

# 优化培养条件和裂解方法提高 hRANKL 在大肠杆菌内的表达

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**摘要:** RANKL/RANK/OPG 轴在骨代谢过程中起到中心调节作用, 也是近年来骨相关疾病治疗研究的热点之一。RANKL 蛋白在 RANKL/RANK/OPG 轴信号传递过程中起到关键作用, 在骨代谢相关实验研究中用途广泛。但是, 使用大肠杆菌 *Escherichia coli* 可溶表达重组人源 RANKL 蛋白 (hRANKL) 时产量远低于鼠源 RANKL (mRANKL)。本研究通过将 LB 培养基 pH 值调整并稳定在 7.5、降低诱导表达温度至 16 °C 并优化细菌裂解条件, 成功地将可溶 hRANKL 产量增加到了对照组的 5-12 倍。该方法有效提高了 hRANKL 在大肠杆菌中可溶表达的产量, 同时也是研究重组蛋白在大肠杆菌内的可溶表达策略的有益尝试。

**关键词:** RANKL, 大肠杆菌, 蛋白表达, 骨质疏松

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# Enhancing hRANKL production in *Escherichia coli* by optimizing culture conditions and lysing program

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**Abstract:** RANKL/RANK/OPG axis is important in bone metabolism regulation, and becomes a popular research area in bone diseases. RANKL is a critical part of RANKL/RANK/OPG axis, and widely required in bone metabolism research. However, the yield of recombinant soluble human RANKL (hRANKL) in *Escherichia coli* is much lower than mouse RANKL (mRANKL). In this study, by adjusting and stabilizing the pH value of LB medium at 7.5, lowering the inducing temperature to 16 °C and optimizing the lysis program, the yield of soluble hRANKL increased by approximately 5 to 12-fold over the non-adjusted group. Our experiment effectively enhanced soluble hRANKL expression in *E. coli* and might constitute a meaningful attempt to obtain soluble expression of recombinant protein in *E. coli*.

**Keywords:** RANKL, *Escherichia coli*, protein expression, osteoporosis

## Introduction

The incidence and development of osteoporosis are largely due to an imbalance between the bone formation mediated by osteoblast and the bone resorption mediated by osteoclasts, in which the number and functional status of osteoclasts make the difference<sup>[1-2]</sup>. The RANKL/RANK/OPG axis plays a critical role in the regulation of osteoclast differentiation, maturation and activation, and the discovery of this axis is one of the milestones of studies on bone metabolism<sup>[3]</sup>. Receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) is a member of the TNF superfamily (TNFSF), which is a type II transmembrane protein discovered independently by four independent research teams<sup>[4-7]</sup> in the late 1990s. There are three naturally occurring forms of human RANKL, two of which are membrane-bound forms, while the other is an extra-cellular secreted soluble

form, which is a short-term, full-length protein cleaved by metalloproteinase 14 and ADAM 10<sup>[8]</sup>. In bone, RANKL expresses on the surface of stromal/osteoclasts as well as osteocytes. Both membrane-bound and soluble RANKL is able to bind to and cluster RANK, which is expressed on osteoclasts and their precursors. Clustered RANK recruits adaptors, such as TRAF6, by their intracellular regions and initiate downstream signaling pathways, ultimately promoting the proliferation, differentiation and maturation of osteoclasts<sup>[3,9]</sup>. As the natural decoy receptor of RANKL, osteoprotegerin (OPG) can compete with RANK in binding to RANKL and inhibiting the differentiation and function of osteoclasts. Other than its initial role in bone-resorption regulation, RANKL/RANK interaction is also of critical importance in cellular immunity regulation and lactating-form mammary gland formation<sup>[10-11]</sup>.

Because the role of the RANKL/RANK/OPG axis in bone metabolism regulation is important, it can be a promising treatment target for osteoporosis and other bone metabolism-related diseases. Currently, many biopharmaceutical enterprises worldwide have developed several antibody-based drugs, among which the most famous one is denosumab, a monoclonal, fully humanized antibody against RANKL which was developed by Amgen Inc. It was reported that denosumab significantly reduced bone-turnover parameters as well as the number of osteoclasts<sup>[8]</sup>.

In our previous studies related to the RANKL/RANK/OPG axis, we initially determined the affinity between RANKL and RANK<sup>[12]</sup>, obtained crystals of RANK, as well as RANKL/RANK complex<sup>[13]</sup>, and analyzed the three dimensional structural information of RANK and RANKL/RANK complex<sup>[11]</sup>. During the research processes, an unfavorable circumstance always bothered us in that the yield of soluble human RANKL (hRANKL) was very low. Under the same cultural, expressional, and purification conditions, the yield of hRANKL from unit LB medium was much lower than that of mouse RANKL (mRANKL) in our experience.

As a central part of the RANKL/RANK/OPG axis, RANKL is considered as the key target of osteoporosis, bone tumor and other bone-metabolism related diseases, which made it highly valuable in bone-metabolism researches. Meanwhile, as the intersection between bone-metabolism and immune system<sup>[14]</sup>, RANKL is also widely required in NF- $\kappa$ B related immune researches. The construction of human RANKL is slightly different from that of mouse RANKL, the pharmacodynamic and pharmacokinetic properties might be different from each other, hence the preparation of hRANKL is required irreplaceable in almost all researches in relation to characteristic analyses or functional examination of the RANKL/RANK/OPG axis. Moreover, just as denosumab, hRANKL is widely used in anti-osteoporosis bio-therapeutic drug

development<sup>[8]</sup>, large-scale drug screening experiments request a mass of human RANKL protein. High through-expression of RANKL would be meaningful to the commercial process. Therefore, it is important to enhance the yield of hRANKL from unit LB medium.

*Escherichia coli* (*E. coli*) is an ideal and widely used tool to express soluble recombinant protein, but the yield of different recombinant protein expressed in *E. coli* varies drastically. One of the major problem that affect the yield of soluble recombinant protein expression is the inclusion bodies formation within the cell<sup>[15]</sup>. Culture condition (such as pH, inducing cell concentration), inducing temperature and extracting method are considered as the common optimizable factors affect the expression and solubility of recombinant protein<sup>[16]</sup>. Papaneophytou<sup>[17]</sup> and his colleague from Greece reported that the expression of hRANKL can be enhanced by controlling the  $OD_{595}$  before induction, changing IPTG concentration, and adjusting post-induction temperature and time.

In this study, according to reports in relevant articles and referring to our experience, to enhance the yield of hRANKL, the pH of the medium and the expression temperature was adjusted; beyond which, pH of medium was stabled by adding stabilizer during cell growth and induction period, and the lysing conditions were optimized. Finally, significantly increased protein yield from unit LB medium was obtained.

