Exopolysaccharide production and lyophilization preparation from *Streptococcus thermophilus* ST

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Abstract: ST strain, isolated from yogurt purchased from a local market and identified as *Streptococcus thermophilus* based on physiological-biochemical characteristics and 16 S rDNA sequence, produced 55.62 mg·L^{-1} of exopolysaccharides（EPS）. The EPS level produced by strain ST depended upon incubation time（24 h）, temperature（40 ℃）, initial pH（7.0）of the medium and supplemented glucose（20 g·L^{-1}）. The major monosaccharide of EPS formed by this strain was confirmed to be glucose. Moreover, data from the lyophilization preparation of strain ST showed that the optimal medium containing NFMS（100.0 g·L^{-1}）, yeast extract（3.0 g·L^{-1}）, CaCO$_3$（9.0 g·L^{-1}）and whey powder（20.0 g·L^{-1}）promoted its viable cells to a level of 1.05 × 10^9 CFU·mL^{-1}. The combination of NFMS（160.0 g·L^{-1}）, glycerol（30.0 g·L^{-1}）, sodium glutamate（30.0 g·L^{-1}）and Tween 80（5.0 g·L^{-1}）was proved to be a good cytoprotectant for the protection of strain ST against the stresses of freeze-drying. Temperature, pH and rotation speed were important factors for gaining high viable counts in pilot-plant production of strain ST lyophilizator. The optimal combination including fermentation temperature（37 ℃）, rotation speed（90 r·min^{-1}）and pH（5.9）, resulted in a direct-vat-inoculation preparation of strain ST that contained viable cells of 1.7 × 10^{11} CFU·g^{-1} after lyophilization. Use of lyophilized strain ST preparation to directly ferment 80.0 g·L^{-1} reconstituted skim milk to yogurt produced lower whey separation than that of yogurt made with non-EPS strains used as controls.

Key words: *Streptococcus thermophilus*; exopolysaccharides（EPS）; multiplication; cytoprotectant; lyophilization.

产胞外多糖的嗜热链球菌 ST 冷冻干燥制剂的研究

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摘要：从当地市售的传统酸奶中分离出一株产胞外多糖的链球菌，经生理生化和 16 S rDNA 序列分析，将该菌株确定为嗜热链球菌 ST。在补充了 20 g·L^{-1}的葡萄糖，初始 pH7.0 的培养基上，40 ℃，培养 24 h 后，其胞外多糖产量为 55.62 mg·L^{-1}。进一步的结构分析显示，此多糖的主要单糖组成为葡萄糖。在生产嗜热链球菌 ST 冷冻干燥制剂时，其最佳的增殖培养基组成为脱脂奶粉 100.0 g·L^{-1}，酵母膏 3.0 g·L^{-1}，碳酸钙 9.0 g·L^{-1}和乳清粉 20.0 g·L^{-1}，活菌数达到 1.05 × 10^9 CFU·mL^{-1}。由脱脂奶粉 160.0 g·L^{-1}、甘油 30.0 g·L^{-1}谷氨酸钠 30.0 g·L^{-1}和 5.0 g·L^{-1}吐温 80 构成的保护剂能明显改善菌体细胞在冷冻干燥条件下的存活能力。在发酵温度 37 ℃、搅拌转速 90 r·min^{-1}和 pH5.9的条件下培养，经冷冻干燥获得的直投式发酵剂产品的活菌数量可达 1.7 × 10^{11} CFU·g^{-1}。当采用固形物仅为 80.0 g·L^{-1}的还原乳做为原料时，与不产胞外多糖的菌株生产的酸乳相比，采用该产品制备成的产品乳清析较少。

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0　Introduction

Some strains of *Streptococcus thermophilus* share the ability to produce extra-cellular polysaccharides（EPS）. The extra-cellular sugar polymers secreted by lactic acid bacteria strains, including ropy EPS secreted into the surrounding environment and capsular EPS tightly attached to cell surfaces, are being used as bio-thickeners, viscosifiers, stabilizers and gelling agents to modify the rheological properties and texture in fermented milk[1-2].

Recent research has shown that use of EPS-forming *S. thermophilus* can offer various technological advantages to yogurt and Mozzarella cheese. Rodarte et al. observed that the use of ropy EPS starter cultures could reduce syneresis and considerably improve viscosity[3]. Marshall et al. demonstrated that the presence of EPS substantially increased the viscosity of stirred yogurt[4]. According to Hassan et al., use of ropy EPS-producing strain decreased the firmness and syneresis of set yogurt[5]. Perry demonstrated that an increase of the functional properties of low-fat or part-skim Mozzarella cheese depended greatly on the utilization of EPS-forming *S. thermophillus* strains[6]. For this to occur, use of EPS-producing strains as a natural source of food additives has received a lot of interest in the dairy industry in recent decades.

