
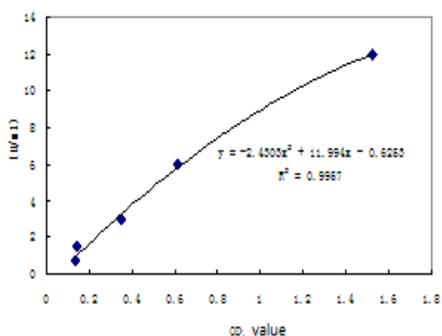


Fig.17 Standard curve of CD4

Fig.18 Variation of CD4 concentration

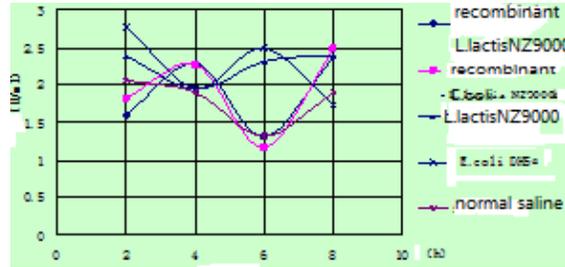
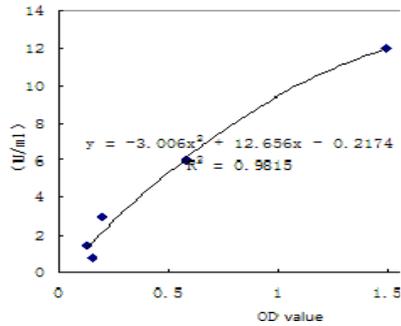


Fig.19 Standard curve of CD8

Fig.20 Variation of CD8 concentration

The ratio of CD4 and CD8 cells from the animals were constant relatively through comparison. And there is no different between them (see Tab 7). The trend of variation is shown in figure 21.

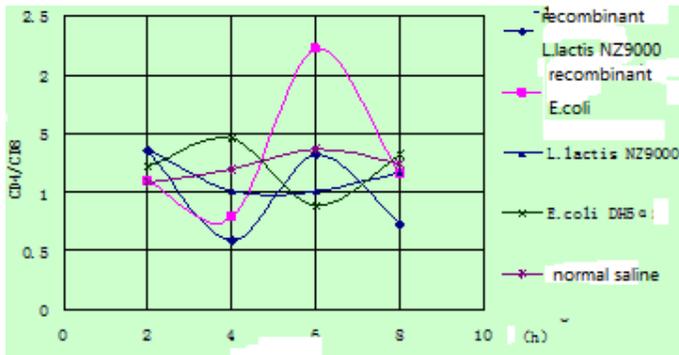


Fig.21 Variation of CD4/CD8

Tab 5. comparison of CD4 among groups(u/ml)

Groups	n	2h	4h	6h	8h
Recombinant <i>L.lactis</i> NZ9000	10	2.175±0.012	1.358±0.012	1.739±0.011	1.739±0.012
Recombinant <i>E.coli</i>	10	2.008±0.009	1.783±0.011	2.609±0.011	2.883±0.014
<i>L.lactis</i> NZ9000	10	3.2256±0.014	1.975±0.013	2.350±0.008	2.803±0.015
<i>E.coli</i> H5α	10	3.378±0.012	2.849±0.011	2.211±0.013	2.289±0.011

NS	10	2.240±0.013	2.289±0.013	1.805±0.012	2.375±0.013
----	----	-------------	-------------	-------------	-------------

Tab 6. comparison of CD8 among groups(u/ml)

Groups		2h	4h	6h	8h
Recombinant <i>L.lactis</i> NZ9000	10	1.601±0.013	2.281±0.010	1.317±0.007	2.377±0.019
Recombinant <i>E.coli</i>	10	1.823±0.011	2.272±0.013	1.174±0.015	2.481±0.018
<i>L.lactis</i> NZ9000	10	2.385±0.016	1.945±0.011	2.322±0.020	2.397±0.016
<i>E.coli</i> H5α	10	2.773±0.012	1.954±0.013	2.483±0.021	1.734±0.023
NS	10	2.059±0.010	1.907±0.012	1.317±0.022	1.907±0.022

Tab 7. comparison of CD4/ CD8 among groups(u/ml)

Groups		2h	4h	6h	8h
Recombinant <i>L.lactis</i> NZ9000	10	1.359±0.075	0.595±0.053	1.320±0.075	0.732±0.091
Recombinant <i>E.coli</i>	10	1.044±0.081	0.785±0.069	2.222±0.083	1.162±0.076
<i>L.lactis</i> NZ9000	10	1.369±0.073	1.015±0.080	1.012±0.081	1.70±0.085
<i>E.coli</i> H5α	10	1.218±0.036	1.458±0.079	0.887±0.064	1.667±0.078
NS	10	1.088±0.054	1.200±0.062	1.543±0.053	1.245±0.081

### Survival of the mice

Five days after the treatment, the mice with *L.lactis* NZ9000 and recombinant *L.lactis* NZ9000 were more active than that with *E.coli* DH5α.

