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Characteristics for the hybrid cell Foaz obtained from the protoplast fusion between yeast and photosynthetic bacteria in soybean processing wastewater*

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Abstract—The optimal pH and temperature, maximum specific degradation rate, half rate constant and flocculation rate for the hybrid cell Foaz were measured in the reaction for the degradation of soybean processing wastewater (SPW) in this study. The optimal pH and temperature for Foaz in SPW were the same as those of its parental strains *Saccharomyces cerevisiae* Y9407 and *Rhodobacter sphaeroides* P9479, but the flocculation rate, the maximum specific degradation rate and the half rate constant for Foaz were higher than those of its parental strains. The results suggest that the characteristics of the inter-kingdom fusant Foaz constructed from the protoplast fusion between the eukaryote cell yeast *S. cerevisiae* and the prokaryote cell photosynthetic bacteria *R. sphaeroides* may favor degrading organic pollutant in SPW and removing biomass from the effluent.

Keywords: *Saccharomyces cerevisiae*; *Rhodobacter sphaeroides*; soybean processing; wastewater.

1 Introduction

Microorganism protoplast fusion has been established as an effective approach in genetic manipulation to construct new strains in fungi or bacteria (Fang, 1990; Taohoun, 1993). In yeast fermentation and pharmacy industries, the technique of protoplast fusion is now often used to create ideal strains for the improvement of product quality and quantity, and it also appeals to environmentalists to develop a new biotechnique in wastewater treatment and in organic toxic pollutants biodegradation (Chen, 1987; Limtong, 1988; Puntambekar, 1995). The process of photosynthetic bacteria (PSBs) used in the treatment of high organic wastewater has been one of the most important achievements in the industrialization field of environmental biotechnique since 1970's. PSBs can purify polluted water in nature. This principle has been applied in designing pilot plants. PSBs have various metabolic pathways for the degradation of organic wastes in the circumstances either with light or without light and either with aerobic or anaerobic habitats. Being favorable to wastewater treatment, the biomass not only served as source of food, but also increased fish weight, increased egg production in hens and improved the quantity and quality of citrus fruit when applied as an organic fertilizer (Bei, 1972; Toya, 1992; Zhou, 1983). *Rhodobacter sphaeroides* is one of PSB species often used in the treatment of organic wastewater. However, the size of PSBs is too small to be separated easily from wastewater. It usually costs a lot to remove

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PSBs cells from effluent with chemical flocculating agents or with a centrifugal separator. Yeasts are fungi that exist predominately in the treatment of organic wastewater for the production of single cell protein(SCP). At least there are 14 genes concerning flocculation found in *S. cerevisiae* including FLO1, FLO2, FLO4, FLO5, FLO8, flo3, flo6, fusel, fsul2, TUP1, flox, LUP1, and amp2. Cloning of FLO1, FLO5 and FLO8 genes to improve. *S. cerevisiae* genetic engineering flocculation has been successful (Speer, 1992; Watari, 1991). Flocculation of yeast is an important brewing property not only in fermentation industry but also in organic wastewater treatment. The size of yeast *S. cerevisiae* cell is about 30 fold of that of *R. sphaeroides*. So the cells of yeast *S. cerevisiae* should be easier to eliminate from effluent than *R. sphaeroides* in a secondary sedimentation tank. The adaptability of *S. cerevisiae* for various organic wastewater and its metablic pathway for the degradation of organic pollutants are not as good as those of *R. sphaeroides*. The purpose of fusing the *S. cerevisiae* Y9407 and *R. sphaeroides* P9479 protoplasts is to construct a new strain which may obtain multiple superiorities from its parental stains in such aspects as flocculation, acid tolerance, thermal tolerance, adaptability to organic wastewater, SCP yield and quality. Eukaryote microorganism and prokaryote microorganism had a same origin about one million years ago(Guo, 1990). Nevertheless, there have been many differences occurring between them during the long evolutionistic process. Eukaryote cell and prokaryote cell should still share some basis in physiology and biochemistry now, so it is possible to construct a new strain through their protoplast fusion, and its hybrid may be different from its parental strains in features and more favorable to the treatment of organic wastewater.