Temperature, pH and composition of medium are important factors affecting EPS production by yoghurt starter cultures. It was proved that prolonged incubation times resulted in the reduction of EPS formed by lactic acid bacteria strains. This might be attributed to an enzymatic existence from cells which leads to the degradation of EPS.

To maintain uniformity in the quality of the end product, certain preservation and production technologies for the starter culture are necessary in order to improve bacterial viability and activity. It is well-known that lyophilization preparations of yogurt cultures display more advantages than those made with other techniques due to long-term storage, marketing and application. Freeze-drying has been considered as a suitable dehydration process for lactic acid bacteria in order to achieve a solid and stable final preparation. Carvalho suggested that growth factors such as compatible solutes and EPS production, drying medium, storage and re-hydration affected the final viability and activity of freeze-dried lactic acid bacteria preparations. Skim milk or whey supplemented with yeast extract or hydrolyzed protein are good growth and suspension media for the preservation of a freeze-dried culture[7]. In addition, milk solids are widely accepted as very good cryogenic agents for the preservation of starter cultures within the whole lyophilization process.

Currently, there is an ever-growing tendency for Chinese dairy producers to use concentrated freeze-dried starter cultures for the production of yoghurt, especially when using adjunct starters or tailor-made blended starters to confer desirable characteristics or health-promoting effects to yoghurt products. Therefore, the objectives of this present study were to: ① investigate the production and characteristics of EPS by *S. thermophilus* ST isolated from local yogurt products; and ② deal with the pilot-plant production and application of lyophiliaztion preparation from EPS-forming strain ST.

1　Materials and methods

1.1　Isolation and identification of ST strain

20 isolates were obtained from yogurt products made by a local dairy producer in Baoding City of Hebei Province, China, using the selective medium M17（Oxoid）. Among these isolates, strain ST showing interesting EPS production was identified based on morphological, physiological, biochemical characteristics plus 16S rDNA sequence. The 16S rDNA sequence was detected by ABI PRISM 377XL DNA sequencer as *S. thermophilus*.

1.2　Bacterial strains and cultivation

Non-EPS forming strains used in this study included *Streptococcus thermophilus* ST2, *Lactobacillus delbrueckii* subsp. *bulgaricus* LB1 and *L. delbrueckii* subsp. *bulgaricus* LB2 obtained from the China Center of Industrial Culture collection（CICC）. Stock cultures, first inoculated into 120 g·L^{-1} reconstituted milk autoclaved at 117 ℃ for 15 min, were propagated at 37 ℃ for 16 h, followed by two sub-culturings in the same medium for later use. The inoculum for sub-cultivations and fermentation experi-

ments was $20 \ \text{g} \cdot \text{L}^{-1}$.

1.3　Establishment of standard curve for assay of EPS

A standard curve of glucose concentration was produced according to the following procedure. 10 mg glucose was added to a 100 mL volumetric flask, and then distilled water was used to fill the flask. The glucose solution was homogeneously mixed, and then 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mL solution was sampled and added to dry tubes. The volume of each tube was made up to 2.0 mL by adding distilled water. Next, 1.0 mL of 6% phenol-sulphuric solution and 5.0 mL of 98% sulfuric acid were added to each tube (kept for 10 min). The solution in each tube was homogenized and kept at 25 ℃ for 20 min. Then, the absorbance was monitored at 490 nm, and 2.0 mL distilled water was used for the control (the standard curve of glucose is shown in **Figure 1**).
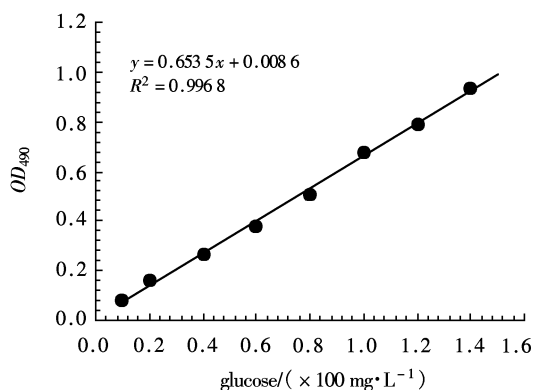