### Discussion

*Lactococcus lactis* is commonly used in fermented industry, especially in fermented dairy products. In addition, *Lactococcus lactis* is one type of host bacteria which could induce expression of common, important research and application value. And *Lactococcus lactis* is relatively simple on rapid growth and metabolism, with

separated decomposition synthesis metabolism. As the small genome beneficial genetic engineering such as separation and purification of extracellular proteins, these advantages make *Lactococcus lactis* has major research value in the regulation of metabolism. At present, *Lactococcus lactis* in milk products have been widely used as a leavening agent for sour cream, yogurt, yogurt, soy milk beverage and dairy production (Rochat *et al.*, 2012; Van de Guchte *et al.*, 2002,).

Report demonstrated the parameter requirement for successful transformation of *L. lactis* NZ9000 strain with electroporation (Gerber, S.D. *et al.* 2007) . Electroporation transformation was used in the study. It is better than chemical transformation in this study, and the concentration of 2-3% glycine in the media also will provide the optimal growth for the transformation efficiency. Also we investigated the cloning and expression of catalase from *E. coli* in *L. lactis* and the role of oxidation and salt stress in the expression of the catalase of *L. lactis* subsp. The transformants were screened for catalase activity on brain heart infusion (BHI) containing tannic acid (BHI-tannic acid medium) and ampicillin (150 µg/ml), Tannic acid could induce the activity of catalase it is identified in the experiment (Osawa, R. 1990.), and this method for cloning catalase gene is feasible.

Many problems were found when genome of *E. coli* was cut with Sau3AI. The intact catalase gene was to be obtained only by random partly cutting. The fragments molecular weights more than 2000Dt were insured to be collected. And the restricted enzyme BamHI which could produce the same cohesive 5'ends as Sau3A was used in the test. For many fragments, the time should be controlled when the plasmid of pUC18-kat was cut with BamHI because of the sites of BamHI in the kat gene in order to ensure the integrity of gene. In this work, catalase is induced either by nisin. Moreover, this induction occurs at the appropriate time in anaerobic culture. It may be transcriptional time and results in enhanced catalase expression. The kat gene was successfully expressed in the heterologous host *E. coli*. It was also identified by western-blot. Of the recombinant strain tested, only recombinant *L. lactis* exhibited greater salt sensitivity compared to the parent culture NZ 9000. This observation suggests that engineering bacteria may be involved in the response of *L. lactis* to salt stress. After 20 min incubation, 70% of the NZ9000 cells remained viable. The effects associated with the catalase insertion were due specifically to changes in recombinant *L. lactis*, strain which was exposed to salt, like the other strains. This strain was more resistant to salt than normal strain NZ9000. This may be the result of high catalase levels as a consequence of the high copy number of the plasmid harboring the catalase gene (Hikmate A, and Anette H, 2002 and Le Loir *et al.*, 1998).

In some cases, catalase genes exhibit sequence homology between the different bacteria strains but they are regulated differently (Schellhorn & Hassan, 1988). It was shown in this study.

The report demonstrated that antigen transport would be increased in the absence of the intestinal microflora, it indicates that the gut microflora is an important constituent

in the intestines defense barrier. For example production of IgE antibodies, it is upon oral antigen administration.

Abrogation of oral tolerance was due to the absence of intestinal flora. The aberrant IgE response could be corrected by reconstitution of the microflora at the neonatal stage, but not at a later age (Sudo *et al.* 1997). However, the interest in gastric bacteria has increased only since the isolation of *H. pylori* from the gastric mucosa of human patients with gastro duodenal diseases (Gerber, S.D.,*et al.*2007,). While immunization with recombinant *L.lactis* containing catalase gene merely induces slightly higher levels of B-cell epitope specific IgG, It is suggest that recombinant *L.lactis* NZ9000 containing catalase gene could stimulate humoral immunity of the animals. Whether contribution, if any, of the T cell response to humoral response leads to qualitatively superior specific IgG antibodies is an interesting consideration to explore in future studies.