## 1 Materials and methods

### 1.1 hRANKL expression and purification

The pGEX-6p-1 vectors containing cDNA encoding glutathione S-transferase (GST)-fused extracellular domain (ECD) of hRANKL or mRANKL were currently maintained in our lab. The vectors are transformed and expressed in BL21 (DE3).

Both host cells were cultured in 5 mL of LB medium (Shanghai Aladdin) containing 0.1 mg/mL

ampicillin (Sigma) overnight (37 °C, 200 r/min). One milliliter of both overnight cultured media was transferred into a 5-L flask containing 2 L of fresh medium with 0.1 mg/mL ampicillin separately and was cultured for 8 hours (37 °C, 125 r/min) until the  $OD_{595}$  of both flasks reached 0.4, after which the incubator was cooled to 20 °C, and the cells grew freely to  $OD_{595}=0.6$ . Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) (Sigma) was added at a final concentration of 0.5 mmol/L as an inducer to induce protein overexpression overnight (20 °C, 125 r/min). The cells were harvested by centrifugation (Backman 8 500 r/min, 4 °C, 8 min) and lysed by Ultrasonic Cell Disruption (Ningbo Xinzhi JY92-IIDN) (225 W, 2-s pulse, 5-s gap, total time 23 min) in lysis buffer (PBS buffer containing 1% Triton X-100, pH 7.4). Both the hRANKL- and mRANKL-disrupted liquids were centrifuged (Backman, 8 500 r/min, 10 min, 4 °C), and the supernatant was purified with glutathione-Sepharose fast flow 4B beads (GE Healthcare) according to the protocol, and the GST-tags were cleaved by PreScission Protease (PSP) (GE Healthcare) overnight (4 °C). The filtrate was further purified with AKTA Avant (GE Healthcare) by size exclusion chromatography (SEC) (Superdex 200) in PBS buffer (pH 7.4). The purified protein was collected, and the yield of each protein was calculated by the Coomassie brilliant blue method<sup>[18]</sup>.

### 1.2 Adjustment of the pH of LB medium

*E. coli* DE3 with pEGX-6p-GST-hRANKL was cultured in 5 mL of LB medium overnight. Three milliliters of cultured-medium were divided equally into six 5 L flasks containing 2 L of fresh medium with different pH values (pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5) and then was cultured to  $OD_{595}=0.6$  (cooling to 20 °C when  $OD_{595}=0.4$ ), as described previously. The amplification speed of *E. coli* in different pH conditions varied, but all of them reached  $OD_{595}=0.6$  at 8 hours ( $OD_{595} \bar{x}=0.576$ ,  $s^2=0.058$ ). IPTG (final concentration 0.5 mmol/L) was added, and induction

was performed overnight (20 °C, 125 r/min). pH and  $OD_{595}$  variation were monitored hourly in all the steps. Subsequent manipulation was similar to that described previously. The experiment was repeated three times.

### 1.3 Stabilization of the expression of pH

Cells were amplified as described previously. Two milliliters of cultured medium was divided equally into four 5 L flasks containing 2 L of fresh medium (flasks were numbered No. 1 to No. 4) and then was cultured until  $OD_{595}=0.6$ , as described before. Flask No. 1 was mixed with 25 mL of 40× PB buffer (108 mmol/L KCl, 400 mmol/L  $Na_2HPO_4$ , 72 mmol/L  $KH_2PO_4$ , pH 7.5) and 25 mL of fresh LB medium (final PB buffer concentration was 1×), flask No. 2 was mixed with 50 mL of 40× PB buffer (final PB buffer concentration was 2×), and both flasks No. 3 and No. 4 were mixed with 50 mL of fresh LB medium. The pH of flasks No.1 to No. 3 flask was adjusted to 7.5, while flask No. 4 was non-adjusted. IPTG was added to all the flasks, and the subsequent manipulation was similar to that described previously. The experiment was repeated three times.

### 1.4 Adjustment of expressional temperature

Cells (hRANKL) were amplified as described previously. Three milliliters of cultured medium were divided equally into six 5 L flasks containing 2 L of fresh medium (0.1 mg/mL ampicillin pH 7.5) and then were cultured until  $OD_{595}=0.6$  as described previously. IPTG was added (final concentration is 0.5 mmol/L), and the flasks were placed in incubators at different temperatures (22 °C, 20 °C, 18 °C, 16 °C, 14 °C and 12 °C). All the flasks were shaken overnight, and the subsequent manipulation was similar to that described previously.

### 1.5 Optimization of lysing program

Cells (hRANKL) were cultured in 5 L flasks containing 2 L of fresh medium (37 °C 125 r/min, pH 7.5) and expressed (0.5 mmol/L IPTG, 16 °C, 125 r/min, overnight) similarly as before. Cells were collected by centrifugation, and the cell pellets were divided equally into 4 pieces designated pellets No. 1

to No. 4. Pellets No. 1 and No. 2 were lysed by osmotic shock. Briefly, the pellets were suspended in 30 mL of PBS sucrose buffer (PBS buffer with 20% (*m/V*) sucrose, 1 mmol/L EDTA, 0.5% (*V/V*) Triton X-100, pH 7.4), afterward were placed on ice for 15 min and then were centrifuged at 8 500 r/min for 5 min. The supernatant was removed, the sediment was suspended in 30 mL of PBS buffer (pH 7.4), and placed on ice for another 15 min. After an osmotic shock procedure, pellet No. 1 was centrifuged as described previously, while pellet No. 2 was lysed again by Ultrasonic Cell Disruption as described before and was subsequently centrifuged. Pellets No. 3 and No. 4 were suspended in 30 mL of PBS sucrose buffer and PBS buffer, respectively, lysed by Ultrasonic Cell Disruption and centrifuged as described previously. All four supernatant pieces following centrifugation were collected and subsequently purified with glutathione-Sepharose fast flow 4B beads, and the subsequent manipulation was similar to that described previously.

### 1.6 Comparison of hRANKL yield according to the literature and that after optimization of induction and cell lysis

Cells (hRANKL) was amplified overnight as described previously, three milliliters of cultured medium was divided equally into two 5 L flasks containing 2 L of fresh medium (flasks were numbered No.1 and No. 2, No. 1 flask containing 2 L of fresh medium, and the pH of the medium was not adjusted; No. 2 flask containing 2 L of fresh medium with 2× PB buffer, and the pH was adjusted to 7.5). Both flasks were cultured to  $OD_{595}=0.6$ , and cooling to 25 °C and 16 °C respectively, IPTG was added (final concentration 0.3 mmol/L in flask No. 1 and 0.5mmol/L in flask No. 2), and pH of flask No. 2 were adjusted to 7.5. Cells in flask No. 1 were harvested at 6.5 h after induction, and cells in flask No. 2 were induced overnight. cell pellets of flask No. 1 were lysed by lysozyme and traditional ultrasonic cell disruption according by the literature, while the cell pellets of flask No. 2 were lysed by

osmotic shock procedure following by ultrasonic cell disruption. The subsequent manipulation was similar to that described previously.