$y = 0.653\,5x + 0.008\,6$
$R^2 = 0.996\,8$

Fig.1　Standard curve for the quantification of glucose

1.4　Quantification of EPS

The extraction of EPS formed by strain ST was adapted from the procedure of Gancel and Novel with slight modification[8]. Approximate 5.0 mL of culture grown in M17-broth at 37 ℃ for 24 h was sampled, and added to a 50 mL centrifuged tube and then heated in a boiling water bath for 10 min to inactivate enzymes potentially capable of producing polymer degradation. The treated culture cooled to room temperature and was mixed with 50 μL of $50 \ \text{g} \cdot \text{L}^{-1}$ pronase solution. Next, 125 μL of 80% tri-chloroacetic acid was added to the mixture which was incubated at 37 ℃ for 1 h in a water bath shaker. The mixture was lowered to 4 ℃ for another 30 min in order to further remove cells and protein. Then the treated mixed solution was centrifuged at 8 000 g for 20 min, and the supernatant was dialyzed with a molecular weight cutoff of $8\,000 \ \text{g} \cdot \text{mol}^{-1}$ for 48 h with eight changes of distilled water. Two volumes of 95% cold ethanol were added to the

dialyzed solution overnight at 4 ℃ to precipitate EPS, followed by separation by centrifugation (8 000 g, 20 min at 4 ℃). The precipitated EPS concentration collected was quantified using the phenol-sulphuric method of Dubois and expressed as glucose equivalent according to the standard curve of glucose concentration previously given.

1.5　EPS formation by strain ST

Different parameters affecting EPS formation by strain ST are listed in **Table 1** according to $L_9(3^4)$. In each condition, the level of EPS produced by strain ST was checked according to the above method.

Table 1　Fermented parameters for high EPS production from strain ST according to $L_9(3^4)$

Level	Factor			
	Time/h	Temperature/℃	Initial pH	W(supplemented glucose)/ $(\text{g} \cdot \text{L}^{-1})$
1	8	30	5.0	20
2	16	35	6.0	40
3	24	40	7.0	60

1.6　Composition analysis of EPS collected

4% sulfuric acid solution was first added to collected EPS precipitates. Next, the EPS precipitates treated was sealed and incubated at 100 ℃ for 4 h in a water bath. Barium carbonate was used as a buffer to adjust the EPS precipitates to pH of 7.0, and the composition of the supernatant was analyzed by capillary electrophoresis. Electrophoresis conditions covered: ① buffer with 5×10^{-5} $\text{mmol} \cdot \text{L}^{-1}$ Cetyltrinethyl ammonium bromide (CTAB) and 130mM borax at pH 9.55; ② voltage with -12 kV pressure and $68.75 \ \text{kPa} \cdot \text{S}$ (detective wave length with 195 nm as well as segregation column temperature at 25 ℃); ③ before sample injection, the capillary was repeatedly washed with distilled water, $1.0 \ \text{mol} \cdot \text{L}^{-1}$ hydrochloric acid and $1.0 \ \text{mol} \cdot \text{L}^{-1}$ sodium hydroxide. Eventually, the capillary was washed with distilled water for 1 min and then with the buffer for 2 min; and ④ a quartz capillary of 50 μm × 50 cm was used to analyze the composition of EPS in the present study.

1.7　The optimal media for the proliferation of strain ST

Media for the multiplication of strain ST according to $L_9(3^4)$ are given seen in **Table 2**, and the media including different levels of non-fat milk solids (NFMS), yeast, $CaCO_3$ and whey powder was studied to maximally increase the live cell counts of this organism. Cell numbers

（CFU·mL^{-1}） were determined by plating the diluted samples on M17-agar medium incubated at 37 ℃ for 48 h. The combination having the maximum viable counts was regarded as the optimal enriched media for strain ST.

Table 2　Media for the multiplication of strain ST according to L$_9$(3^4)

level	Factor			
	NFMS/ (g·L^{-1})	Yeast extract/ (g·L^{-1})	CaCO$_3$/ (g·L^{-1})	Whey powder/ (g·L^{-1})
1	100.0	8.0	3.0	10.0
2	110.0	5.0	6.0	15.0
3	120.0	3.0	9.0	20.0

1.8　The detection of the maximal biomass of strain ST

Strain ST was grown in the optimal multiplication media at 37 ℃ for 24 h. Its growth was monitored by measurement of optical density（OD）at 600 nm at regular intervals, and the optimal harvest time was the time at which the OD value reached the maximum.