Lymphocytes are anti infection and tumor immune cells, which mainly contain CD4 molecules and CD8 molecules. CD4 cells play a messenger role. CD8 cells can kill infected cells and cancer cells. In the paper, the relative number of CD4 cells was no significantly ( $p>0.05$ ) increased in the probiotic group. No difference was observed between mouse strains in regard to the presence only in the blood of the experimented animals. It was shown that no influence of recombinant *L. lactis* NZ9000 containing catalase gene for cellular immunity of animal could be detected in the blood. It is known that the immune type of humoral and cellular immunity, complement each other, but different diseases showed different types of immune cells. Only the enzyme change in the cells and this change is not a foreign antigen, so that the humoral and cell mediated immunity level will not change. The researchers found that genetically modified mice with catalase deficiency were still normal phenotypes, suggesting that catalase is essential for animals only under certain conditions.

[M8]The genetic improvement of *L. lactis* is a matter of biotechnological interest in the food industry and in the pharmaceutical and medical fields. The main metabolism of *L. lactis* is through the anaerobic pathway, fermentation, which produces lactic acid from the available carbohydrates and is used for industrial food production (Martirosyan A.O. *et al.*, 2004,[M9] Boon EM, *et al.* 2007, Abriouel *et al.*, 2004, Percy, 1984).The vast majority of aerobic microorganisms contain catalase, However *Lactococcus lactis* do not have catalase activity or little, resulting in the presence of oxygen decreased antioxidant capacity (Boon EM, *et al.* 2007, Abriouel *et al.*, 2004). The purpose of this study is to insert the catalase gene from the bacteria into *Lactococcus lactis* by gene engineering methods to induce its expression in order to improve the utilization degree of *Lactococcus lactis*[M10]. This shows that some enzyme activity could make the organism have a selective advantage over *S. bovis* when grown in the presence of tannins (Engesser, D. M *et al* 1994)

The result in genetic performance of *L. lactis* reveals another industrial advantage for producing new protein by genetic engineering. It is shown that the recombinant *L*

*lactis* containing catalase is beneficial by the test of animals' survival. It is not only important in dairy production, but also has potential for use as oral vaccine, and foreign protein production.[M11]

### Acknowledgements

We thank Dr. Alexandra Gruss from Institute of National Agriculture Research in France for providing the plasmids. This research[M12] was supported by Guandong Natural Science Fund Committee (06029354) .

[M13]My colleagues and I have no financial and non-financial competing interests in relation to this paper.

### References:[M14]

- Abriouel, H., Herrmann, A., Starke, J., Yousif, N. M., Wijaya, A., Tauscher, B., Holzapfel, W. & Franz, C. M. Cloning and heterologous expression of hematin-dependent catalase produced by *Lactobacillus plantarum* CNRZ 1228. *Applied and environmental microbiology*. 2004, 70: 603-606.
- Arockiaraj, J., Easwvaran, S., Vanaraja, P., Singh, A., Othman, R. Y. & Bhassu, S. Molecular cloning, characterization and gene expression of an antioxidant enzyme catalase (MrCat) from *Macrobrachium rosenbergii*. *Fish & shellfish immunology*. 2012, 32: 670-682.
- Bernhard Tauscher,<sup>2</sup> Wilhelm Holzapfel,<sup>1</sup> and Charles M. A. P. Franz<sup>1\*</sup> Cloning and Heterologous Expression of Hematin-Dependent Catalase Produced by *Lactobacillus plantarum* CNRZ 1228 *Appl Environ Microbiol*. 2004; 70(1): 603–606.
- Boon EM, Downs A, Marcey D. "Catalase: H<sub>2</sub>O<sub>2</sub>: H<sub>2</sub>O<sub>2</sub> Oxidoreductase". *Catalase Structural Tutorial Text*. Retrieved 2007-02-11.
- Boucher, I., Parrot, M., Gaudreau, H., Champagne, C. P., Vadeboncoeur, C., Moineau, S. Novel Food-Grade Plasmid Vector Based on Melibiose Fermentation for the Genetic Engineering of *Lactococcus lactis*. *Appl. Environ. Microbiol*. 2002. 68: 6152-6161
- Chung K T, Wong T y,Wei C I,et al.Tannins and human health :A review[J].Critical reviews in food science and nutrition ,1998,(38):421-464.
- Engesser, D. M., and W. P. Hammes.. Non-heme catalase activity of lactic acid bacteria. *Syst. Appl. Microbiol*. 1994,17:11-19.
- Hikmate Abriouel,<sup>1†</sup> Anette Herrmann,<sup>1</sup> Joachim Stärke,<sup>2</sup> Nuha M. K. Yousif,<sup>1</sup> Agus Wijaya,<sup>1</sup> Jacek S, Loewen P C. Diversity of properties among catalases. *Archivers of biochemistry and biophysiscs*, 2002, 401:145- 154.
- Gerbersdorf, SD, Jancke T,Westrich B, Sediment properties for assessing the crosion risk of contaminated riverine sites. *J Soil Sediments* 2007, 7:25-35.
- Le Loir Y, Gruss A, Ehrlich SD, Langella P A nine-residue synthetic peptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*. *Lait* 1998, 81:217-226
- MartirosyanA.O., Sh.L.Mndzhoyan,L.M.Charyan,Antimicrobial Activity of Lactic Acid Bacteria from Sour Milk Products Narine, Karine,and Matsun. *Applied*

