High RNA accumulation in *Candida tropicalis* is affected by specific growth rate and different medium composition

LIN Zhong[1], LUO Zhong-qiu[1], DOU Jie[1], WANG Hui[1], QIU Wei-ran[2], CAO Jing[2], ZHOU Chang-lin[1]*

[1] *School of Life Science and Technology, China Pharmaceutical University, Nanjing 210009;*

[2] *Nantong Qiuzhiyou Bioscience and Biotechnology Co. Ltd., Nantong 226236, China*

Abstract Batch and continuous fermentation were adopted to investigate the effect of specific growth rate and amino acid components on RNA accumulation in *Candida tropicalis* ATCC 20408 in fermentation medium (FM), yeast peptone dextrose medium (YPD), molasses fermentation medium (MFM) and FM without corn steep liquor. The data showed that obvious differences in intracellular RNA accumulation were observed at different cell growth phases in bath fermentation prosess, and RNA level reached 11.8% (g-RNA /g-DCW) during exponential phase, and only 6.9% during stationary phases. It was also found that intracellular RNA accumulation increased with the increase of specific growth rate in continue fermentation prosess, and the highest RNA level reached 15.6% with the glucose conversion rate of 42.8% at the dilution rate of 0.5 h^{-1}. Furthermore, the data showed that RNA lever was notably increased in batch fermentation process when amino acids or peptone was added into the fermentation medium containing no corn steep liquor. Taken together, it was reported for the first time that specific growth rate and amino acid components plays a leading role on the intracellular RNA accumulation in *C. tropicalis*, and specific growth rate is more important.

Key words RNA; *Candida tropicalis*; continuous fermentation; amino acids; specific growth rate

CLC Number Q815 **Document code** A **Article ID** 1000 - 5048(2011)02 - 0169 - 07

菌体比生长速率及不同培养基成分变化对解脂假丝酵母中 RNA 累积的影响

林 忠[1],罗众球[1],窦 洁[1],王 慧[1],邱蔚然[2],曹 静[2],周长林[1]*

([1]中国药科大学生命科学与技术学院,南京 210009;[2]南通秋之友生物科技有限公司,南通 226236)

摘 要 为了研究解脂假丝酵母 ATCC 20408 菌株比生长速率和培养基中氨基酸成分对酵母 RNA 累积的影响。采用分批和连续培养的方法,解脂假丝酵母分别在发酵培养基、YPD 培养基、糖蜜培养基以及不含玉米浆的成纤维细胞培养基(FM)中进行培养。结果表明,在分批培养的不同生长期,菌体细胞内 RNA 累积量有明显区别;在对数生长期时,RNA 含量达干重的 11.8%(g-RNA/g-DCW);稳定期时,RNA 含量仅为 6.9%。连续培养时,酵母 RNA 比生长速率越大,酵母 RNA 积累量越高,当细胞比生长速率为 0.5 h^{-1}时,RNA 含量可达 15.6%,糖的转化率为 42.8%。在不含玉米浆的 FM 培养基中加入不同的氨基酸组分进行有氧分批培养时,添加混合氨基酸或蛋白胨组分均可提高酵母细胞 RNA 的含量。本研究首次报道了高比生长速率和混合氨基酸组分对解脂假丝酵母细胞 RNA 的积累具有明显的促进作用,而比生长速率对酵母 RNA 的累积影响更大。

关键词 RNA;解脂假丝酵母;连续发酵;氨基酸;比生长速率

---

* Received date 2010-09-06 * Corresponding author Tel: 86 - 25 - 83271323 E-mail: cl_zhou@cpu.edu.cn

Foundation Item This project was sponsored by the Industrialization of Scientific Research Promotion Projects of Colleges and Universities in Jiangsu Province (No. JH10-12); the Qing-Lan Project of Educational Department of Jiangsu Province (2008); and the Natural Science Foundation of Jiangsu Province (No. SBK200921231)

Ribonucleic acid (RNA) is high molecular substance in organisms. Nucleotide and ribonucleotide are incomplete hydrolytic products of nucleic acid. Recently, plenty of data strongly suggest that exogenous nucleic acid including RNA, ribonucleotide, and nucleotide is an indispensable nutrient in plants, animals and humans under specific physiological conditions[1-2]. RNA biosynthesis is, in contrast to RNA metabolism, less conserved and totally absent in mammals such as animals. Yeasts, in particular, have shown to be highly active in RNA synthesis, yielding unusually high level per biomass.