1.9　Screening for the cytoprotectants of strain ST cells to be lyophilized

Cytopotectants for the freeze-drying of strain ST according to L$_9$(3^4) are given in **Table 3.** Combinations containing different concentrations of NFMS, glycerol, sodium glutamate and Tween 80 were assayed based on their ability to protect strain ST against damage from the freeze-drying process. Cell numbers（CFU·g^{-1}）were estimated by plating the diluted samples on M17-agar medium incubated at 37 ℃ for 48 h. The combination able to allow strain ST to have the highest bacterial counts during freeze-drying was regarded as the optimal cytoprotectant.

Table 3　Cytoprotectants designed for improving the viability of freeze-dried strain ST

level	Factor			
	NFMS/ (g·L^{-1})	Glycerol/ (g·L^{-1})	Sodium glutamate/ (g·L^{-1})	Tween80/ (g·L^{-1})
1	120.0	20.0	10.0	5.0
2	160.0	30.0	20.0	10.0
3	180.0	50.0	30.0	15.0

1.10　300 L pilot-production of lyophilization preparation of strain ST

Different fermented parameters affecting viable cells counts from strain ST in the 300 L pilot-production are given in **Table 4** according to L$_9$(3^4). In each condition, the viable counts of strain ST were checked according to plate count.

Table 4　Fermented parameters for high viable cells from strain ST according to L$_9$(3^4)

Level	Factor			
	Temperature/℃	Time/h	Rotation speed/ (r·min^{-1})	pH
1	37	15.5	85	5.8
2	38	16.0	90	5.9
3	39	16.5	100	6.0

Cultures of strain ST fermented in its enriched medium for 16 h were first centrifuged（14 000 g）for 15 min at 4 ℃, and then the cell precipitation was mixed with the optimal cytoprotectant. The mixture was freeze-dried at （40 ℃ for 15 h. The live cell counts（expressed as CFU·g^{-1}）after lyophilization were determined on M17-agar media incubated at 37 ℃ for 48 h.

1.11　Whey separation determination of undisturbed set yoghurt

Spontaneous syneresis of undisturbed set yoghurt made with strain ST was determined using a siphon method. The method was based on that described by Amatayakul and Halmos[9]. The level of whey separation from yoghurt was expressed as the percentage weight of whey over the initial yoghurt sample.

1.12　Statistical analysis

All data from the means of three replicates for each experiment were analyzed using one-way analysis of variance at 95 % confidence level with SPSS version 12 for Windows.

2　Results and discussion

2.1　Identification of ST strain

Morphologically, strain ST showed phenotypic characteristics of a Gram-positive, spherical- to ovoid-shaped, catalase-negative organism. **Table 5** shows the growth, physiological and biochemical characteristics of ST strain, which are equal to those of *Streptococcus thermophilus* described by Bergey's Manual of Systematic Bacteriology （the second edition）. Strain ST was primarily identified as *S. thermophilus*.

Moreover, the sequence alignment of ST strain with 16 S rDNA gene of *Streptococcus thermophilus* AY188354.1 showed that there was 100 % homology of the sequences from position 6 to 1 450 between strain ST and strain AY188354.1 from position 37 to 1 481. Thus, strain ST was finally confirmed to be *Streptococcus thermophilus*.

Table 5　Growth, physiological and biochemical properties of
S. thermophilus ST

	Characteristics	*S. thermophilus* ST
	at 10 ℃	−
	at 45 ℃	+
Growth	in 6.5 % NaCl	−
	at pH 9.6	−
	at 60 ℃ for 30 min	+
Hydrolyzation	from starch	+
	from esculin	−
	Arabinose	−
	Cellobiose	−
	Galactose	−
	Glucose	+
	Lactose	+
	Maltose	−
	Mannitol	−
	Mannose	−
Sugar fermentation	Melicitose	−
	Raffinose	−
	Melibiose	−
	Rhamnose	−
	Ribose	−
	Sorbitol	−
	Sucrose	+
	Fucose	−
	Xylose	−
	Synanthrin	−

" + ": positive reaction; " − ": negative reaction

2.2　EPS production

Data from **Table 6** shows that fermentation time and temperature have a significant effect on EPS production by strain ST, compared to initial pH of the medium and supplemented glucose ($P < 0.05$). It was seen that strain ST was able to produce the maximum EPS (reaching 44.13 mg·L^{-1}), when the combination of fermentation parameters was No.9, *i.e.* incubation time of 24 h at 40 ℃, initial pH of 6.0 and 20 g·L^{-1} level of supplemented glucose. However, an analysis by orthogonal test indicated that the optimal fermentation conditions for EPS formation by strain ST should be theoretically 24 h fermentation-time at 40 ℃, initiative pH of 7.0 and in medium supplemented with glucose 20 g·L^{-1}. It was further confirmed that use of the optimal conditions improved the EPS level formed by strain ST to 55.62 mg·L^{-1} (data not shown).

