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Purification of (S)-2-methyl-1,4,5, 6-tetrahydropyrimidine-4 carboxylic acid from Halophiles.

Chen Rui-Feng[1,2], Lv Li-Huo[2], Yao Su[2], Zhu Li-Jun[2], Li Bin[2], Qian Jun-Qing[1*]

[1]Zhejiang University of Technology, Zhejiang Province, 310014, PR China

[2]Zhejiang Apeloa Jiayuan Pharmaceutical Co., Ltd, Zhejiang Province, 322118, PR China

Abstract

Aim: This paper is focused on the techniques of fermentation, separation and purification of (S)-2-methyl-1,4,5, 6-tetrahydropyrimidine-4 carboxylic acid generated by *Halomonas elogate* 1A01717.
Methods: This study take *Halomonas elogate* 1A01717 to ferment to produce a total of 97.05 Kg fermented liquid, and carry out purification process including ceramic membrane microfiltration, electrodialysis desalination, diatomaceous earth filter, upper column, crude extraction and refining.
Results: Three experimental processes are stable, getting total Ectoine 634.7 g, total yield: 45.17%. The yield of 6.04 g/L is calculated according to material coefficient 70%, 35 L of 50 L fermentation canned. Finally, the products getting in the three tests quality conforms to the standard.
Conclusion: This process can be used for a large-scale production and purification of ectoine after further optimization and adjustment.

Keywords: Halomonas elongate, Fermentation, Purification, Ectoine.

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Introduction

Microbes in highly saline environment must have mechanism avoiding moisture loss by resisting osmotic pressure, and adapt to osmolality need the cytoplasm has the capacity to produce the same osmolality as the surrounding matrix. On the one hand, cell confront extracellular hypertonic environment through accumulation of K+ and pump out Na+ by Na+/K+ reverse transportation system or purple membrane light driven H+ proton on cell membrane; on the other hand, halophilic microorganisms can produce or accumulation low molecular weight osmotic soluble substances (compatible solute) to hold sensitive area of intracellular protein molecules and stabilize combined water on biological macromolecular surface. The main characteristic of this kind of small molecules is: it usually does not participate in intracellular center cycle metabolism, even which can be accumulated to a high intracellular concentration. Therefore it is called compatible solute. The accumulation of the solute could help cell to maintain cell multiplication elements, such as osmotic balance, cell volume and electrolyte concentration [1].

Many compatible solutes had been discovered, such as glycine betaine, pyrimidine and its derivatives, amino acids and its derivatives, sugar and its derivatives [2,3]. Ectoines class compatible solutes (Ecs) is one of the most common compatible solutes in halophilic bacteria, which could provide protection for cell, protein, cell membranes and nucleic acid to outside extreme conditions stimulation, for example high temperature, freezing, radiation, drying, therefore Esc received extensive attention of the researchers from all over the world. Esc have been found so far in the organism include (S) -2-methyl-1, 4, 5, 6-tetrahydropyrimidine- 4-carboxylic acid, ectoine (Ec) and (S,S) -2-methy-l-5-hydroxyl -1,4,5,6 -tetrahydropyrimidine -4-carboxylic acid, Hydroxyectoine (HE).

Structure characteristic

From the point of view of structure, Ectoine is cyclic amino acid derivatives, which can't reflect unique molecular characteristics of cyclic amino acids for its intramolecular electron delocalization. Ec ($C_6H_{10}N_2O_2$, CAS: 96702-03-3) and HE are hydrophilic and facultative ion biological small molecules. Ec is inert in biology and does not interfere in most of intracellular enzyme and binding reaction. Ec has intracellular high tolerance (1 M), thermal stability and not easy to be decomposed. Ec is highly water-soluble, without static charges, with intracellular balance ability of improve osmotic pressure by accumulation of high concentrations.