## 2 Results

### 2.1 The enhancement of pH stabilizer and the yield of hRANKL

RANKL is highly conserved during evolution<sup>[6]</sup>. Full-length hRANKL and mRANKL share 84.6% identity in their protein sequences, while the identity of their ECDs is 90% (sequence alignment ExpAsy<sup>®</sup>; see Table 1). According to our previous research, the similarity of structure and function between hRANKL and mRANKL has been proved by obtaining the crystal structure of hRANKL/mRANK complex<sup>[19]</sup>. However, the difference in yield between hRANKL and mRANKL from unit LB medium was 5-fold–12-fold (Fig. 1) according to our experiment.

In this study, the pGEX-6P-1 plasmid vectors contained hRANKL cDNA and mRANKL cDNA, which were both maintained in our lab and were both transformed with the same *E. coli* BL21 (DE3) strain, and the expression and purification conditions were same between the two types of RANKL.

Though the sequence of the two kinds of RANKL is highly conserved, the pI value, which is one of the most important influence parameters of protein yield and solubility, of two kinds of RANKL is of great difference ( $pI_{\text{mouse}}=6.39$ ,  $pI_{\text{human}}=8.03$ ). So we suspect that the pH might be of critical importance on the yield and solubility of hRANKL. To determine the optimal pH for hRANKL expression, we set the pH of LB medium between 6.0 and 8.5 and compared the output in each condition, and the results showed that the yield of hRANKL from unit medium increased along with the increase in pH. The output reached a peak at pH 7.5 and then decreased with the further increase in pH (Fig. 2A). Compared with pH 6.0, 6.5, 7.0, 8.0 and 8.5, the output at 7.5 increased by 246%, 126%, 10%, 75% and 155%, respectively.

**Table 1** Sequence comparison of human (Homo) and mouse RANKL

Sequence name	Size	Sequence
Homo	1	MRRASRDYTKYL R GSEEMGGPGAPHEGPLH - APPPPAPH
Mouse	1	MRRASRDYGKYLRSSEEMGSGPGVPHEGPLHPAPSAPAPA
Identity		***** ** * ***** ** **
Homo	40	QPPAASRSMFVALLGLGLGQVVC SVALFFYFRAQMDPNRI
Mouse	41	PPPAASRSMFLALLGLGLGQVVC SIALFLYFRAQMDPNRI
Identity		***** ** * ***** *****
Homo	80	SEdGTHCIYRILRLHENADfQDTTLESQDTKLIpDSCRRI
Mouse	81	SEdSTHCFYRILRLHENADLQDSTLESEDT—LPDSCRRI
Identity		** * ** * ***** ** * ***** *****
Homo	120	KQAFQGA VQKELQHIVGSQH IRAEKAMVDG SWLDLAKRSK
Mouse	119	KQAFQGA VQKELQHIVGPQRF S GAPAMMEGSWLDVAQRGK
Identity		***** * * * ***** ** * ***** * * *
Homo	160	LEAQPFAHLTINATDIPSGSHKVSLS SWYHDRGWAKISNM
Mouse	159	PEAQPFAHLTINAASIPSGSHKVTLS SWYHDRGWAKISNM
Identity		***** ***** ***** ***** *****
Homo	200	TFSNGKLI V NQDGFYLYANICFRHHETS GD LATEY LQLM
Mouse	269	TLSNGKLR V NQDGFYLYANICFRHHETS GSVPTDY LQLM
Identity		* ***** ***** ***** ***** ***** * *****
Homo	240	VYVTKTSIKIPSSHTLMKGGSTKYWSGNSE FHFYSIN VGG
Mouse	239	VYVVKTSIKIPSSHNL MKGGSTKNWSGNSE FHFYSIN VGG
Identity		** * ***** ***** ***** ***** ***** *****
Homo	280	FFKLRSGEEISIEVSNPSLLDPDQDATYFGAFKVRDID
Mouse	279	FFKLRAGEEISIQVSNPSLLDPDQDATYFGAFKVDID
Identity		***** ***** ***** ***** ***** ***** *****

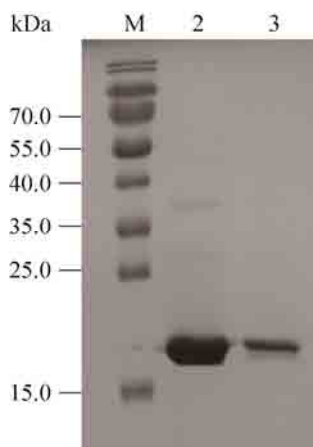


Fig. 1 Comparison of mouse RANKL (mRANKL) and human RANKL (hRANKL) on 15% SDS-PAGE. Both proteins were from equal volumes and were purified from equal volumes of bacteria, and the condition of bacterial growth and protein expression was same. M: protein marker; lane 2: mRANKL; lane 3: hRANKL. The yield of mRANKL was significantly higher than that of hRANKL.

It is well known that, accompanying the fermentation of *E. coli*, accumulation of cell-metabolite (acetic acid, in particular) alters the pH of medium constantly, and it ultimately affects the bacterial growth and overexpression of protein. To determine the pH change in the medium, we monitored the pH values of LB medium with different initial pH values. The result showed that, with the growth of *E. coli*, the pH values of all groups presented a decreasing trend (Fig. 2B), in agreement with the literature published previously<sup>[20]</sup>. IPTG was added at 8 hours to induce the expression of protein; the pH values of all the groups declined slightly and increased afterward. The end-point pH values of all the groups were approximately 7.5 ( $\bar{pH} = 7.38$ ,  $s^2 = 0.267$ ), except for the group with  $pH_{initial}$  6. Did the pH values affect the output of hRANKL by impacting the bacterial volume alone or additional parameters? To answer this question,

we monitored the  $OD_{595}$  value alterations in all the groups constantly (see Fig. 2C). The results showed that all the groups had similar growth trends, and when IPTG was added, the growth rates of all the groups decreased, indicating that IPTG inhibited bacterial growth. The highest end-point  $OD_{595}$  value appeared in the groups with  $pH_{initial}$  7.0 and  $pH_{initial}$  7.5, consistent with the literature published previously<sup>[20]</sup>. The comparison of  $OD_{595}$  and the yield of hRANKL in medium with different pH values is shown in Fig. 2D.