The maximum EPS occurs among most strains of lactic acid bacteria during the exponential growth phase and is reached during the early decline stage of growth. Gancel[8] observed that EPS-forming *S. thermophilus* S22 produced no EPS when the strain grew for 0 ~ 6 h, and the highest EPS level formed by this strain occurred between an incubation time of 14 h and 18 h. Additionally, *S. thermophilus* S22 produced much more EPS when cultivated in pH 7.0 culture media than 5.5, since a rapid growth of strain S22 occurred in pH 7.0. *L. casei* subsp. *casei* NCIB 4 114 was reported to produce 110 mg·L^{-1} EPS when growing in the NFMS medium to which 50 g·L^{-1} glucose was added. Pham et. al.[10] indicated that *L. rhamonosus* R was able to produce 438 mg·L^{-1} EPS when grown in glucose BMM and with glucose continually supplemented, *L. delbrueckii* subsp. *bulgaricus* NCFB2772 produced the most polymers when cultivated at 45 ℃[11]. Clearly, the results obtained in the present study are in agreement with those given by other authors.

Table 6　The combinations of different fermentation parameters which affected the EPS production by ST strain*

WKTrial	A(time)	B(temperature)	C(initiative pH)	D(supplemented glucose)	EPS/(mg·L^{-1})
1	1(8 h)	1(30 ℃)	1(5.0)	1(20.0 g·L^{-1})	22.40
2	1(8 h)	2(35 ℃)	2(6.0)	2(40.0 g·L^{-1})	15.21
3	1(8 h)	3(40 ℃)	3(7.0)	3(60.0 g·L^{-1})	34.80
4	2(16 h)	1(30 ℃)	2(6.0)	3(60.0 g·L^{-1})	7.41
5	2(16 h)	2(35 ℃)	3(7.0)	1(20.0 g·L^{-1})	14.90
6	2(16 h)	3(40 ℃)	1(5.0)	2(40.0 g·L^{-1})	15.21
7	3(24 h)	1(30 ℃)	3(7.0)	2(40.0 g·L^{-1})	25.46
8	3(24 h)	2(35 ℃)	1(5.0)	3(60.0 g·L^{-1})	36.17
9	3(24 h)	3(40 ℃)	2(6.0)	1(20.0 g·L^{-1})	44.13
K1	72.41	55.27	73.78	81.43	215.69
K2	37.52	66.28	66.75	55.88	
K3	105.76	94.14	75.16	78.38	
k1	24.14	18.42	24.59	27.14	
k2	12.51	22.09	22.25	18.63	
k3	35.25	31.38	25.05	26.13	
R	22.74	12.96	2.80	8.51	

* Notes: K stands for the sum of the level, k represents the average of the level and R stands for k_{max}-k_{min}

2.3　EPS composition

The main monosaccharide composition of EPS formed by strain ST are shown in **Figure 2** The major moiety of the EPS was confirmed to be glucose by internal standard.
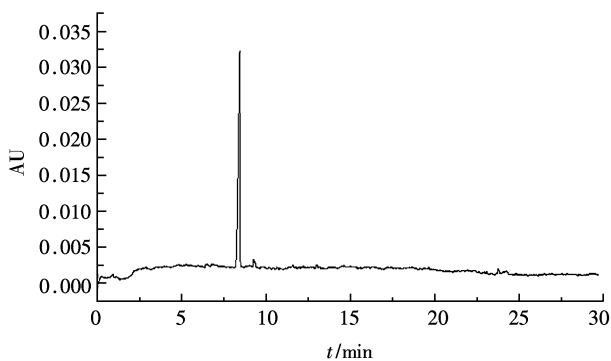

Fig.2　Analysis of the composition of EPS formed by strain ST

Vuyst showed that the composition of the EPS produced by *Streptococcus mutans* was glucans[12]. Our results support the conclusion of Vuyst. Lemoine demonstrated that the EPS structure produced by *S. thermophilus* was galactose and glucose at a ratio of $1:1$[13]. Cerning et al. reported that glucose and galactose were the main saccharides of the EPS material from *S. thermophilus*, along with small amounts of xylose, arabinose, rhamnose and mannose[14]. The work of Tamine suggested that the chemical composition of the EPS material produced by starter culture RR was a β-glucans which yielded only glucose after acid hydrolysis. These studies showed that the composition of EPS was directly related to the strains employed. Tamime showed that the EPS produced by yoghurt starter culture could form a web of filaments attaching the microbial cells to the protein matrix of the yoghurt[15]. Presence of EPS would reduce syneresis and thus improve the texture and viscosity of the final products, particularly in fermented milk.