Biological function

In *Sinorhizobium meliloti*, Ec induced a series of the expression of genes related to osmotic pressure in the break down, which makes it synthesize its compatible solute trehalose and glutamate etc. [4]; Schnoor et al. [5] found that protection of Ec for DNA and its huge potential of application in PCR technology. Ec could reduce the PCR reaction temperature by reducing the dependence of double-stranded

DNA melting temperature on the base constitute, stabilize polymerase enzyme activity under the high temperature condition, and also could combine with DNA to make DNA structure cannot be cut by restriction enzyme. Thus Ec greatly improve the amplification efficiency. Ec is especially suitable for amplification trace or difficult DNA fragments in clinical and criminal detection; Ec could play a role as chaperone to identify misfolding protein and suppress the formation of protein polymer, protect cells from harm of toxic inclusion body, reduce the phenomenon of apoptosis death [6]; New study has found that Ec could improve the regeneration ability of skin and delay the aging of skin [7,8], which can be used as the sun screener and moisturizing agent to prevent the skin dry, aging and sunburn, etc. In addition, Ec has certain curative effect for neurological diseases such as Alzheimer's disease, Parkinson's disease and so on. Therefore, Ec will have broad application prospects in such fields as pharmaceutical, food, cosmetics, biological agents, enzymes, agricultural, chemical synthetic drug and organic electronic materials [9-11].

Identification method

The molecular structure, physical and chemical characteristics of Ec and HE are very similar, so it is difficult to identify. Galinski et al. [12] firstly detected Ec by nuclear magnetic resonance (NMR) and mass spectrometry method, and identified the molecular structure using isotope labeling and Nuclear Magnetic Resonance (NMR) method, qualitative to quantitative analysis of Ec usually use High Performance Liquid Chromatography (HPLC) in research in recent years. Nagata et al. [13] and Eshinimaev et al. [14] did qualitative and quantitative analysis of Ec synthesized by *Brevibacterium* sp. JCM 6894, *Methylobacter marinus* 7 C and *Methylomicrobium alcaliphilum* 5 s respectively with HPLC and ultraviolet detector, the results show the maximum absorption peak appear at 220-230 nm uv wavelength, and Ec could be accurately quantified to concentration of 10 mg/L through comparison with standards. Kunte et al. [15] can nimbly detect trace amounts of Ec using FMOC pre-column derivatization HPLC method. Even the single plaque synthesis of Ec on agar plate can be analyzed by this method. In addition to the HPLC method, anion exchange chromatography pulse amp method also could be used to determine the most compatible solutes including Ec, but sample processing of which is more complex [16]. The molecular structure of Ec is usually analysed by nuclear magnetic resonance (NMR), Nagata et al. [17], Zhu et al. [18] identified Ec and HE structure of salt-tolerant bacterium *Brevibacterium* sp. JCM 6894 and moderate Halomonas ventosae DL7 with NMR hydrogen spectrum and carbon spectrum analysis. Current most common and convenient method of detection and identification of compatible solutes are mainly HPLC and NMR methods.

Synthetic pathway

HE, as one of the important types of compatible solutes, exists widely in chemical and heterotrophic aerobic, anti-salt or moderate halophilic bacteria. Ec is primary compatible solutes in some extreme halophilic bacteria, most moderate halophilic bacteria and some anti-salt bacteria [19]. Especially almost all species of Halomonas genus could synthetize Ec as the main cell compatible solute [20,21]. Since it has chiral model, it is difficult to use chemical method to synthetize. Currently, it is generated by halophilic bacteria fermentation, then make HE release by osmosis from intracellular into extracellular, which is extracted for HE products. Ec synthesis pathway has thorough research at the genetic level and enzyme levels, the synthesis is regulated by three enzymes coding by three gene (*ect A*, *ect B* and *ect C*) [22]; HE is synthetized by hydroxylation enzyme encoding by *ect D* or *thp D* gene (*ect*) [23]. Peters et al. [24] firstly expounded the synthesis pathway of Ec in *Ectothiorhodospira halochloris* and *Halomonas elongata* [25], which as follows: Oxaloacetic acid → Aspartic acid → Aspartic acid -β-semialdehyde → L - 2, 4-2 aminobutyric acid → Nγ - acetyl 2 aminobutyric acid → HE.

Material

Strains

Halomonas elogate 1A01717 (Marine Culture Collection of China (MCCC) provide)

Medium

MG medium: L - glutamic acid sodium (Sigma) 3.74%, potassium hydrogen phosphate (Sigma) 1.18%, potassium dihydrogen phosphate (Sigma) 0.30%, manganese sulfate (Sigma) 0.001%, magnesium sulfate (Sigma) 0.04%, Select Yeast Extract 0.10%, NaCl 5.85%, pH7.0.

Reagent

TB phosphate buffer: Mix potassium hydrogen phosphate (Sigma) 164.3 g/L, potassium dihydrogen phosphate (Sigma) 23.1 g/L and basal culture medium with proportion of 1:9.