Since the highest output appeared in the group with initial pH 7.5, and the final pH of all the groups was approximately 7.5 ( $pH_{\bar{x}}=7.38$ ,  $S^2=0.267$ ) in our study, we hypothesized that the optimal pH of hRANKL expression might be approximately 7.5. Therefore, pH

stabilizer, which is expected to maintain the pH of the medium at approximately 7.5, was used to determine whether maintaining the pH of the medium could increase the output of hRANKL. In the subsequent experiment, different concentrations of pH stabilizer (PB buffer 2 $\times$ , 1 $\times$  and 0 $\times$ ) were added to the medium to keep the pH at approximately 7.5. The results showed that, when pH stabilizer was added, the pH of the expressional term was very stable (Fig. 3A), and the pH values at all the monitoring points varied little over repeated experiments ( $S^2_{1\times PB}=0.016$ ,  $S^2_{2\times PB}=0.014$ ). The outputs of the pH-adjusting-only group, 1 $\times$ PB buffer-added group, and 2 $\times$ PB buffer-added group increased by 18.6%, 37% and 39%, respectively, compared to the non-adjusted group (Fig. 3B).

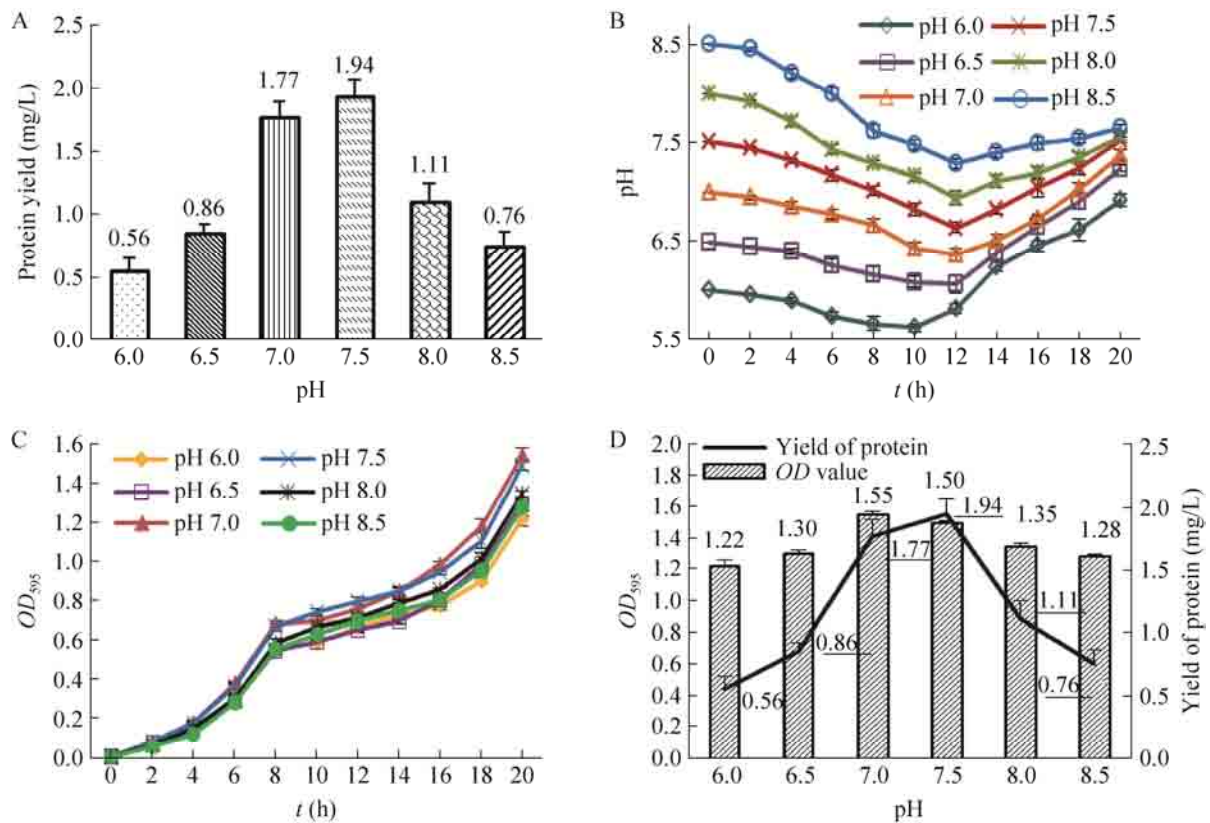


Fig. 2 The influence of hRANKL expression by pH. (A) Yield of hRANKL in ration medium with different pH values; the yield at pH 7.0 and pH 7.5 was much higher than in the remainder of the groups. (B) The pH curve of hRANKL in medium of different pH values. (C) The growth curve of *E. coli* in medium of different pH values; the inducer IPTG was added at 8 h, and growth of all groups started to slow after induction. The final  $OD_{595}$  of group pH 7.5 and pH 7.0 was much higher than in the remainder of the groups. (D) Comparison of final  $OD_{595}$  and the protein yield of hRANKL in LB medium of different pH values.

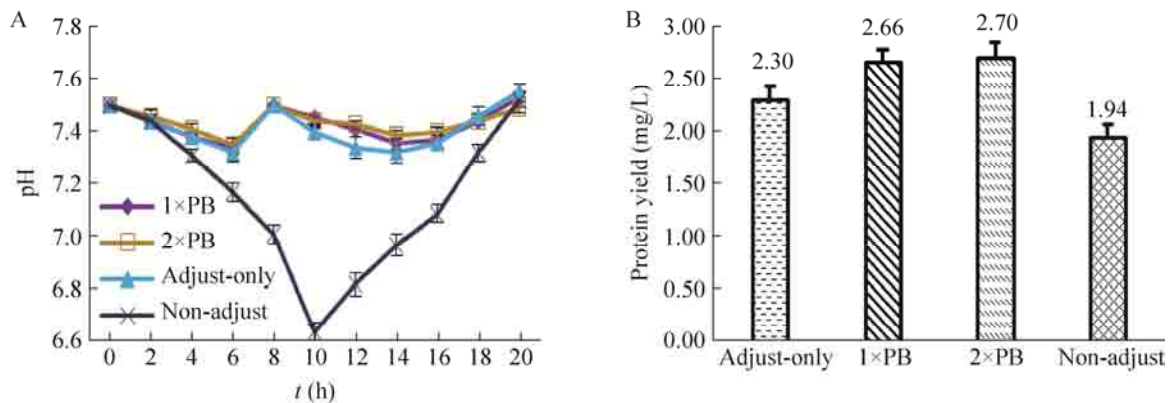


Fig. 3 The enhancement of hRANKL expression by pH stabilizer. (A) pH change in LB medium with different treatments. Compared with the non-adjusted group, adjusting the pH to 7.5 after induction could effectively stabilize the pH after induction. (B) Protein yield in LB medium with different treatments. The yield of hRANKL in groups with PB stabilizer added were much higher than in the non-adjusted group.