On the basis of the researches on PSB interspecific protoplast fusion and yeast intergeneric protoplast fusion(Cheng, 1993; 1995), the author of this paper has succeeded in conducting inter-kingdom protoplast fusion between the yeast *S. cerevisiae* Y9407 and the PSB *R. sphaeroides* P9479 (Cheng, 1996a). The hybrid Foaz identified was used as the starting strain for this study. The objective is to describe the optimal pH and acceptable pH range, the optimal temperature and acceptable temperature range, the flocculation rate, the maximum specific degradation rate and the half rate constant measured for the Foaz in SPW. Although many researchers described the results for purification of SPW by use of PSBs, yeast or other microorganism strains(Liu, 1991; Ru, 1989; Zhang, 1987), but it is the first time to report the degradation kinetic parameters for the fusant Foaz in SPW. The results demonstrate once more that the hybrid Foaz is a stable karyogamy fusant strain, which has a variety of characteristics different from those of either of its parental strains and the same as those of itself both in LLM medium and in monosodium glutamate wastewater(Cheng, 1996a; 1996b).

2 Materials and methods

2.1 Strains

Rhodobacter sphaeroides P9479 (Nt^rSm^s) obtained from Shanghai Institute of Plant Physiology, Chinese Academy of Sciences was a highly flocculent strain through the treatment of 15 mmol/L(Cheng, 1996a). Sm is the abbreviation of antibiotic streptomycin (Sm) and Nt is that of nystatin.

Saccharomycee cerevisiae Y9407 (Nt^sSm^r) was purchased from Dongguan Sugar Mill in Guangdong Province with high activity in metabolism.

Foaz (Nt^rm^r) was the starting strain which had been identified with stable characteristics in morphology, biochemistry and genetics from the products of the inter-kingdom protoplast fusion

between *Saccharomyces cerevisiae* Y9407 (Nt^sSm^r) and *Rhodobacter sphaeroides* P9479(Nt^rSm^s) (Cheng, 1995).

2.2 Media(Ormerod, 1961; Toya, 1992)

LMM is a liquor medium for the cultivation of Foaz. The prescription of LMM is 3g K_2HPO_4, 1g KH_2PO_4, 0.5g NH_4NO_3, 0.1g Na_2SO_4, 10mg $MgSO_4 \cdot 7H_2O$, 1mg $MnSO_4 \cdot 4H_2O$, 0.5g $CaCl_2$, 1mg $FeSO_4 \cdot 7H_2O$, 0.1g yeast extract, 10g dextrose and 5g sodium acetate, dissolved in 1000 ml distilled water and the regulated to pH 7.0 after autoclaving at 121℃ and 100kPa for 15min.

SMM is solid medium for Foaz. SMM = LMM + 1.5% agar.

2.3 Wastewater

The soybean processing wastewater (SPW) was sampled from NJ soybean product mill. The detailed data for the SPW quality are shown in Table 1.

Table 1 The quality for the raw soybean processing wastewater

Item	Concentration, mg/L	Item	Concentration, mg/L	Item	Concentration, mg/L
Al	0.855	Fe	2.910	Sr	0.178
Ba	0.308	K	331	Ti	0.042
Be	ND*	Li	ND	V	ND
B	0.137	Mg	122	Zn	1
Ca	202	Mn	0.184	pH**	4.8
Cd	ND	Mo	0.109	TN***	583
Co	0.008	Ni	0.564	TP****	73
Cr	0.035	Na	12	COD_{cr}	20832
Cu	0.344	Pb	ND	BOD_5	14346