- biochemistry and Microbiology 2004; 40(2):178-180.
- Osawa, R. Formation of a clear zone on tannin-treated brain heart infusion agar by a Streptococcus Streptococcus sp. isolated from feces of koalas. Appl. Environ. Microbiol. 1990, 56:829-831
  - Percy, M. E. Catalase: an old enzyme with a new role? Can. J. Biochem. Cell Biol. 1984. 62:1006-1014.
  - Powell, IB, Achen M G. A simple and Rapid Method for Genetic Transformation of Lactic Streptococci by Electroporation. Appl Environ Microbiol,1988,54: 655-660.
  - Rochat, T., Boudebouze, S., Gratadoux, J. J., Blugeon, S., Gaudu, P., Langella, P. & Maguin, E. Proteomic analysis of spontaneous mutants of Lactococcus lactis: Involvement of GAPDH and arginine deiminase pathway in H<sub>2</sub>O<sub>2</sub> resistance. Proteomics. 2012, 12: 1792-1805.
  - Sambrook J, Fritsch E and Maniatis T Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. (1989).
  - Schellhorn, HE & Hassan, HM Transcriptional regulation of KetE in Escherichia coli K-12. J bacterial 1988, 170:4286-4292.
  - Sudo S, Fujikawa T, Nagakura T, Ohkubo T, Sakaguchi K, et al. Structures of mollusc shell framework proteins. Nature. 1997, 387: 563–564
  - Van de Guchte, M., van der Vossen, J. M., Kok, J. & Venema, G. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in Lactococcus lactis subsp. lactis. Applied and environmental microbiology. 1989, 55: 224-228.
- [Y Yuzugullu](#) , [T Chi](#) , [AR Pearson](#) , [MA Smith](#) , [S Phillips](#). [Crystallization of bifunctional catalase-phenol oxidase \(CATPO\) from Scytalidium thermophilum](#), [Acta Crystallographica](#) , 2001 , 65 (a1) :137-138

## Appendix: sequence detail

```

atgtcgcaakataaacgaaagaacccakatcagcaccagtcaccactacacgattccagc... ..60
gaagcgaaaccgggatggactcactggcacctgaagacggatctkatcgtccagcggct... ..120
gaaccaacaccgcctggtgcacaacctaccgcccaggagcctgaagcccctgatacg.....180
cgtaacgaaaaactaattctctggaagacgtacgcaaaggcagtgaaattatgcgctg.....240
accactaatcaggcgtgkcatcgccgacgatcaaaactcactgcgtgcccgttagccgt..... 300
ggtccaactctgctggaagattttattctgcgagaaaatcactcactttgackatgag.....360
cgkatcccgaacgtattgttkatgcacgcatcagcagctcacggttattccagcca.....420
tataaaagcttaagcgatatcaccaaagcggatttctctcagatacgaacaaaatcacc..... 480
ccagttattgttagtttctaccgttcaggcggcgctggctctgccgataccgtacgt.....540