During recent years, RNA and its hydrolytic products have drawn much of our attention because of many proposed benefits on health when used as food additives because it can promote infants intestinal tract function and fat metabolism, maintain livers normal function, strengthen learning and memory power, and strengthen human growth[3-4].

Data also strongly suggested that cytidine and uridine could be used as drug intermediates for the treatment of cancer, hepatitis and coronary heart disease[5-7].

However, it is not easy to reach the required levels by the diet only and new strategies have to be developed to increase the RNA production. It is very difficult to synthesize nucleotide and ribonucleotide artificially. Therefore, naturally produced RNA and its hydrolytic products seem to be more rational for fortification purposes. High producing strains should be selected for biotechnological production of natural RNA, and different yeast strains can be used for RNA accumulation. Mutational *Candida tropicalis* (ATCC 20005) has high RNA content ranging from 10 g to 11 g per 100 g dry cell[8-9].

Model bacteria have shown that RNA content was highly correlated to growth rate, e. g. in *Escherichia coli* and *Salmonella typhimurium*, stable RNA cell content increases by one order of magnitude with a four to five-fold increase in growth rate[10]. The regression of (RNA: DNA)/RNA_{max}: DNA_{max} on μ was highly significant ($r^2 = 0.76$ for data pooled across four isolates) perhaps reflecting an underlying common relationship between RNA content and growth rate[11].

Data also strongly suggested that the RNA content of *Aerobacter aerogenes* organism varied with the potassium content[12]. The higher protein and RNA content of the cells due to the more rapid growth rate of *Ceratocystis ulmi* with arginine as the nitrogen source had been confirmed by Kulkarni *et al*[13].

Here, we found for the first time that high RNA accumulation in *Candida tropicalis* was notably affected by specific growth rate and medium composition and RNA content reached 15.6% which was 42% higher than reported before. This is of considerable importance in the development of a production process for yeast RNA and for increasing RNA accumulation in yeast-fermented food.

1 Materials and Methods

1.1 *Microorganisms and medium compositions*

C. tropicalis (ATCC 20007, ATCC 20408) were from American Type Culture Collection (ATCC); *Saccharomyces. cerevisiae* (CGMCC 1012, CGMCC 1226) were from China General Microbiological Culture Collection Center (CGMCC); *Pichia pastoris* was kept in our lab. All the microorganisms were maintained in 15% glycerol (wt/vol) at −80 °C.

For fermentation, the following culturing media were used. YPD, a complex yeast medium consisting of (per liter) yeast extract, 10 g; peptone, 20 g and glucose, 50 g at pH 4.0. Fermentation medium (FM) was prepared as described (per liter): glucose, 50 g; $(NH_4)_2SO_4$, 5 g; $MgSO_4 \cdot 7H_2O$, 1 g; $ZnSO_4 \cdot 7H_2O$, 20 mg; $CaCl_2 \cdot 2H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 20 mg; H_3PO_4, 3 mL; KCl, 1.5 g; NaCl, 1 g; yeast extract, 3 g; corn steep liquor, 3 g; at pH 4.0, for the batch and continuous fermentation. FM medium did not contain corn steep liquor in order to eliminate the effect of amino acids on corn steep liquor. Molasses fermentation medium (MFM) was prepared with raw molasses from sugar beets, in which saccharose content is 68 g/L, supplemented with (per liter), $(NH_4)_2SO_4$, 5 g; $MgSO_4 \cdot 7H_2O$, 12 g; $ZnSO_4 \cdot 7H_2O$, 20 mg; $FeSO_4 \cdot 7H_2O$, 20 mg; NaCl, 1 g; H_3PO_4, 3 mL, adjusted pH 4.0 with sodium hydroxide.

For the continuous fermentation, the initial glucose concentration in FM and in feed was 50 g/L during batch and continuous phases.

For experiments in flasks, 50 mL of the different medium was used. To study the effect of specific compounds in peptone on *C. tropicalis* RNA accumulation, different components were added to FM without corn steep liquor at the same concentration (per liter) as in 20 g peptone (according to Difco, Becton Dickinson, USA); methionine, 0.2 g; histidine, 0.1 g; glutamic acid, 2 g; glycine, 3.1 g; and serine, 0.6 g. One

experiment, in which all amino acids at described concentrations were added to FM medium without corn steep liquor, was also performed. Antifoam agent DF-104 (0.1 mL) was added to 1 L medium.