2.4　Multiplication media of ST strain

It is seen that NFMS had a large influence on the live cells of strain ST from **Table 7**, since its R-value was 3.2. Moreover, the highest live cells reached 1.05×10^9 CFU·mL^{-1}, if No.3 combination was used to enrich strain ST. However, an analysis by orthogonal test indicated that the optimal medium for enriching of strain ST theoretically included 100.0 g·L^{-1} NFMS, 5.0 g·L^{-1} yeast extract, 9.0 g·L^{-1} CaCO$_3$ and 20.0 g·L^{-1} whey powder. Use of the optimal medium promoted strain ST to produce the highest number of live cells with 0.90×10^9 CFU·mL^{-1}. From **Table 7**, it is clear that No.3 combi-

nation with NFMS （100.0 g·L^{-1}）, yeast extract （3.0 g·L^{-1}）, CaCO$_3$（9.0 g·L^{-1}）and whey powder （20.0 g·L^{-1}）was the optimal media that could promote better strain growth.

Table 7　Media available for the multiplication of ST strain *

Trial	NFMS	Yeast extract	CaCO$_3$	Whey powder	cell counts （×10^8 CFU·L^{-1}）
1	1	1	1	1	9.20
2	1	2	2	2	8.70
3	1	3	3	3	10.50
4	2	1	2	3	7.10
5	2	2	3	1	6.80
6	2	3	1	2	4.90
7	3	1	3	2	8.90
8	3	2	1	3	10.20
9	3	3	2	1	7.80
K1	28.40	25.20	24.30	23.80	
K2	18.80	25.70	23.60	22.50	
K3	26.90	23.20	26.20	27.80	
k1	9.47	8.40	8.10	7.93	
k2	6.27	8.57	7.87	7.50	
k3	8.97	7.73	8.73	9.27	
R	3.20	0.84	0.86	1.77	

　*　Notes: K stands for the sum of the level, k represents the average of the level and R stands for k_{max}-k_{min}

Tamime suggested that 16 % total solid （TS）in the growth medium is regarded as a realistic level for a mixed strain from yoghurt starter culture[15]. Alaeddinoglu suggested that skimmed milk or whey supplemented with yeast extract or hydrolyzed protein were good growth and suspension media for the preservation of freeze-dried cultures[16]. Moreover, the survival rate during freezing and freeze drying of lactic acid bacteria was improved when growing in the presence of a calcium culture medium. Also, neutralization of the growth medium to pH range 5 to 6 was recommended. Yoghurt cultures could be preserved for 150 day at 0 ℃ when the growth medium was supplemented with 5.0 g·L^{-1} CaCO$_3$（15）. The results obtained in the present study are in agreement with those given by other authors.

2.5　Cytoprotectants for the preservation of strain ST

Data from **Table 8** shows the important role of NFMS in improving the survival of strain ST. Use of NFMS as a protective agent was effective in gaining maximal bacterial counts. The role of Tween 80 in preventing strain ST from damage during freeze-drying was the weakest of the cyto-

protectants, but no statistical differences were found among the four chosen protective agents in improving the viability of lyophilized cells of strain ST. No.5 was considered as the optimal cytoprotectant-combination due to higher viability of strain ST. Use of No.5 as cytoprotectants would maintain the maximal viability having 4.72×10^{11} CFU·g $^{-1}$ after the completion of freeze-drying. Moreover, an analysis by orthogonal test suggested that the optimal composition consisted of cryoprotectants for strain ST ideally included 160.0 g·L^{-1} NFMS, 50.0 g·L^{-1} glycerol, 30.0 g·L^{-1} sodium glutamate and 10.0 g·L^{-1} Tween 80. Use of the ideal cytoprotectant-combination would maintain the bacterial live counts to a level of 4.65×10^{11} CFU·g^{-1}. From data in **Table 8**, it is clear that No.3 combination, including NFMS (160.0 g·L^{-1}), glycerol (30.0 g·L^{-1}), Tween 80(5.0 g·L^{-1}) and sodium glutamate (30.0 g·L^{-1}), would be the optimal cytoprotectants for the highest protection of strain ST cells against the injury of freeze-drying.