* ND: not detected; ** pH value; *** TN: total nitrogen; **** : total phosphorus

The inductively coupled plasma spectrometer (ICP-AES) J-A1100, USA was used for the analysis of metal elements. All the values of metal element or heavy metal element concentration are lower than that in " Sanitary standards for drinking water(GB5749-85)", " Standards for fishery water quality" (GB11607-89) and "Standards for irrigation water quality"(GB 5084-92) (China Standard Press, 1985; 1989; 1992). It means that the concentration of metal elements in SPW is safety to mankind and micro organism. COD_{cr} and BOD_5 was analysed according to "Standard methods for the examination of water and wastewater", 15th Edition, 1980, APHA published, USA(APHA, AWWA, WPCF, USA, 1980). TN(total nitrogen) and TP(total phosphorus) were measured by an oxidation method (Qian, 1987).

The raw soybean processing wastewater sterilized by autoclaving at 121℃ and 100kPa for 20 minutes was diluted with distilled water and then its pH was regulated with 0.1 mol/L NaOH or 0.1 mol/L HCl to meet the requirement of the experiment.

2.4 Testing the optimal pH and the optimal temperature (Qi, 1988)

The hybrid Foaz was incubated for 4 hours separately in 150 ml diluted SPW samples (BOD_5 =2000mg/L) at 30℃ with their pH values ranging from 3 to 11 for the measurement of the optimal pH for the hybrid growth, the inoculation liquor of Foaz was cultivated at 25 ℃ in LMM. Foaz was introduced into new diluted SPW samples with pH 7 at 15℃ to 45℃ for the measurement of the optimal temperature for the growth, the inoculation liquor of Foaz was cultivated at 35℃ in

LMM. The values of the specific growth rate μ to various habitats were used to indicate the adaptability of Foaz growth in SPW, which mean the rate (%) of biomass enhancement per hour (h^{-1}); $\mu = (\ln Xn - \ln Xo)/\Delta t$. Here Xn is the amount of Foaz biomass in SPW after the reaction; Xo is the amount of Foaz biomass before the reaction; and Δt is the reaction time of 4 hours for this experiment shaking at 250 r/min.

2.5 Measuring the flocculation rate(Spencer, 1989)

The optical density of SPW sample containing Foaz after the shaking was tested at 660 nm before and after setting for 10 minutes respectively, the flocculation rate $P(\%) = (A^b660 - A^a660)/A^b660$. Here A^b660 is the value of optical density before static setting of 10 minutes; A^a660 is the value of optical density after setting of 10 minutes.

2.6 Testing the maximum specific degradation rate and the half rate constant

The kinetic parameters of the maximum specific degradation rate q_{max} and the half rate constants were tested through a shaking reaction with 5 group samples whose concentrations of BOD_5 varied from 147 mg/L to 2449 mg/L with its pH 7.0 and its temperature at 35℃ for each strain. The data obtained for the experiment were treated by statistically and described as a regression equation according to that of Monod(Monod, 1942; Chen, 1989). The Monod equation is $1/q = (Ks/q_{max})\ (1/Se) + (1/q_{max})$. Here the symbol of q is the specific degradation rate (h^{-1}), equal to the rate of organic pollutant degraded per unit of biomass per hour; Ks is the BOD_5(mg/L) concentration of wastewater for microorganisms to meet the specific degradation rate at 1/2 q_{max}; and q_{max} is the maximum specific degradation rate. The normal value of the regression equation is equal to $1/q_{max}$, so the value of q_{max} can be first worked out after the regression equation is obtained; the slope value of the regression equation is equal to Ks/q_{max}, so the Ks value can be worked out after the value of q_{max} is obtained(Huang, 1983).

3 Results

The optimal pH value for the growth of the hybrid Foaz and its parental strains in SPW samples with BOD_5 2000 mg/L at 30℃ are shown in Table 2.