1.2 *Growth conditions*

Cells were precultured on YPD plates, containing agar, 20 g/L, for 24 h. Inoculation cultures were prepared in 250 mL shake flasks containing 50 mL of the same medium as used for the experiment (YPD, FM, FM without corn steep liquor or MFM) inoculated with one colony from the fresh plates and grown at 30 °C on a rotating shaker, 200 r/min for 12 h.

For batch fermentation, 15 L fermentor (BIOSTAT®C, B. Braun Biotech, Germany) was used at a working volume of 10 L. The temperature was kept constant at 30 °C and pH was adjusted to 4.0, maintained by automatic addition of 0.5 mol/L ammonia solution. The dissolved oxygen was set to 30% of saturation by stirring rate and air flow.

The termertation coudition were maintained as batch fermentation stated above. The feed of FM medium started immediately after the cells had finished the carbon source. Dilution rates varied from 0.3 h^{-1} to 0.7 h^{-1}. After three residence times, it was assumed that the culture had reached stability, which was confirmed with green weight measurements and stable oxygen concentration levels.

1.3 *Determination of green weight, dry weight, residual glucose and glucose conversion rate*

Green weight was measured by centrifugation of 5 mL of culture broth for 10 min at 8 000 r/min. Then the cells were dried for 24 h at 110 °C and cooled in a desiccator prior to weighing.

Residual glucose was determined using the 3, 5-dinitrosalicylic acid (DNS) method[14].

1.4 *Determination of intracellular RNA*

Samples were centrifuged at 8 000 r/min at 4 °C and washed once with saline water. Analysis of RNA in the yeast cells was performed by an ultraviolet spectrophotometricmethod[15]. 10 mL of zymotic fluid samples were centrifuged at 8 000 r/min, 10 min at 4 °C, the supernatant was removed and the cells were blended with 10 mL of saline water and boiled for 20 min in 70 °C shaking water bath. 1 mL of mixture above was centrifuged at 8 000 r/min, 4 °C for 10 min, and 100 μL of supernatant was added into 4.9 mL of distilled water, diluted to 50 times. The RNA accumulation was quantified with UV detection at 260 nm and was expressed as RNA percent in 100 g dry yeast cells. The samples were analyzed in triplicate.

1.5 *Determination of special growth rate (μ)*

Determination of special growth rate (μ) according to Monod equation model which was proposed to describe microbial growth in batch cultures[16], defined as follows:

$$\mu = \frac{1}{X} \cdot \frac{dX}{dt} \quad (1)$$

In which, dX is the increased biomass, dt is time of process and X is biomass. The dilution rate (D) was calculated as the inlet medium flow rate divided by the total liquid volume in the bioreactor and the filter unit. D was changed from 0.3 h^{-1} to 0.7 h^{-1} in continuous fermentation. And the process of continuous fermentation conformed to Monod equation.

2 Results

2.1 *RNA accumulation during batch fermentation in FM medium*

The data on RNA accumulation in different yeast strains during batch fermentation in FM medium are presented in Figure 1A. The results showed that the highest RNA accumulation was observed in *C. tropicalis* ATCC 20408, which reached approximately 11.8% and the lowest (4.6%) in *S. cerevisiae* CGMCC 1012, and *Pichia pastoris* reached the middle level of 7.4%. So *C. tropicalis* selected for RNA accumulation investigated not only batch fermentation but also continuous fermentation.

The data on RNA accumulation in *C. tropicalis* during batch fermentation in FM are presented in Figure 1B. Large variations in RNA accumulation at different growth phases were observed. During the first 10 hours, in the exponential phase of growth, the RNA accumulation was at the stable level of 12.2% . When glucose was depleted and the cells entered to the stationary growth phase, the RNA accumulation rapidly decreased. These results showed that RNA accumulation in *C. tropicalis* was closely related to glucose degrading. The specific growth rate began to decrease rapidly from 0.5 h^{-1} to 0.01 h^{-1} during this phase, the highest level of specific growth rate reached 0.5 h^{-1} at the early exponential phase. It was also observed that cells began to lyse after 16 h cultiva-

tion, and low intracellular RNA accumulation observed during the stationary phase may, to some extent, be due to the decrease of specific growth rate.