## 2.2 The optimization of expressional temperature

It is well known that temperature greatly affects output of soluble recombinant protein prepared from an *E. coli* expression system<sup>[21]</sup>.

We next attempted to determine the optimal temperature of hRANKL expression by inducing the protein in different temperatures and comparing the output of soluble hRANKL. According to our previous research, the ratio of inclusion bodies forming was increased when the inducing temperature was higher than 20 °C (unpublished data); therefore, the top temperature was selected at 22 °C. As shown in Fig. 4, with the same inducing time (12 h) and IPTG concentration (0.5 mmol/L), the output of RANKL increased with the decrease in inducing temperature, and the peak output appeared at 16 °C (Fig. 4A). The yield of soluble hRANKL stopped increasing and began to decline with the continual lowering of the inducing temperature. Compared to those at 22 °C, 20 °C, 18 °C, 14 °C, and 12 °C, the yield of soluble hRANKL at 16 °C increased by 88%, 73%, 29%, 22% and 74%, respectively.

Considering that the variation in the yield of hRANKL might be caused by different final bacterial concentrations in different temperatures, we monitored the  $OD_{595}$  alteration in all the inducing temperature conditions, and the results are shown in Fig. 4B. With

the lowering of the inducing temperature, the terminal bacterial concentration decreased continuously and was significant at 16 °C. Compared with that at 16 °C, the terminal bacterial concentration at 14 °C and 12 °C decreased by 12.1% and 25.8%, respectively. Therefore, the decreased outputs in temperatures lower than 16 °C might partly be due to the decrease in final bacterial concentration.

## 2.3 The enhancement of hRANKL yield by the combination of osmotic shock with ultrasonic cell lysis

The most widely used method for lysis of hRANKL fusion protein extraction is Ultrasonic Cell Disruption (UCD)<sup>[11-13,19,22-28]</sup>. However, according to our experience, the yield of protein is affected by the running time and the power of ultrasonic devices. The efficiency of cell lysis is low with a short running time or low power; however, the protein might be damaged when the running time is too long, or the power is too high.

To enhance the extraction efficiency while protecting the protein from being damaged, we attempted to combine the UCD with osmotic shock (termed Combined Cell Lysis, CCL). During the osmotic procedure, the cells were split by a rapid change in cross-membrane osmotic pressure, and slight ultrasonic shock was subsequently performed to cause the cytolysis drastically. The yield of hRANKL by using different lysing methods is shown in Fig. 5.



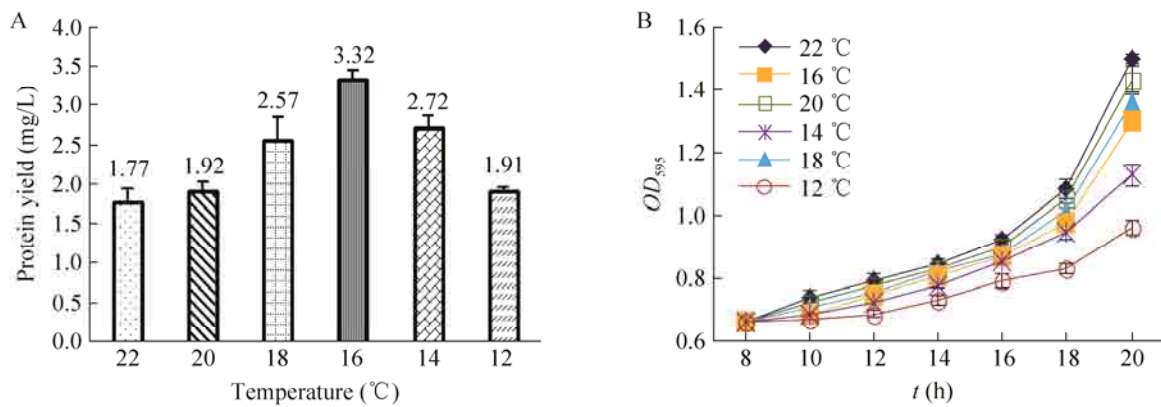


Fig. 4 The influence of inducing temperature on hRANKL expression. (A) hRANKL yields with different inducing temperatures. The highest yield appeared in 16 °C. (B) The growth curve of *E. coli* at different inducing temperatures. The final  $OD_{595}$  of each group decreased as the temperature decreased.

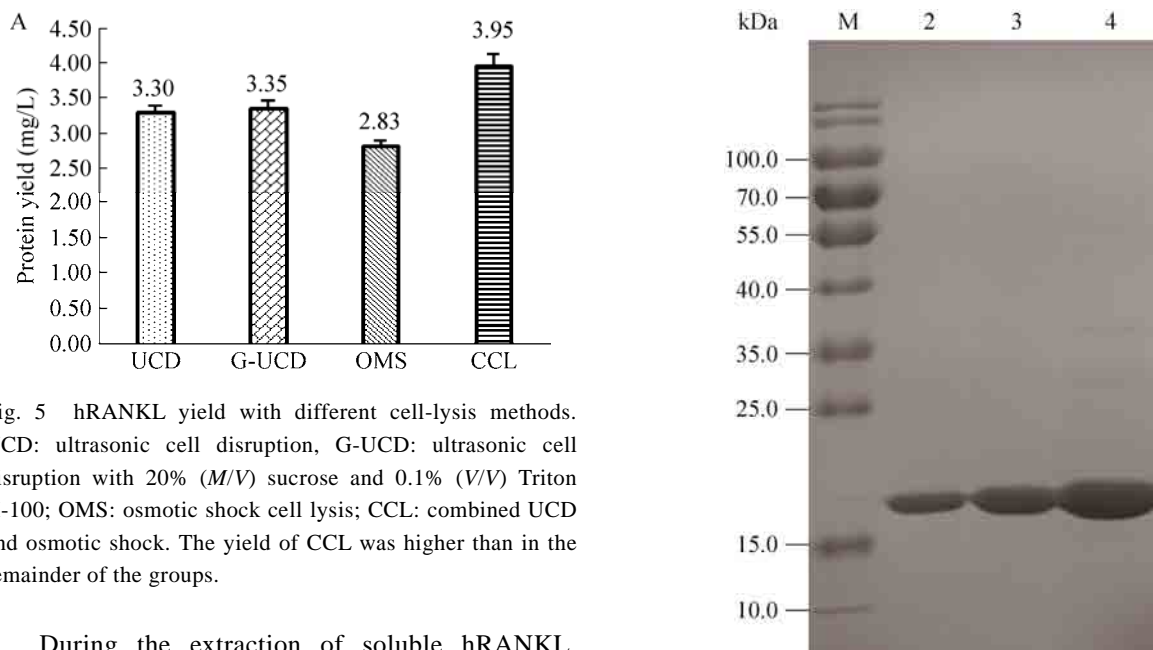


Fig. 5 hRANKL yield with different cell-lysis methods. UCD: ultrasonic cell disruption, G-UCD: ultrasonic cell disruption with 20% (*M/V*) sucrose and 0.1% (*V/V*) Triton X-100; OMS: osmotic shock cell lysis; CCL: combined UCD and osmotic shock. The yield of CCL was higher than in the remainder of the groups.