Table 2 Comparison of acid tolerance between Foaz and its parental strains in SPW

Strain	μ values for each strain at different pH, h^{-1}				
	pH value				
	3.0	5.0	7.0	9.0	11.0
Y9407	0.076	0.220	0.261	0	0
P9479	0	0	0.189	0.054	0
Foaz	0.083	0.184	0.253	0	0

Notes: Each value is the mean of 5 samples

Table 2 shows that the acceptable pH range for Foaz growth in SPW ranged from pH 3.0 to pH 7.0 being the same as that of *S. cerevisiae* Y9407, but the optimal pH values for both Foaz and its parents were the same, being pH 7.0, at which three strains had their highest specific growth rate respectively. However, their specific growth rate were different. The value of the specific growth rate μ for Foaz in SPW at the optimal pH was 0.253 h^{-1}, which was higher than that of *R. sphaeroides* P9479 and lower than that of *S. cerevisiae* Y9407 which enables the hybrid Foaz

to treat organic wastewater, because the values of pH for most organic wastewater are in the range of low level.

The optimal temperature for Foaz growth in the SPW with BOD_5 2000 mg/L sample at pH 7.0 is shown in Table 3.

Table 3 Comparison of thermal tolerance between Foaz and its parental strains in SPW

Strain	μ values for each strain at different temperature, h^{-1}						
	T, ℃						
	15	20	25	30	35	40	45
Y9407	0.139	0.208	0.399	0.481	0.491	0.452	0.113
P9479	0.029	0.061	0.228	0.355	0.422	0.206	0
Foaz	0.095	0.153	0.294	0.447	0.462	0.430	0

Notes: Each value is the mean of 5 samples

Table 3 shows that the acceptable temperatures for Foaz growth in SPW samples ranged from 15℃—40℃ the same as that of *R. sphaeroides* P9479. The values of μ for Foaz at 30℃ with pH 7 shown in Table 3 are higher than those in Table 2, but the μ value sequence for the three strains at 30℃ with pH 7 in Table 3 was the same to that in Table 2. It may be that the inoculation liquor of the three strains were cultivated at different temperature, the higher the cultivation temperature was, the higher the initial activity would be. The cultivation temperature for the inoculation liquor in Table 3 was 35℃ and that in Table 2 was 25℃. All the values of μ for Foaz in the range of the acceptable temperature were higher than those of *R. sphaeroides* P9479 and lower than those of *S. cerevisiae* Y9407. The optimal temperature for all the three strains, Foaz, *S. cerevisiae* Y9407 and *R. sphaeroides* P9479, were 35℃ when the specific growth rates reached their highest levels, 0.462 h^{-1}, 0.491 h^{-1} and 0.422 h^{-1}, respectively.

The values of the maximum specific degradation rate q_{max} and the half rate constant *Ks* measured for Foaz and its parental strains in SPW samples which BOD_5 concentration was from 147 mg/L to 2449 mg/L with the optimal pH 7.0 and the optimal temperature 35℃ are shown in Table 4.

Table 4 The values of q_{max} and *Ks* for Foaz and its parental strains in SPW samples

Strain	Regression equation for $1/q$ to $1/Se$	Equation of relativity* between $1/q$ and $1/Se$	q_{max}, h^{-1}	$Ks(BOD_5)$, mg/L
Y9407	$1/q = 523(1/Se) + 0.886$	$r = 0.987 > r_{(3)}0.001$, ++	1.129	590
P9479	$1/q = 406(1/Se) + 1.580$	$r = 0.993 > r_{(3)}0.001$, ++	0.634	257
Foaz	$1/q = 489(1/Se) + 1.295$	$r = 0.976 > r_{(3)}0.001$, ++	1.295	633

Notes: ++: the positive reality between $1/q$ and $1/Se$ is very significant; * The regression coefficient of $r_{(3)}0.01$ is 0.959

The value of the maximum specific degradation rate q_{max} for Foaz was 1.259 h^{-1}. It means that the amount of the pollutant degraded by Foaz was equal to its biomass of 125.9% per hour, which was higher than that of its two parental strains, and the value of the half rate constant Ks for Foaz was 633 mg/L while its specific degradation rate was equal to 1/2 q_{max} which was also higher than that of its parental strains. These imply that the hybrid Foaz got a better degradation capacity of organic pollutants than its parental strains and it needed higher concentration of BOD_5 for its growth to meet 1/2 q_{max}.