TES solution: Dissolve 0.5844 g NaCl (Sigma) in 80 ml double distilled water, add 1 ml of 0.5 mol/l EDTA (Sigma), 0.2 ml Tris - HCl (pH=8.0) (Sigma), add water to volume of 100 ml.

KS30 salinity regulating solution: Potassium chloride 20 g, Sodium chloride 3000 g, Magnesium sulfate heptahydrate130 g, calcium chloride dihydrate 0.2 g, iron sulfate heptahydrate 0.2 g and TES 20 ml.

Ceramic membrane, diatomite, dow resin, sulfuric acid (Sigma), methanol (Sigma)

Equipment

Fermentation tank, ceramic membrane circulating tank, electrodialyzer

Steps

Preparatory work: Correct pH and DO electrode according to the standard calibration procedure (see fermentation equipment manual).

Prepare stronger ammonia water needed in the fermentation process, prepare 3 linter mass bottle (including flow pipe, gauze, kraft paper and silicone tube), sterilize for 20 min at 115°C ±1°C, add stronger ammonia water in sterilized inter mass bottle, let stand for 2 h. Set aside. Sterilize defoaming agent and ammonia water.

Prepare inter mass medium and KS30 according to culture medium formula, sterilize in an autoclave for 20 min at 115°C ± 1°C, natural cooling to reserve.

Preparation of medium and sterilization: Measure about 22.5 L tap water after insert of pH and dissolved oxygen electrode, dissolve the formula composition and pour in fermentation tank, adjust pH to 7.2 ± 0.1 with liquid alkali, sterilize in an autoclave for 20 min at 121°C ± 1°C, then cool to 25°C ± 1°C, save at constant temperature. Control volume of culture medium in 25 ± 0.5 L after sterilization cooling.

Control of fermentation

Seed culture: Inoculate sterile strain 1A01717 in MG medium, shaking culture about 24 h under the condition of 25°C and 200 rpm. Detect thallus status.

Fermentation: Take moderate halophilic bacterium seed liquid (1% volume of culture medium) and inoculate into fermented liquid of fermentation tank by sterile operation. Culture conditions are as follows: Temperature: 28°C, throughput: 0.8 L/min, rotation speed: 200 rpm; tank pressure: 0.03 MPa. Increase rotation speed when dissolved oxygen is lower than 30%, the highest rotation speed does not exceed 800 rpm. pH: whole-process control pH 7.5 ± 0.1. Starting tank pressure is 0.03 MPa, increase the dissolved oxygen by raising the tank pressure after rotation speed reach 800 RPM, but the highest tank pressure cannot be more than 0.05 MPa.

Began inter mass and KS30 regulating fluid salinity at the same time (speed slightly faster than the inter mass medium) when dissolve oxygen rises quickly, keep sugar concentration at 1 ± 0.5 g/L, adjust the inter mass speed according to the dissolved oxygen.

When OD value reach 90 around, stop adding KS30, adding solid NaCl and take sample every two hours and test OD and content, stop fermentation when content of Ectoine reach 15 g/L tank, control impurity (Hydroxyectoine content/Ectoine content) ≤ 5% in the process. Control total salt concentration about 15%, continue to ferment for 2 h after salt concentration reach 15%, then finish the fermentation.

Note: Fermentation temperature could not be too high; otherwise it will lead to a dramatic increase of HE, affecting product quality. It is best to pass film immediately after finish fermentation. Refrigerated storage temperature cannot be lower than 5°C, as too low temperature could cause cell broken and Ectoine lossing with supernatant after released from cell will affect yield.

Analysis of fermentation samples: Sugar concentration of fermentation liquid: Ferment for 10 h, sampling and lead to QC to test glucose content. OD of fermentation liquor: Sample and test absorbance value under 600 nm according to the requirements of fermentation control. When OD ≥ 50, sample and test content each 4 h.

Pretreatment of measure Ec content in fermentation liquor: Take 1.5 ml fermentation liquor to high-speed centrifuge for 3 to 5 mins, move supernatant, add water to 1.5 ml, let stand for more than 30 minutes after mixing, high-speed centrifuge for 3 to 5 mins, take supernatant to test.

Ceramic membrane microfiltration: It is best to pass film immediately in ceramic membrane cycle tank after finish fermentation. Control temperature 10°C-20°C in process of passing membrane, stop filtering after dialysis of fermentation supernatant 2/3, discharge the fermentation supernatant into the sewage treatment system, heat the concentrated solution to denature. The temperature is raised to 90°C- 95°C, keep constant temperature for 1 h. Continue passing ceramic membrane after cooling to below 50°C, add purified water to wash after filtering to the minimum volume, wash to Ectoine content in residue: ≤ 2 g/L, dialyze volume to 1-1.5 times of fermentation liquor volume.