During the extraction of soluble hRANKL, Ultrasonic Cell Disruption was more efficient than osmotic shock alone. By using CCL, the yield of hRANKL was remarkably enhanced. Compared with that of lysed by osmotic shock alone and UCD alone, the yield of hRANKL that lysed by CCL increased by 40% and 18%, respectively. And the output can be slightly enhanced by adding 20% (*M/V*) sucrose, as well as 0.1% (*V/V*) Triton X-100, into the ultrasonic lysing buffer; but there was no statistical difference.

Fig. 6 15% SDS-PAGE comparison of hRANKL expression according to the literature, hRANKL expression after optimization of induction and cell lysis, and mouse RANKL. All the proteins were equal volumes and purified from equal volumes of bacteria. M: protein marker; Lane 2: hRANKL yield before optimization; Lane 3: hRANKL yield following the method reported by literature previously; Lane 4: hRANKL yield after optimization of induction and cell lysis. The yield of hRANKL after optimization was significantly higher than that of expressed following the method reported by literature previously.

## 2.4 Comparison of hRANKL yield according to the literature and that after optimization of induction and cell lysis

As described previously, it is reported<sup>[17]</sup> that the yield of hRANKL could be significantly enhanced at following conditions:  $OD_{595}$  before induction 0.55, IPTG concentration 0.3 mmol/L, a post-induction temperature 25 °C and the post-induction time 6.5 h. To examine the efficiency of our optimization method, we compared the hRANKL yield of the two optimization method. After purification, the hRANKL yield following the method reported by literature previously was 3.3 mg/L while the hRANKL yield by our new method was 5.2 mg/L, which is a 58% increase.

Finally, by adjusting the pH of the LB medium to 7.5, adding 2×PB solution as pH stabilizer, readjusting the pH of the LB medium to 7.5 after the induction period, lowering the inducing temperature to 16 °C, and lysing the cells by combining UCD with osmotic shock (CCL), the yield of soluble hRANKL is significantly enhanced. As shown in Fig. 7, supernatant after cell lysis was collected as internal reference, and

the concentrate of GST-RANKL fusion protein is similar among all groups. However, the yield of soluble protein varied widely after cleavage purification, the yield of soluble hRANKL after optimization reached 5.2 mg/L of LB medium, approximately 12-fold improvement over the non-adjusted group.

## 3 Discussion

*Escherichia coli* (*E. coli*) is the earliest and most widely used strains for recombination protein expression<sup>[29-31]</sup>. As a mature expression system, the genetic and culturing features of *E. coli* have been well discussed, and many widely used expression tools based on *E. coli* have been developed<sup>[32-34]</sup>. The efficiency of recombinant proteins expressed in *E. coli* was affected by many factors: the internal characteristics of recombinant genes; the metabolism of host cells; the interaction between foreign genes and the host cells; and moreover, the expression and purification conditions, that is, the pH value of the LB medium, the expression temperature, and the protein extraction method<sup>[32]</sup>.

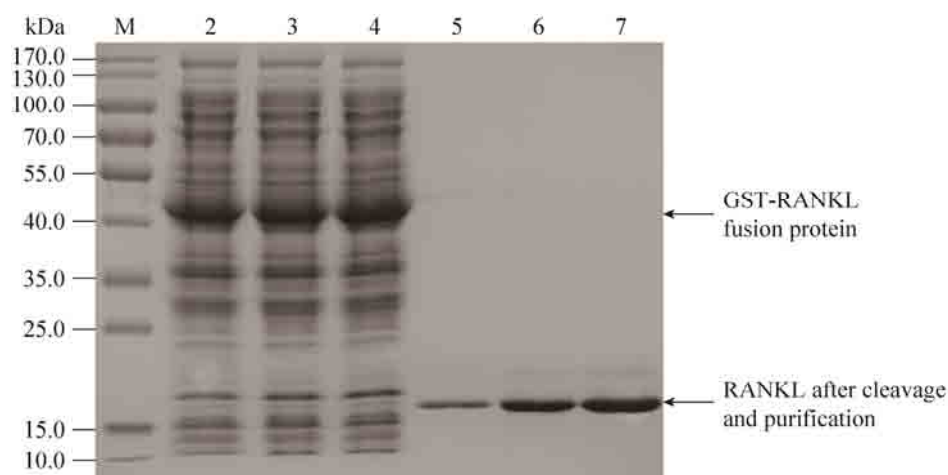


Fig. 7 15% SDS-PAGE comparison of human RANKL, mouse RANKL and supernatant after cell lysis (as internal references). M: protein marker; lane 2: supernatant after cell lysis of human RANKL before optimized; lane 3: supernatant after cell lysis of human RANKL after optimized; lane 4: supernatant after cell lysis of mouse RANKL; lane 5: human RANKL before optimized; lane 6: human RANKL after optimized; lane 7: mouse RANKL. All proteins are equal volume of purified from equal volume of bacteria and all supernatant are of similar concentration according to the internal references. But the protein yield of human RANKL after optimized is significant higher comparing to that before optimized and similar with mRANKL. Positions which the arrow pointed to are GST-hRANKL fusion protein.

In our study, the yield of mRANKL ranged between 5 and 6 mg/L of LB medium. It was reported that, when using *E. coli* as a host, mRANKL fused with MBP-tag, and the yield of mRANKL was approximately 2.5 mg/500 mL of LB medium<sup>[22]</sup>, similar to our experience. In contrast, the reported yield of hRANKL was much lower, 31 mg/15 L of medium (2 mg/L)<sup>[23]</sup>, and it ranged between 0.6 and 1 mg/L in our study.