The flocculation rate of Foaz and its parental strains in SPW with BOD_5 2000 mg/L measured

are shown in Table 5.

Table 5 shows that Foaz has the highest value of flocculation rate among the three strains, 3.5%. It means that the optical density of SPW sample cultivated with Foaz decreased 3.5% after setting for 10 minutes.

Table 5 Comparison of flocculation rates between Foaz and its parental strains

Strains	Flocculation rate P, % *
Y9407	3.1
P9479	1.0
Foaz	3.5

* : From the mean value of 5 samples

4 Discussion

The hybrid Foaz obtained from the inter-kingdom protoplast fusion between the eukaryote cell of *S. cerevisiae* Y9407 and the prokaryote cell of *R. sphaeroides* P9479 was identified in resistance to antibiotics of strepmycin (Sm) and nystatin (Nt), cellular morphogenesis, biochemistry and genetics to be a real karyogamy fusant cell with stable genetic and function characteristics in LMM media and in monosodium glutamate wastewater (Cheng, 1996b; 1996c). Foaz gained the two kinds of resistance to both of the antibiotics, Sm and Nt, while one of its parents has only the resistance to one of the two antibiotics; Foaz had a cellular nucleus similar to that existing in the cell of *S. cerecisiae* Y9407 appearing in its own cell after 4 day's protoplast fusion; The volume of the cell and the DNA content of the cell were higher than those of *R. sphaeroides* P9479 but lower than *S. cerevisiae* Y9407; The structural gene pucBA of light harvesting protein from *R. spaheroides* P9407 and structure gene FLO1 of flocculation protein from *S. cerevisiae* Y9407 both existed in the hybrid cell Foaz, which were demonstrated by polymerase chain reactions(PCR) and DNA sequencing experiments (Cheng, 1995; Cui, 1996). Although the characteristics for Foaz cell are affected obviously by their parental strains, they are quite different from either *S. cerevisiae* Y9407 or *R. sphaeroides* P9479. So, it is possible for Foaz to show some new characteristics which may be different from those of its parental strains in SPW in this study.

The optimal pH and the optimal temperature for Foaz growth in SPW were the same as those of its parental strains, but the values of the specific growth rate μ for Foaz were different from those of *S. cerevisiae* Y9407 and *R. spahaeroides* P9479. It illustrated that the capacity of Foaz growth was affected by the factors from its parental strains, even if Foaz and its parental strains needed the same optimal pH and the same optimal temperature for growth.

The value of flocculation rate for Foaz cultivated in SPW was higher than either of its parental strains. It is a very important feature to be able to remove biomass of Foaz easily from the effluent of treated wastewater. The hybrid Foaz containing flocculation genes at least included FLO1 gene from *S. cerevisiae* Y9407 and it had a bigger cellular volume, which could both enhance the flocculation of cell Foaz.

The value of the maximum specific degradation rate q_{max} for Foaz in SPW was higher than that of its parental strains. It is a very useful character for the hybrid Foaz in order to treat organic wastewater effectively. The parameters of q_{max} and Ks are the key data for designing the process of organic wastewater treatment. The higher the value of q_{max} is, the higher the efficiency of treatment will be.

The conclusion for evaluation of the results for measurement of the optimal pH and temperature, the acceptable range of pH and temperature and flocculation rate in SPW for Foaz growth is the same as that of Foaz in LMM medium. And the results obtained for the measurement of q_{max} and Ks for Foaz in SPW are similar to that of Foaz in monosodium glutamate (MSG)

wastewater. It is very clear that the hybrid Foaz was a real karyogamy fusant strain with stable characteristics which may favor degrading organic pollutant in wastewater effectively and removing the biomass from effluent of organic wastewater treated.

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