Table 8　Cytoprotectants available for improving the viability of freeze-dried strain ST*

Trial	NFMS	glycerol	sodium glutamate	Tween 80	cell counts ($\times 10^{10}$ CFU·g^{-1})
1	1	1	1	1	9.9
2	1	2	2	2	12.0
3	1	3	3	3	32.5
4	2	1	2	3	18.7
5	2	2	3	1	47.2
6	2	3	1	2	47.0
7	3	1	3	2	40.2
8	3	2	1	3	31.8
9	3	3	2	1	31.2
K1	54.4	68.8	88.7	88.3	
K2	112.9	91.0	61.9	99.2	
K3	103.2	110.7	119.9	83.0	
k1	18.1	22.9	29.6	29.4	
k2	37.6	30.3	20.6	33.1	
k3	34.4	36.9	40.0	27.7	
R	19.5	14.0	19.4	5.4	

* Notes: K stands for the sum of the level, k represents the average of the level and R stands for k_{max}-k_{min}

Milk solids are widely accepted as a very good cytogenic protective agent for the preservation of the starter cultures, and use of levels up to 20 to 25 % total solids was recommended by Tamime and Robinson[15]. Castro[17] suggested that skim milk powder was the optimal dry medium. Skim milk is suggested to provide a coating for the

treated cell, and thus stabilizes the cell membrane and prevents cellular injury. In addition, it forms a porous structure to make re-hydration easier[18]. It was proved that such permeable compounds as glycerol made the cell membrane more plastic and bound water which suppresses excess dehydration, reduced salt toxicity and prevented formation of ice crystals within the cell during freezing[7]. Carvalho[19] found, for the majority of lactic acid bacteria, that survival rate during storage was improved when sodium glutamate was added as a protectant to skim milk. The protection might be due to stabilization of their protein structure via reactions between the amino group of the protectant and the carboxyl groups of bacterial proteins, coupled with the ability to retain greater amounts of residual moisture[20]. Tween 80 was suggested to lead to changes of the fatty acid composition of the lactic acid bacteria cells, giving bacterial cells more resistance to freezing[21]. These study conclusions proved practicable for the appropriate selection of cytoprotectants in our present study.

2.6　The optimal harvested time of strain ST cells

Strain ST grew rapidly in its enriched medium at 37 ℃ for the first 4 h, and the whole exponential phase lasted up to 8 h (shown in **Figure. 3**). The largest viability occurred at 16 h-incubation time, and thus the optimal time for harvesting the high-density cells of strain ST was 16 h.
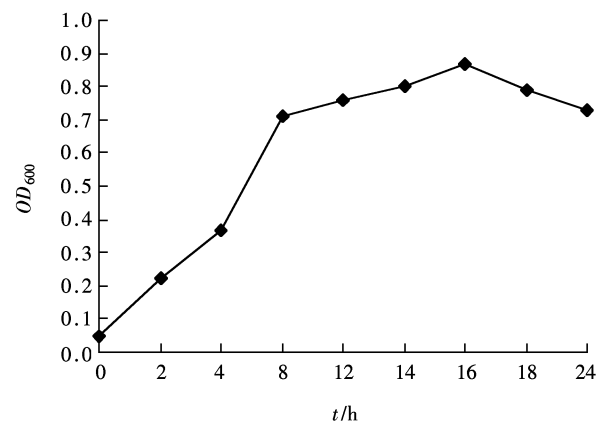

Fig.3　The growth pattern of strain ST incubated at 37 ℃

2.7　A 300 L pilot-plant production for the DVS of strain ST

From **Table 9**, it is seen that rotation speed and temperature had significant effects on bacteria live cells, compared to pH of the medium and fermentation time ($P < 0.05$). An analysis by orthogonal test showed the idea combination should be fermentation temperature at

37 ℃, 16 h incubation time, rotation speed of 90 r/min and pH at 6.0. The bacterial live counts reached 1.5×10^9 CFU·mL^{-1}, if the idea combination of fermenting parameters was used. Of 9 combinations associated with the optimal fermented parameters, the No.2 combination was clearly the best for DVS production of strain ST due to the maximal live cell counts of 1.6×10^9 CFU·mL^{-1}. It is clear from **Table 9** that the optimal parameters from No.2 combination were fermentation temperature at 37 ℃, 16 h cultivation time, 90 rpm·min^{-1} of rotation speed and pH controlled at 5.9.