It is reported that the yield and solubility of soluble protein expressed in *E. coli* is influenced mainly by over twenty independent parameters including isoelectric point (pI), molecular weight and proportion of specific kinds of amino acid (e.g. aliphatic fraction, Arginine fraction, hydrophilicity index, proportion of individual amino acid, et al.)<sup>[15]</sup>. As described previously, the sequences of the ECDs of hRANKL and mRANKL are highly conserved during evolution, the molecular weight of each sequence are similar (hRANKL ECD: 17946; mRANKL ECD: 17781). The proportion of individual amino acid of two kind of RANKL is displayed in Table 2, the percentage of each amino acid is similar. The most variable amino acids is Glutamine (2.52% in hRANKL ECD, while 3.77% in mRANKL ECD). The aliphatic indexes (AI) of each sequence were calculated according by reported in literature<sup>[15]</sup>. The AI of hRANKL ECD is 74.21%,

which is similar to that of mRANKL ECD (73.60%); while the hydrophilicity index of two kind of RANKL is almost identical (62.89% in hRANKL ECD, 62.26% in mRANKL ECD). Hence we suspected that the difference of yield and solubility between hRANKL and mRANKL is mainly due to the variation of pI value.

The *E. coli* metabolism in LB medium is quickly affected by changes in pH and acetate concentrations<sup>[34]</sup>. The initial pH of the LB medium affected the metabolite accumulation and nutrient absorption of bacteria<sup>[16,34]</sup>, ultimately affecting the yield of soluble protein<sup>[15,35]</sup>.

The most important influence of pH and accumulated metabolites on protein expression was inclusion body formation, which is a type of misfolded and insoluble form of expressed protein<sup>[20,36]</sup>. Andrea M et al<sup>[20]</sup> reported that inclusion bodies were far fewer, and their size was far smaller under a controlled medium pH at 7.5, compared with uncontrolled pH.

In our results, the highest protein output appeared at pH 7.5, and the end-point pH value of all the groups was approximately 7.5, so pH 7.5 might be the optimal pH value of hRANKL expression, while when the pH value was maintained at 7.5 by stabilizer, the yield of soluble hRANKL increased by 39% compared to the uncontrolled group. We suspected that inclusion bodies played an important role in these phenomena.

**Table 2 Proportion of individual amino acid of hRANKL and mRANKL ECD**

Amoni acid	hRANKL ECD (%)	mRANKL ECD (%)	Amoni acid	hRANKL ECD (%)	mRANKL ECD (%)
A	5.03	5.66	L	7.55	6.91
R	2.52	2.52	K	6.29	6.29
D	5.66	5.03	M	1.89	1.89
C	0.63	0.63	F	6.29	5.66
Q	2.52	3.77	P	3.14	4.40
E	4.40	3.14	S	11.95	11.95
H	4.40	4.40	T	6.29	5.03
I	6.92	6.29	W	1.89	1.89
G	7.55	8.16	Y	5.66	5.03
N	5.03	6.29	V	4.40	5.66

The influence of temperature on the expression of recombinant protein in *E. coli* has been reported widely<sup>[21,36-38]</sup>. The optimal growing temperature of *E. coli* in LB medium is 37–39 °C, under which conditions most of the recombinant protein is expressed as misfolded and inactive inclusion bodies<sup>[16,32,35]</sup>. It has been proven that the solubility of protein was increased greatly by lowering the inducing temperature<sup>[3,10-13,16,19,22-23,36]</sup>.

Luan et al<sup>[24]</sup> reported that hRANKL could be obtained by induction with 0.5 mmol/L IPTG in 16 °C for 20 h. In contrast, Harper<sup>[25]</sup> reported that long-term culture could cause bacteriolysis, and the expressed protein might be enzymolized by the protease released from the broken cells. We used to obtain stable hRANKL successfully by induction with 0.5 mmol/L IPTG in 20 °C<sup>[19]</sup> and 22 °C<sup>[12]</sup> overnight, although the productivity was low.

In this study, we obtained the cell concentration curve, as well as the yield curve, of soluble hRANKL in different inducing temperatures. The end-point cell concentration ( $OD_{595}$ ) decreased as the temperature decreased, while the output of protein increased in contrast. The peak yield of soluble hRANKL appeared at 16 °C, and both  $OD_{595}$  and protein output began to decrease with the decreasing temperature subsequently. We suspected that the yield of hRANKL in low temperatures was a cross effect of two processes: increased protein solubility and decreased protein expression efficiency. When the temperature was too low (in this study <14 °C), the increasing protein expression was too low to compensate for the decreased cell growth and protein expression efficiency, and the yield of hRANKL decreased.

At the same time, *E. coli* grew slowly in low temperatures, and the relevant metabolite that inhibited protein expression, such as acetate, accumulated slowly<sup>[35]</sup>; lower temperatures could prevent the enzymolysis of recombinant proteins by heat shock proteases or other proteases secreted by *E.*

*coli*<sup>[27,7]</sup>.

However, the low expression of the recombinant protein in low temperatures is a relative concept because it was reported that the insolubility problem of proteins could be resolved by lowering the temperature to 6–10 °C in a special, cold-resistant *E. coli* strain called “ArcticExpress”<sup>[21]</sup>. In other words, the expression of recombinant protein at low temperatures is not “inhibited” but relatively “delayed” because the protein expression period in 6 °C was longer than one week<sup>[21]</sup>. We did not select the lower temperature because the “time cost” had to be considered.

The recombinant protein is expressed in periplasm, and the bacteria need to be broken down to release the protein. UCD was highly efficient, but sometimes the power of it was so heavy that the proteins were broken down for a while.

There are many cell lysis methods, such as thermal treatment, lysozyme methods, osmotic shock, etc. Osmotic shock is a classic and efficient method, the principle of which is changing the intracellular osmotic pressure rapidly by altering the extracellular fluid, the cells will be bursted by a large volume of water that enters the cells. Because there was no mechanical disruption during this period, no protein damage occurred. In our study, although osmotic shock alone was not sufficiently efficient in hRANKL extraction, combination with UCD could significantly enhance the yield of soluble hRANKL; the lower time and power of UCD would result in less damage to the protein.

During Ultrasonic Cell Disruption (UCD), the parameters of the ultrasonic instrument should be adjusted according to the circumstances. Importantly, the gap between the two pulses should be sufficiently long to avoid thermal injury to the protein. According to our experience, the circulation of a 2-s pulse with a 5-s gap at 225 W would be safe; however, the time of pulse and gap should be adjusted with the change in power.

It was reported that proteolysis could be efficiently avoided by adding a moderate enzyme inhibitor, such as PMSF, leupeptin, and pepstatin, during the cytolysis, affinity chromatography and size exclusion chromatography (SEC) periods<sup>[25]</sup>, which were repeated successfully in our lab. Nonetheless, it must be noted that no enzyme inhibitor should be added during the PSP cleavage period because the PSP would be disabled, and the digestion efficiency would be affected.