Note: Control temperature 10°C-20°C when firstly pass ceramic membrane to dialyze fermentation clear liquid, too high temperature can lead to Ectoine released from cell, lossing along with the fermentation supernatant and affecting the yield.

Electrodialysis desalination: Adjust ceramic membrane dialysis fluid pH 5.5, pump feed liquid into the electrodialyzer, then do electrodialysis, Control material fluid level and strong water level consistent in electrodialysis process, control pH 5.5, finish electrodialysis when the feed liquid conductivity dropped to below 4000 us/cm.

Note: feed liquid level and strong water level should be consistent as far as possible, control pH 5.5 around in the process; otherwise it will increase loss of Eetoine in thick water.

Diatomite filtration: Add 0.05% EDTA disodium salt into the electrodialysis desalination feed liquid, stir well, adjust pH 2.0 ± 0.2 mm with sulfuric acid, then a small amount of floccule is separated out, add 0.5% volume of feed liquid diatomite filtration .

Upper column: Put filtrate to pass Dow resin regenerated with sulfuric acid at flow speed of 1.5-2 BV/hr, upper column amount is 75-75 g per 1 L wet resin. Wash impurity mixed with 3 - 4 times of resin volume acid water (pH 1.5) at 1.5-2 BV/hr flow speed after the end of upper column. After pickling, wash column with purified water, at flow speed 3 BV/hr, wash to pH 3.0-3.5. Water quantity is about 10 times of resin volume. After water washing, elute with 0.2 mol/L sodium hydroxide with elution speed of 1.5-2 BV/hr, stop collecting when collecting buffer pH above 8.0.

Note: Drying rate of coarse product reach more than 97%, too low would increase Ectoine solubility in the methanol, affecting yield.

Crude extract: Adjust Ectoine eluent pH 7.0 using sulfuric acid, concentrate at 85°C, decolor for 30 min at 60°C-85°C with 0.2% volume of eluent of activated carbon after concentrating to 1/5 volume, filter, concentrate the filtrate to dry crude products.

Note: Calculate methanol amount accurately. Too much methanol can lead to more digestion impurities, affecting the product quality, Too less methanol make Ectoine not completely dissolved, affecting the yield.

First refinement: Take crude products, calculate methanol addition according to the Ectoine solubility in methanol (11.36 g/100 g) , stir and reflux for 2 h at 60°C after adding methanol, immediately filter, top wash filter cake in 1 times weight of methanol, concentrate filtrate at 70°C-80°C to 30%-35% Ectione concentration, cool below 4°C and crystallize, centrifuge for first refined wet product.

Note: Drying to the best high, too low will affect the product quality.

Second refinement: Take first refined wet product, calculate methanol addition according to the Ectoine solubility in methanol (10 g/100 g), heat to 60°C to dissolve, decolor for 30 min with 2% of wet mass weight activated carbon, then filter, wash carbon with methanol of 1 times weight of filter cake, concentrate filtrate at 70°C-80°C to 25%-30% Ectione concentration, cool below 4°C, centrifuge for second refined wet product.

Note: Drying to the best high, too low will affect the product quality.

Third refinement: Take second refined wet product, calculate methanol addition according to the Ectoine solubility in methanol (10 g/100 g), heat to 60°C to dissolve, then filter, concentrate filtrate at 70°C- 80°C to 25%-30% Ectione concentration, cool below 4°C, centrifuge for third refined wet product. Vacuum dry wet product at 70°C-75°C for Ectoine finished product.

Stock solution recycling

First refined stock solution recycling: Decompress concentrate first refined mother liquor to recycle methanol at 70-80°C, the remaining residual liquid return electrodialysis process in the ceramic membrane dialysis fluid after recovery of methanol recovery was completed.

Second and third refined stock solution recycles: Second and third refined stock solution were mixed and concentrated to 25%-30% Ectoine concentration at 70°C-80°C, cool below 4°C for crystallization, centrifuge for rough wet product, stock solution was merged into first refined stock solution, rough wet product repeat refined step 2 times, dry wet product for finished product.