Table 9　The optimal fermented parameters for the pilot-production of strain ST DVS preparations*

Trial	temperature	Time	rotation speed	pH	cell counts ($\times 10^8$ CFU·mL^{-1})
1	1(37 ℃)	1(15.5 h)	1(85 rpm/min)	1(5.8)	12
2	1(37 ℃)	2(16.0 h)	2(90 rpm/min)	2(5.9)	16
3	1(37 ℃)	3(16.5 h)	3(100 rpm/min)	3(6.0)	13
4	2(38 ℃)	1(15.5 h)	2(90 rpm/min)	3(6.0)	15
5	2(38 ℃)	2(16.0 h)	3(100 rpm/min)	1(5.8)	11
6	2(38 ℃)	3(16.5 h)	1(85 rpm/min)	2(5.9)	9
7	3(39 ℃)	1(15.5 h)	3(100 rpm/min)	2(5.9)	8
8	3(39 ℃)	2(16.0 h)	1(85 rpm/min)	3(6.0)	10
9	3(39 ℃)	3(16.5 h)	2(90 rpm/min)	1(5.8)	14
K1	41.000	35.000	31.000	37.000	
K2	35.000	37.000	45.000	33.000	
K3	32.000	36.000	32.000	38.000	
k1	13.667	11.667	10.333	12.333	
k2	11.667	12.333	15.000	11.000	
k3	10.667	12.000	10.667	12.667	
R	3.000	0.666	4.667	1.667	

* Notes: K stands for the sum of the level, k represents the average of the level and R stands for k_{max}-k_{min}

Concentrated yoghurt cultures (i.e. single and mixed strain) were centrifugated at 11 000 g for 15 min at 4 ℃ at the end of the log phase in a batch system with the pH controlled by adding 10 mol·L^{-1} NaOH, the starter culture did not have a significant decrease in the following storage stage[15]. Wolff reported that vacuumed freeze-drying was more suitable for *S. thermophilus* than atmospheric pressured freeze-drying[22].

In short, we designed the following procedure to produce the DVS preparation of strain ST, i.e. fermentation temperature at 37 ℃, rotation speed with 90 r/min, pH at 5.9, inoculation size of 3 %, supplemented with 15.0 g·L^{-1} NMFS, and 16 h fermentation time. Then, the cells were harvested by centrifugation at 14 000 g for 15 min at 4 ℃. Next, the harvested cells were mixed with their optimal cryoprotectants, and lyophilized at −40 ℃ for 15 h. The detected viable bacteria in the final freeze-dried powder products reached 1.7×10^{11} CFU·g^{-1}. Clearly, the bacterial counts of strain ST DVS preparations from the present study were near to those of commercial DVS products[15].

2.8　Whey separation of the yoghurt products manufactured with strain ST DVS culture

Yogurt product made with EPS-forming strains ST showed significant lower level of whey separation ($P <$ 0.01) than those made with non-EPS strains ST2, LB1 and LB2 (see **Figure 4**). The amount of separated whey from the yoghurts produced with strain ST decreased by 6 %, 8 % and 12 % as compared to the products manufactured with strain ST2, LB1 and LB2, respectively. A lower level of whey separation might be attributed to the water-binding ability of EPS.
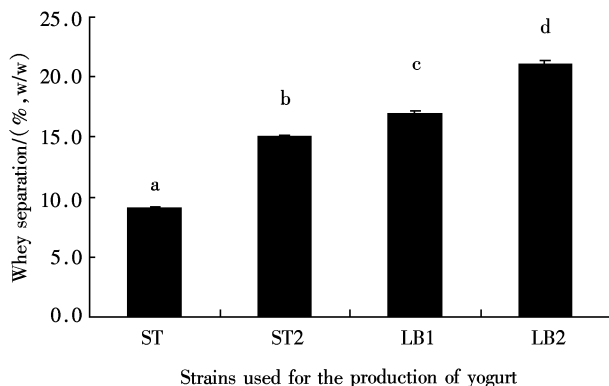

Fig.4　Whey separation of set yoghurt produced with EPS-forming strain ST as well as non-EPS strains (ST2, LB1, LB2). Error bars represent standard deviation ($n = 3$). Bars that do not share same letters (a, b, c, d) are significantly different ($P < 0.01$)

3　Conclusions

The present study showed the ability of strain ST to produce the maximal EPS $(55.62 \ \text{mg} \cdot \text{L}^{-1})$ when fermenting conditions were 24 h incubation time at 40 ℃, initial pH at 7.0 and in medium supplemented with glucose of $20.0 \ \text{g} \cdot \text{L}^{-1}$. The major monosaccharide of the EPS formed by strain ST was confirmed to be glucose. Moreover, the present study provided information available for the pilot-plant production of the EPS-producing DVS culture. Use of the EPS-forming strain to make set yoghurt products produced significant lower whey separation, showing that the DVS preparations produced according to our design procedure are practical for the manufacture of fermented dairy products such as yogurt.

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