## REFERENCES

- [1] Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature*, 2003, 423(6937): 337–342.
- [2] Yu B, Wang CY. Osteoporosis: the result of an “Aged” bone microenvironment. *Trends Mol Med*, 2016, 22(8): 641–644.
- [3] Wada T, Nakashima T, Hiroshi N, et al. RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med*, 2006, 12(1): 17–25.
- [4] Anderson DM, Maraskovsky E, Billingsley WL, et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature*, 1997, 390(6656): 175–179.
- [5] Wong BR, Rho J, Arron J, et al. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J Biol Chem*, 1997, 272(40): 25190–25194.
- [6] Lacey DL, Timms E, Tan HL, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*, 1998, 93(2): 165–176.
- [7] Yasuda H, Shima N, Nakagawa N, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA*, 1998, 95(7): 3597–3602.
- [8] Lacey DL, Boyle WJ, Simonet WS, et al. Bench to bedside: elucidation of the OPG-RANK-RANKL pathway and the development of denosumab. *Nat Rev Drug Discov*, 2012, 11(5): 401–419.
- [9] Lomaga MA, Yeh WC, Sarosi I, et al. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev*, 1999, 13(8): 1015–1024.
- [10] Theill LE, Boyle WJ, Penninger JM, et al. RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu Rev Immunol*, 2002, 20: 795–823.
- [11] Liu CZ, Walter TS, Huang P, et al. Structural and functional insights of RANKL-RANK interaction and signaling. *J Immunol*, 2010, 184(12): 6910–6919.
- [12] Zhang SQ, Liu CZ, Huang P, et al. The affinity of human RANK binding to its ligand RANKL. *Arch Biochem Biophys*, 2009, 487(1): 49–53.
- [13] Walter TS, Liu CZ, Huang P, et al. Crystallization and preliminary X-ray analysis of mouse RANK and its complex with RANKL. *Acta Crystallogr Sect F Struct Biol Cryst Commun*, 2009, 65: 597–600.
- [14] Walsh MC, Choi Y. Biology of the RANKL-RANK-OPG system in immunity, bone, and beyond. *Front Immunol*, 2014, 5: 511.
- [15] Harrison RG, Bagajewicz MJ. Predicting the solubility of recombinant proteins in *Escherichia coli*. *Methods Mol Biol*, 2015, 1258: 403–408.
- [16] Gräslund S, Sagemark J, Berglund H, et al. The use of systematic N- and C-terminal deletions to promote production and structural studies of recombinant proteins. *Protein Expr Purif*, 2008, 58(2): 210–221.
- [17] Papanephytou CP, Rinotas V, Douni E, et al. A statistical approach for optimization of RANKL overexpression in *Escherichia coli*: purification and characterization of the protein. *Protein Expr Purif*, 2013, 90(1): 9–19.
- [18] Krauspe R, Scheer A. Coomassie brilliant Blue G-250 dye-binding technique for determination of autolytic protein breakdown in *Euglena gracilis* and comparison to other methods of autolysis

- measurement. *Anal Biochem*, 1986, 153(2): 242–250.
- [19] Liu CZ, Zhao YF, He W, et al. A RANKL mutant used as an inter-species vaccine for efficient immunotherapy of osteoporosis. *Sci Rep*, 2015, 5: 14150.
- [20] Castellanos-Mendoza A, Castro-Acosta RM, Olvera A, et al. Influence of pH control in the formation of inclusion bodies during production of recombinant sphingomyelinase-D in *Escherichia coli*. *Microb Cell Fact*, 2014, 13: 137.
- [21] Song JM, An YJ, Kang MH, et al. Cultivation at 6–10 °C is an effective strategy to overcome the insolubility of recombinant proteins in *Escherichia coli*. *Protein Expr Purif*, 2012, 82(2): 297–301.
- [22] Park SJ, Lee SH, Kim KJ, et al. Soluble expression and purification of receptor activator of nuclear factor-kappa B ligand using *Escherichia coli*. *J Microbiol Biotechnol*, 2015, 25(2): 274–279.
- [23] Willard D, Chen WJ, Barrett G, et al. Expression, purification, and characterization of the human receptor activator of NF- $\kappa$ B ligand (RANKL) extracellular domain. *Protein Expr Purif*, 2000, 20(1): 48–57.
- [24] Luan XD, Lu QY, Jiang YA, et al. Crystal structure of human RANKL complexed with its decoy receptor osteoprotegerin. *J Immunol*, 2012, 189(1): 245–252.
- [25] Harper S, Speicher DW. Expression and purification of GST fusion proteins. *Curr Protoc Protein Sci*, 2008, Chapter 6: Unit 6.6 (6.6.1–6.6.26).
- [26] Lam J, Nelson CA, Ross FR, et al. Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. *J Clin Invest*, 2001, 108(7): 971–979.
- [27] Ito S, Wakabayashi K, Ubukata O, et al. Crystal structure of the extracellular domain of mouse RANK ligand at 2.2-Å resolution. *J Biol Chem*, 2002, 277(8): 6631–6636.
- [28] Nelson CA, Warren JT, Wang MWH, et al. RANKL employs distinct binding modes to engage RANK and the osteoprotegerin decoy receptor. *Structure*, 2012, 20(11): 1971–1982.
- [29] Blount ZD. The unexhausted potential of *E. coli*. *eLife*, 2015, 4: e05826.
- [30] Baneyx F. Recombinant protein expression in *Escherichia coli*. *Curr Opin Biotechnol*, 1999, 10(5): 411–421.
- [31] Chen R. Bacterial expression systems for recombinant protein production: *E. coli* and beyond. *Biotechnol Adv*, 2012, 30(5): 1102–1107.
- [32] Baneyx F, Mujacic M. Recombinant protein folding and misfolding in *Escherichia coli*. *Nat Biotechnol*, 2004, 22(11): 1399–1408.
- [33] Sezonov G, Joseleau-Petit D, D’Ari R. *Escherichia coli* physiology in Luria-bertani broth. *J Bacteriol*, 2007, 189(23): 8746–8749.
- [34] Losen M, Frölich B, Pohl M, et al. Effect of oxygen limitation and medium composition on *Escherichia coli* fermentation in shake-flask cultures. *Biotechnol Prog*, 2004, 20(4): 1062–1068.
- [35] Leone S, Sannino F, Tutino ML, et al. Acetate: friend or foe? Efficient production of a sweet protein in *Escherichia coli* BL21 using acetate as a carbon source. *Microb Cell Fact*, 2015, 14: 106.
- [36] Jia BL, Jeon CO. High-throughput recombinant protein expression in *Escherichia coli*: current status and future perspectives. *Open Biol*, 2016, 6(8): 160196.
- [37] Bird PI, Pak SC, Worrall DM, et al. Production of recombinant serpins in *Escherichia coli*. *Methods*, 2004, 32(2): 169–176.
- [38] Sørensen HP, Mortensen KK. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microb Cell Fact*, 2005, 4: 1.

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