Results

Fermentation

The mean OD value at end of three experimental fermentation is 92.9, average value of pH is 7.5, average Ectoine content is 1.45%, which means the average Ectoine content is 468.3 g (Table 1).

Table 1. *Fermentation result.*

Test	OD value	pH	Fermentation liquor weight (kg)	Ectoine content	Ectoine (g)	Ectoine Content (g/Kg)
1	108.0	7.5	30.5	1.64%	499.9	16.39
2	87.1	7.3	32.15	1.41%	454.5	14.14
3	83.5	7.7	34.4	1.31%	450.6	13.10
Average	92.9	7.5	32.35	1.45%	468.3	14.48

Table 2. *Electrodialysis desalination result.*

Test Number	Weight (kg)	Ectoine content	Chromatographic purity	feed liquid after desalting (kg)	Ectoine content	Ectoine (g)	Yield
1	51	0.70%	84.28%	55.4	0.54%	300.3	84.11%
2	51	0.70%	84.28%	50.4	0.53%	265.1	74.26%
3	51	0.70%	84.28%	52.7	0.54%	282.2	79.05%
Average	51	0.70%	84.28%	52.8	0.54%	282.5	79.14%

Table 3. *Dow resin column result.*

Test Number	Eluent (kg)	Ectoine content	Ectoine (g)	Yield
1	63	0.44%	276.4	92.07%
2	42.3	0.60%	255.2	96.26%
3	29.8	0.93%	276.1	97.83%
Average	45	0.66%	269.2	95.39%

Ceramic membrane filtration

A total of 3 batch fermented liquid mixture pass ceramic membrane, because fermented liquid of an experiment in the process of dialysis is not enough to pass ceramic membrane cycle. 153 kg total liquid was got from dialysis after wash, Ectoine content is 0.7%, the weight was 1071 g, and Ectoine chromatographic purity is 84.28%. Ectoine overall yield of experimental procedure is calculated, 76.23%.

Electrodialysis desalination

Ceramic membrane washing dialysate was divided into three parts, each 51 kg, conducting electrodialysis desalination. feed liquid 55.4 Kg, 50.4 Kg and 52.7 Kg were got after desalination, the content of Ectoine were 0.54%, 0.53% and 0.54% respectively, Ectoine yield was calculate, 84.11%, 74.26% and 79.05% respectively, the average was 79.14%, mean 282.5 g Ectoine (Table 2).

Table 4. Crude product extracts result.

Test Number	Crude product dry weight (g)	Ectoine content	Ectoine Chromatographic purity	Ectoine (g)	Yield
1	780.0	34.44%	93.91%	268.6	97.17%
2	650.0	36.68%	95.60%	238.4	93.43%
3	453.7	58.65%	95.37%	266.1	96.37%
Average	627.9	43.26%	94.96%	257.7	95.66%

Table 5. Refining result.

Test Number	Third refining yield	Stock solution quantity (g)	Ectoine content	Ectoine proportion	Ectoine balance rate
1	90.37%	382.2	4.86%	10.36%	100.73%
2	89.48%	452.5	3.24%	8.73%	98.21%
3	100.10%	350.0	3.69%	8.18%	108.28%
Average	93.32%	394.9	3.93%	9.09%	102.40%

Table 6. Extraction result of second and third refined stock solution.

Ectoine (g)	First refined product Dry weight (g)	Ectoine content	HE	Ectoine (g)	Residue	Specific Rotation	Yield	Ectoine proportion
113.8	91.8	96.47%	3.24%	88.9	0.17%	+143.5°	78.13%	23.18%

Dow resin column

Eluent 63 Kg, 42.3 Kg and 29.8 Kg were got respectively after upper column. Its Ectoine content is 0.44%, 0.60% and 0.93% respectively, meaning Ectoine 276.4 g, 255.2 g and 276.1 g. The step yield of three tests were 92.07%, 96.26% and 97.83%, respectively (Table 3).

Crude product extract

Crude product was got after enrichment decoloration and drying, dry weight of crude products in three experiments are: 780.0 g, 650.0 g and 453.7 g, Ectoine content respectively was: 34.44%, 36.68% and 36.68%, the average of 43.26%. Ectoine chromatographic purity was measured: 93.91%, 95.60% and 95.60%, the average of 94.96%. Ectoine weight was calculated, 268.6 g and 238.4 g and 266.1 g, with the mean of 257.7 g. The yield was 97.17%, 93.43% and 96.37%, with average of 95.66% (Table 4).

Refining

The refining results are as shown in Table 5.