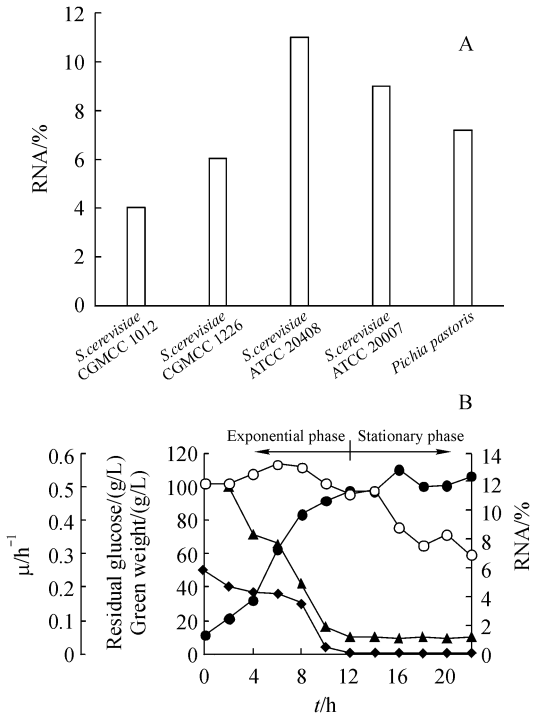

Figure 1 (A) RNA level in different yeast strains cultured in fermentation medium for 12 hours; (B) Growth, specific growth rate and ribonucleic acid content and residual glucose during batch fermentation of *C. tropicalis* ATCC 20408 in fermentation medium. Specific growth rate is calculated by Monod equation

2.2 *RNA accumulation in continuous fermentation*

Specific growth rate plays an important role in RNA accumulation in *C. tropicalis*. It was found that RNA accumulation was increased to 15.6% when specific growth rate was changed from 0.3 h^{-1} to 0.5 h^{-1} in continuous process. The results are shown in Figure 2. It was also found that RNA accumulation increased linearly with increasing growth rate (Figure 2, C), The glucose conversion rate can keep stable level of 42.8% during the entire process of continuous fermentation when dilution rate was from 0.3 h^{-1} to 0.7 h^{-1} (Figure 2, B). When dilution rate started from 0.3 h^{-1}, the RNA accumulation also began to increase and the green weight was kept at 115.6 g/L. However, the RNA accumulation and the cell green weight started to decrease rapidly (Figure 2-A), and the residual glucose increased rapidly at dilution rate of 0.6 h^{-1} (Figure 2, B). It indicated that the critical dilution rate was 0.5 h^{-1}, confirming the results from batch experiment in FM medium (Figure 1).