```

gatatccgtggcttfgccaccaagtctatactgaagagggtatTTTTgacctgttggc.....600  
aataacacgccaatctctttatccaggatgcgkataaatccccgattttgttkatgcg..... ..660  
gtaaaaccagaaccgactgggcaattccacaagggcaaagcgccacgatactttctgg.... ..720  
gattatgttctctgcaacctgaaactctgcacaacgtgatgtggcaatgtcggatcgc.....780  
ggatcccgcgcagttaccgactatggaaggcttcggattkacacctccgcctgatt..... ..840  
aatgccgaagggaaggcaacgttcgtactgttccactggaaccactggcaggtaaagcc... ..900  
tctactgtttgggatgaagcacaactaaccggacgtgacccggactccaccgccgc... ..960  
gagttgtgggaagcgattgaagcaggcgattttctgaatacgaactgggcttccagttg..... 1020  
attcctgaagaagacgaattcaagttcacttcgatcttctgatccaaccaaacttacc.....1080  
ccggaagaattggtcctgttcagcgtgtcggcaaatggtgctcaatcgtaatccggat.....1140  
aactctttgctgaaaacgaacaggtggcttckatccaggkcatattgtcccggctg..... 1200  
gattcaccacgacccgttattgcagggcggtttgttcttataccgatacacaatc..... ..1260  
agtcgtcttggtggccgaatttckatgaattccgattaaccgccgacctgccttac..... ..1320  
kataattccagcgtgacggkatgkacgtatgggatcgcactaaccggcgaattac.... ..1380  
gaaccgaactcgatcaacgataactggccgcgcgaaacaccgccggggccgaaacgcggc..1440  
ggtttgaatkataccaggaacgcgtggaaggaaataaagttcgcgagcgcagtcakatg.....1500  
tttggcgaatattatcckatccgcgtctgttctggctaagtacagacgckattcgagcag.....1560  
cgkcatattgtcgtatggttcagtttgagtaagcaagtagttcctccgtatattcgt.....1620  
gagcgcgttgtgaccagctggcgkatattgatctactctggcccaggcgggtggcgaac.... ..1680  
aatctcggatcgaactgactgatgaccagctgaatcacccacctccggacgtcaac... ..1740  
ggctgaaaaaggatckatccttaagtctgactckattctgacggatgtgaaaggt..... ..1800  
cgcgtgtagcaattttgcttaatgatgaagtgagatcggcagaccttctggckattctc..... ..1860  
aagcgcgtgaaggccaagcgttkatgccaactgctctactcccgaatgggggaagtg. ...1920  
actgcggatgacggtacgggtgttgcctatagctgctaccttgcgggtgcgcttcgctg..... ..1980  
acggctgatgacgkattgtcccttgcggcaatattgcggatcgcgtgacaacggcgtat... ..2040  
gccactactatctgatggaagcctacaaacacctaaaccgattgcgctggcaggagac..... ..2100  
gcgcgcaagttaaagcaacaatcaaggctcgtgaccagggtgaagaagggttgcggaa... ..2160  
gctgacagcggcgtgtagttttatggatgaactgtaacgctgatgacagcacaccgc..... ..2220  
gtgtggtcacgkattcctaagattgacaaaatccccggcgtaa.....2262

Fig.10 Determination of nucleotide sequence of kat katE gene (bt sequence)

MSQHNEKNPHQHQSPLHDSSEAKPGMDSLAPEDGSHRPAAEPTPPGAQPTAPGSLKAPDT  
RNEKLSLEDVRKGSENYALTTNQGVR IADDQNSLRAGSRGPTLLEDFILREKITHFDHERIPER  
IVHARGSAAHGYFQPYKSLSDITKADFLSDTNKITPVFVRFSTVQGGAGSADTVRDIRGFATKF  
YTEEGIFDLVGNNTPIFFIQDAHKFPDFVHAVKPEPHWAIPQGQSAHDTFWDYVSLQPETLHNV  
MWAMSDRGIPRSYRTMEGFGIHTFRLINAEGKATFVRFHWKPLAGKASLVWDEAQKLTGRDP  
DFHRRELWEAIEAGDFPEYELGFQLIPEEDEFKFDLDDPTKLIPEELVPVQRVGKMVLNRNP  
DNFFAENEQVAFHPGHIVPGLDFTNDPLLQGRLFSYTDTQISRLGGPNFHEIPINRPTCPYHNFQ  
RDGMHRMGIDTNPANYEPNSINDNWPRETTPPGPKRGGFESYQERVEGNKVRERSPSFG EYYSH  
PRLFWLSQTPFEQRHIVDGFSELSKVVRPYIRERVVDQLAHIDLTLAQAVAKNLGIELTDDQLN  
ITPPPDVNGLKKDPSLSLYSIPDGDVKGRVVAILLNDEVRSADLLAILKALKAKGVHAKLLYSR  
MGEVTADDGTVLP IAAATFAGAPSLTVDAVIVPCGN IADIADNGDANYYLMEAYKHLKPIALAG  
DARKFKATIKVADQGEEGIAEADSADGSFMDELTLMTAHRVWSRIPKIDKIPA.[M15]

Fig 11. Sequence of amino acids from KAT protein \*The sequence was from Blast search