Stock solution extraction

Refined product was retrieved after mixing three group of second and third refined stock solution (Table 6).

Refine of first refined product extracted from stock solution: Three experimental processes are stable, totally getting Ectoine 634.7 g. Total yield: 45.17% (6.04 g/L). The

yield of 6.04 g/L is calculated according to material coefficient 70%, 35 L of 50 L fermentation canned (Table 7).

Finally, appearance, solution appearance, pH, chloride content, specific rotation, Ectoine purity and HE content of the product from three tests are conform to the prescribed standards, the results are as in Table 8.

Table 7. First refined product result.

Feed liquid (g)	Ectoine (g)	Refined dry weight (g)	Ectoine (g)	Ectoine content	HE content	Residue	Specific Rotation	Refining yield	Ectoine protortion
123.9	85.76	77.37	75.03	96.30%	3.14%	0.10%	+143.5°	87.49%	10.60%

Table 8. Ectoine extraction result.

Test items	Standard code	Test Number		
		1	2	3
Appearance	White to white crystalline	White powder	White powder	White powder
Solution appearance	Solution should be clarified, almost colorless.	Meet the requirement	Meet the requirement	Meet the requirement
PH (2%)	6.0-8.0	6.38	6.56	6.74
Chloride	≤ 0.05%	<0.05%	<0.05%	<0.05%
Specific Rotation (1%, water)	+139°~+145°	+141.9°	+142.6°	+142.1°
Purity (HPLC)	≥ 96.0%	97.3%	98.4%	99.0%
Related substances (HPLC)	HE ≤ 5.0%	2.60%	2.65%	2.52%

Discussion

Intracellular Ec is usually extracted by cracking cells with 80% ethanol solution, another extraction method protecting cell activity is based on the osmotic regulation mechanism of secretion channels, adopting the method of down shock, which shift bacterial cell in high osmotic pressure environment suddenly to low osmotic pressure in a short period of time, making solute rapidly released from intracellular [26]. Bacteria milking method adopts the means of the low permeability impact to produce compatible solutes, which means culturing bacteria under high osmotic pressure, releasing solute by low permeability impact, then make bacteria culture in high permeability again, then release solute by low permeability impact. The circulation repeated 8-9 times, getting the product. Ec higher yield is obtained by the high density fermentation method [27]. Doan et al. [28] develop two steps fed-batch fermentation basising on it, getting Ec maximum yield 6 g/L every day. However, the processes of these methods are relatively complex, the purpose of this paper is to optimize the purification of Ec.

Because Ec can protect biological activity of biological macromolecules such as enzyme, nucleic acid, cell membrane in high temperature, freezing, drying, ray protective free radicals and other adverse conditions. In this process, Ectoine yield was 6.04 g/L, comparing with the existing reports: Halomonas ventosae DL7 (404. 8 mg/L) [29], *Halomonas elogate* has application development potential for certain high yield of Ectoine, but which has yet to grope and optimize the fermentation culture conditions (such as salinity, nutrient composition, temperature metal ions, pH and other factors).

HE in cell has the effect of osmoprotectant to resist extracellular high osmotic pressure [30]. Hence, Control for osmotic pressure of fermentation liquor has a great influence on fermented result. Higher the osmotic pressure within a certain scope, more HE amount was accumulated. But with the improvement of HE content, the ratio of HE/Ec increased rapidly, and could end up more than control standards (5%). Therefore, Ec content should be mesured timely during late period of fermentation. The end of fermentation should be judged well, in case of HE ratio exceed standard.

Conclusion

Ec will have a wide range of uses in fine chemical industry, biological medicine, biological manufacturing and other fields, which makes the synthetic purification very important. The purpose of this paper is to study purification method of Ec. The innovation point lies in after extracting crude products from the column eluent directly, desalting using methanol, which improves yield and reduces the amount of waste water; The original bacteria concentrate passed ceramic membrane after heating for denaturation, then electrodialysis, reducing the equipment corrosion and the salt incidence, which was conducive to improve product quality; Cooling crystallization after enrichment was used in the process of refining, improving the yield, which reached 93.32%; Rcycling of the fermentation supernatant and electrodialysis water reduced the high salt waste water emissions. This process can be used for a large-

scale production and purification of ectoine after further optimization and adjustment.

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***Correspondence to**

Qian Jun-Qing

Zhejiang University of Technology

Zhejiang Province

PR China

Email: qjq@zjut.edu.cn