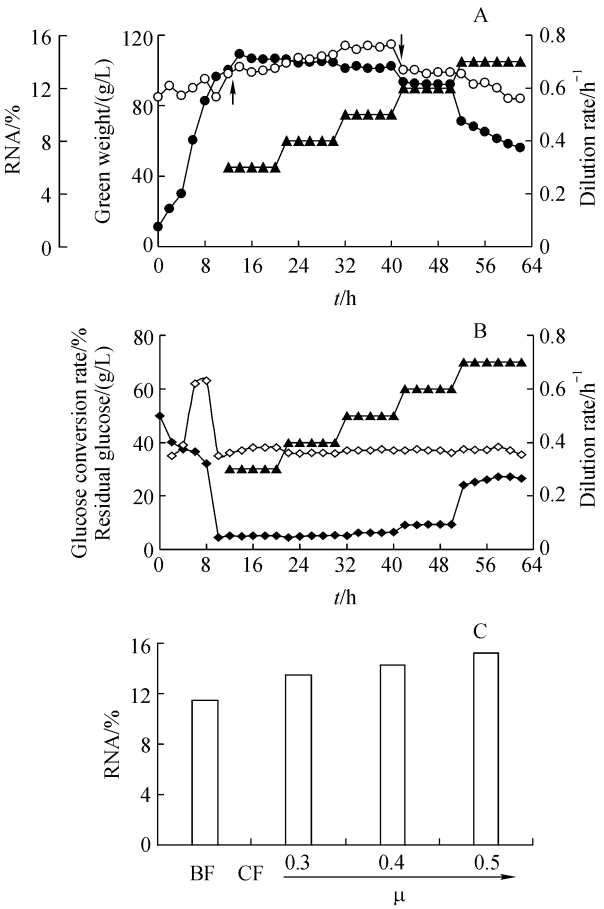

Figure 2 (A) Growth and ribonucleic acid content during continuous fermentation of *C. tropicalis* ATCC 20408 in fermentation medium; (B) Residual glucose and glucose conversion rate during continuous fermentation of *C. tropicalis* ATCC 20408 in fermentation medium. Glucose conversion rate is the ratio of *C. tropicalis* dry cell weight (g/L) to glucose consumption (g/L); (C) Ribonucleic acid content in batch fermentation (BF) and at different growth rates in a continuous fermentation (CF)

2.3 *Comparison of RNA accumulation during batch fermentation in FM medium without corn steep liquor, YPD or MFM medium*

RNA accumulation was directly related to growth rate as described above, but growth medium composition also played an important role on RNA accumulation and cell growth. FM medium without corn steep liquor, YPD and MFM medium were investigated in batch fermentation in *C. tropicalis* (Figure 3). Compared to FM medium without corn steep liquor, RNA accumulation in YPD medium was increased 90% and reached the RNA level of 12%. In MFM medium, RNA accumulation was increased 60% and reached the RNA level of 9.5%. Moreover, YPD and MFM

medium were favorable for yeast growth and increased green weight. The results showed that YPD medium have some positive factors on RNA accumulation.

2.4 *Effect of amino acid and peptone composition on RNA accumulation*

To elucidate the effect of components in YPD media on RNA accumulation, experiments in flasks were performed. Based on our understanding of cellular RNA accumulation, some components present in YPD that might be involved in increasing the RNA accumulation were added to FM medium without corn steep liquor. However, YPD medium only had more peptone compared to FM medium without corn steep liquor and MFM medium, a 90% increase in RNA accumulation was observed (Figure 3, A), showing that one or more components in peptone were involved in increasing the cellular RNA accumulation.

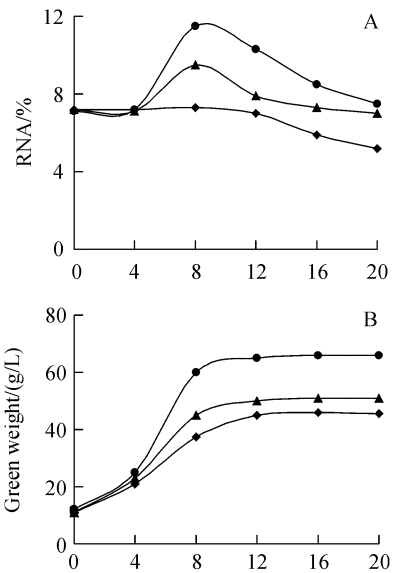

Figure 3 Ribonucleic acid content (A) and growth (B) during controlled batch fer mentation of *C. tropicalis* ATCC 20408 in YPD, MFM and FM without corn steep liquor

The next experiment was designed to elucidate which components in peptone gave the increased RNA accumulation in *C. tropicalis* (Figure 4, A). Components in peptone tested were methionine, glutamine, serine, histidine, glycine and a mix of them. Increase in RNA levels was observed when glutamine (42%), serine (56%), methionine (45%), glycine (18%) or mix of amino acids (85%) were present, whereas histidine decreased RNA levels by 16%. Green weight was decreased and RNA accumulation was increased. Mixture of amino acids increased RNA level to the same extent as peptone. Thus amino acids or a combination of several factors in peptone, as well as in molasses, yield dramatic increasing effect on *C. tropicalis* RNA accumulation, which would be investigated in future studies.

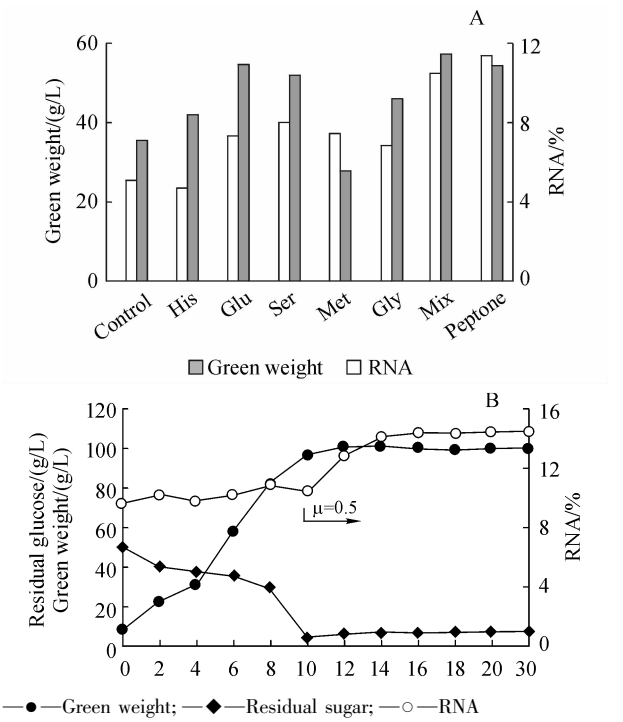

Figure 4 (A) Ribonucleic acid content in *C. tropicalis* ATCC 20408 cultured in corn liquor deprived medium supplemented with peptone, histidine, glutamine, glycine, serine or methionine and mix of them. Control means no supplementation. Cells were harvested after 12 h cultivation; (B) Growth, residual glucose, RNA level of *C. tropicalis* ATCC 20408 strain cultured in YPD medium in continuous fermentation when dilution rate was 0.5 h^{-1}

Our experiment was also designed to elucidate whether *C. tropicalis* cultured in FM and YPD medium have any difference about RNA accumulation in continuous fermentation. The data showed that the RNA accumulation and green weight have little difference between FM and YPD medium at the dilution rate of 0.5 h^{-1} (Figure 4, B; Figure 2). So we concluded from data above that, specific growth rate plays the leading role in continuous fermentation in contrast to amino acids.

3 Discussion

In the present study, we investigated the influence of fermentation media and specific growth rate on RNA accumulation in *C. tropicalis*. The RNA accumu-

lation started to increase immediately after inoculation and a fairly stable level was rapidly reached. Highest RNA level was found when the cells were fermentatively degrading glucose, and the growth rate was highest ($\mu_{max} = 0.5\ h^{-1}$, Figure 1, B) during this phase.

A rapid drop in RNA level was observed when the glucose was consumed mostly and the stationary growth phase started. This was probably due to the cells concentration which was kept constant and RNA biosynthesis productivity which was decreased during this phase.

The RNA level, increased rapidly during the exponential phase reflecting a high net RNA biosynthesis. To some extent, the RNA biosynthesis may have been distributed to the newly formed cells earlier during the phase. The data obtained from our research showed that the amount of RNA presented in a yeast cell seemed highly correlated with specific growth rate. However, the growth conditions are continuously changing during batch fermentation process. A chemostat for concluding evidence is needed[17].

Thus, it seemed possiblly to regulate specific RNA accumulation in the cells by controlling specific growth rate. It was also found that, compared with amino acids composition, specific growth rate was the dominating determiner for RNA accumulation in *C. tropicalis*, because continuous fermentation in YPD ($D = 0.5\ h^{-1}$) would not yield even higher RNA accumulation (Figure 4, B).

Since yeast can utilize amino acids from its surroundings, endogenous biosynthesis may be expected to increase when amino acids were added to FM without corn steep liquor, which was deprived from FM medium in order to eliminate the effect of amino acids. The exact composition of molasses is also unknown, but since it is a product from sugar beets, i. e. a plant source, it most likely contains amino acids, too. Molasses was usually used because it is one of the favored media for the production of Baker's yeast in the yeast manufacturing industry. So we focused our attention on investigating the effects of adding amino acids[18].

The RNA level was increased by 90% when yeast cells were cultured in FM medium without corn steep liquor in the presence of peptone. Peptone is a mixture of free amino acids and peptides which obviously affects RNA accumulation to a large extent. Amino acids, tightly involved in the RNA metabolism or as growth factors, are methionine, serine, glycine, glutamine and histidine. RNA accumulation was increased when a mixture of them or methionine, glycine, glutamine, serine was separately added to FM medium without corn steep liquor, but the effectiveness was not as large as peptone added.

We may conclude that RNA accumulation in *C. tropicalis* varies extensively depending on medium composition, specific growth rate and different growth phases. Amino acids (except histidine) are favorable to RNA accumulation. Furthermore, YPD is a favorable medium for RNA accumulation directly used as food additives in batch fermentation. However, specific growth rate plays a leading role in continuous fermentation. Therefore, *C. tropicalis* seems to be a good alternative to RNA accumulation. High RNA level through controlling medium composition and specific growth rate.

Reference

[1] Grimble GK. Why are dietary nucleotides essential nutrients[J]. *Br J Nutr*, 1996, **76**(4): 475 – 478.

[2] Jane DC, Allan WW. The role of nucleotides in human nutrition [J]. *J Nut Biochem*, 1995, **6**(2): 58 – 71.

[3] Rudolph FB, Van Buren CT. The metabolic effects of enterally administered ribonucleic acids[J]. *Curr Opin Clin Nutr Metab Care*, 1998, **1**(6): 527 – 530.

[4] Choudhury D, Pal AK, Kumar S, *et al*. Dietary yeast RNA supplementation reduces mortality by Aeromonas hydrophila in rohu juveniles[J]. *Fish Shellfish Immunol*, 2005, **19**(3): 281 – 291.

[5] Kiichiro K, Maruyama H, Kikuchi S, *et a*1. Suppression of lipopolysaccharide-Induced tumor necrosis factor-release and liver injury in mice by naringin[J]. *Eur J Pharmacol*, 1999, **368**(2): 245 – 250.

[6] Lu SC, Mato JM. *S*-Adenosylmethionine in cell growth, apoptosis and liver cancer[J]. *J Gastroenterol Hepatol*, 2008, **23**(1): 73 – 77.

[7] Fioravanti M, Buckey AE. Citicoline in the treatment of cognitive impairment[J]. *Clin Interv Aging*, 2006, **1**(3): 247 – 251.

[8] Yoshio N, Ibaraki, Isao B, *et al*. Process for preparing yeast cells containing an enhanced amount of ribonucleicacid: US, 3411989 [P]. 1968-11-19.

[9] Shinji Y, Yamashita, Yaizu, *et al*. Ribonucleic acid-enriched brewer's yeast cells and process for producing the same: US, 7135327 B2[P]. 2006-11-14.

[10] Bremer H, Dennis PP. *Modulation of chemical composition and other parameters of the cell by growth rate*: *cellular and molecular biology*[M]. Washington DC: ASM Press, 1996: 1 553 – 1 569.

[11] Kemp PF, Lee S, Laroche J. Estimating the growth rate of slowly growing marine bacteria from RNA content [J] *Appl Environ Microbiol*, 1993, **59**(8): 2 594 – 2 601.

[12] Tempest DW, Meers JL. The influence of NaCl concentration of the medium on the potassium content of aerobacter aerogenes and on the inter-relationships between potassium, magnesium and ribonucleic acid in the growing bacteria[J]. *J Gen Microbiol*, 1968, **54**(3): 319 – 325.

[13] Kulkarni RK, Nickerson KW. Nutritiohal control of dimorphism in *Ceratocystis ulmi*[J]. *Exp Mycol*, 1981, **5**(2): 148 – 154.

[14] Bertolini MC, Erlandes JR, Laluse C, *et al*. New yeast strains for alcoholic fermentation of high sugar concentration[J]. *Biotechnol Bioeng*, 1991, **13**(3): 197 – 202.

[15] Ohta S, MauL S, Maul AJ, *et al*. Characterization of a heat-shock process for reduction of the nucleic acid content of *Candida utiLis* [J]. *Appl Microbiol*, 1971, **22**(3): 415 – 421.

[16] Liu Y, Liu QS, Tay JH, *et al*. Initial conditions-dependent growth kinetics in microbial batch culture[J]. *Process Biochem*, 2005, **40**(1): 155 – 160.

[17] Jin W, Fang F, Han Qing Y, *et al*. Substrate consumption and biomass growth of *Ralstonia eutropha* at various S_o/X_o levels in batch cultures[J]. *Bioresour Technol*, 2007, **98**(13): 2 599 – 2 604.

[18] Sofia H, Johan P, Thomas A, *et al*. Growth rate and medium composition strongly affect folate content in *Saccharomyces cerevisiae* [J]. *Int J Food Microbiol*, 2008, **123**(1): 93 – 100.