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A N I N T E R N A T I O N A L J O U R N A L

Mutation Breeding of Lipase-producing Strain *Flavobacterium* sp. by Supercritical CO_2 with Hydrazine Hydrate

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ABSTRACT

This work aimed to obtain an ideal mutant strain with higher lipase yield using hydrazine hydrate (HZH) as a novel additive to treat Flavobacterium *sp. strain YY25 by supercritical CO_2. The survival rate and the positive mutation rate of the tested strain were strongly dependent on the dose of HZH. The treatment by 0.5% HZH in supercritical CO_2 (8 MPa, 35°C) for 30 min provided 58.3% of positive mutation rate and an expected mutant strain with about 76.7% increase in lipase yield compared with the wild strain. Possible mutagenesis mechanisms were further explored. The analysis on pH drop of the treated seed liquid was performed to better understand the interaction. Results proved that the induced mutation with enhanced yield of lipase could be achieved by co-mutagenesis of supercritical CO_2 and HZH.*

Key words: Supercritical CO_2, Hydrazine hydrate, Mutation, pH Drop, Lipase, *Flavobacterium* sp.

INTRODUCTION

Lipases are well-known enzymes extensively used in industrial biotransformation processes, which usually exhibit a good chemioselectivity, regioselectivity, enantioselectivity and possess a broad substrate specificity exhibiting optimum activities over a wide range of temperatures (Bertoldo et al. 2011). *Flavobacterium* sp. strain YY25 produced an intermediate temperature alkaline lipase, which had stable and higher enzyme activity in tert-butanol, a common organic medium. Fermentation for 24 h provided the strain a maximum level of lipase yield, about 16.4 u/mL. Mutations of strains may be induced by the treatment with physical, or chemical mutagens to improve lipase yield for the sake of better applications. It is known that stress is a disturbance to the normal functioning of a biological system and mutagenesis is often increased in the bacterial populations as a consequence of stress-induced genetic pathways (Tenaillon et al. 2004; Foster 2005). A previous study had demonstrated that supercritical CO_2 was a promising environmentally-friendly mutagen, or co-mutagen owing to its distinctive stress (Zhang et al. 2008).

Carbon dioxide (CO_2) is an inert, inexpensive, easily available, odorless, tasteless, environment-friendly, and GRAS (Generally Recognized As Safe) solvent. The supercritical technology is a green sustainable process in which the solvents' power and selectivity can be relatively easy to tune according to the operating conditions (Hegel et al.

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2011). CO_2 is frequently used in near-, or supercritical conditions, wherein it shows excellent mass transfer properties in terms of penetration into matrices (Valverde et al. 2010). Possibly due to assist in the permeability, supercritical CO_2 had been found to enhance the sterilization effects and/or mutagenicity of the additives (co-solvents) such as hydrogen peroxide (H_2O_2) (Zhang et al. 2006), dimethylsulfoxide (DMSO) (Zhang et al. 2008), etc. The solvent properties of supercritical CO_2 could also be drastically changed by the addition of a small amount of polar solvent as a modifier (Sicardi et al. 2000).

Hydrazine derivatives are widely used compounds in the pharmaceutical, agrochemical, polymer and dye industries, and also as precursors in organic synthesis. Many of them show significant biological activity and several are shown to be effective for the treatment of tuberculosis, Parkinson's disease and hypertension (Bredihhin and Mäeorg 2008). Some hydrazine compounds, hydrazine hydrate (HZH), phenylhydrazine (PHZ), hydralazine (HLZ) and nialamide (NLD), have been reported mutagenic in strain WP2 *uvrA*/pKM101. HZH could induce a higher level of revertants, which carried suppressor mutations resulting exclusively from G:C–A:T transitions (Blanco et al. 1998).

In order to obtain a mutation with enhanced yield of lipase, here HZH was employed to treat *Flavobacterium* sp. strain YY25 through adding straight into the seed liquid before treatment by supercritical CO_2. The co-effects of supercritical CO_2 and HZH on the survival rate and positive mutation rate of the tested strain were examined, and possible action mechanisms were explored in this work.

MATERIALS AND METHODS

Strains and culture conditions

Flavobacterium sp. strain YY25, a wild bacterium that produced extracellular lipase, was used as a tested strain. Using a selective medium, it was isolated from the fat soil sample around piggery. The strain was a Gram-negative, rod-shaped bacterium and showed yellow circular colonies on the agar plate. It was identified *Flavobacterium* based on its physiological and biochemical characteristics using automatic microorganism identification instrument. The tested strain was stored in refrigerator on agar slant (beef extract 0.5%, peptone 1%, NaCl 0.5%, agar 2%, pH 7.5).

In order to prepare the seed liquid, a loop of cells from the slant was inoculated into 50 mL of seed medium (beef extract 0.5%, peptone 1%, NaCl 0.5%, pH 7.5) and cultivated with shaking at 30°C, 200 rpm for 16h, which contained a cell concentration of about 10^8 cfu/mL. For the production of lipase, 5.0 mL of seed liquid was inoculated into 50 mL of fermentation medium (peptone 0.5%, yeast extract 0.2%, beef extract 0.3%, K_2HPO_4 0.1%, NaCl 0.1%, $MgSO_4$ 0.01%, olive oil 1%, glycerol 0.5%, Triton X-100 0.1%, pH 7.0) and cultivated with shaking at 30°C, 200 rpm for 24 h.

Mutation treatment

Various doses of HZH were added independently into the seed liquid and shaken up before supercritical treatment. HZH (0.5 mL at 64 wt.% hydrazine) in 50 mL of seed liquid expressed 1.0% content. A batch procedure was used for the treatment of the prepared seed liquid by supercritical CO_2. Figure 1 represented the scheme of the apparatus. After the system was cleaned with 75% (v/v) aqueous ethanol solution and then sterile distilled water to prevent possible contamination, 50 mL of seed liquid with HZH was loaded into the reactor with thermostat and the pipelines were linked tightly. Then CO_2 was pumped into the reactor with the high-pressure plunger-pump. The pressure and temperature were held for 30 min after the system reached 8 MPa, 35°C. Afterwards, gas-valve was opened to let out the CO_2 until a complete discharge of pressure was attained. The samples from different runs of treatments were taken out from the reactor. The control sample without HZH received the same treatment as the treated sample except that it was kept at atmospheric pressure.

Cell viability measurement

In order to explore the survival kinetics, the serial dilution-agar pour plate procedure was employed to quantify viable cells in 50 mL of seed liquid. The treated seed liquid as well as the control was diluted serially with the sterile water by a factor of ten until a moderate dilution was attained. After 1.0 mL of the moderate dilution was added into the Petri-dish, about 15 mL of agar medium (beef extract 0.5%, peptone 1%, NaCl 0.5%, agar 1.5%, pH 7.5) was poured promptly. They were mixed and incubated for 48 h at 30°C. The count of

different seed liquids was repeated in parallel for three times. The number of viable cells was expressed as cfu/mL (cfu: colony forming unit). The values of survival rate were calculated with the arithmetic means of Log (*N*/*No*) of minimum three runs, where *No* was the number of cells in the control sample and *N* was the number of cells in the treated sample.

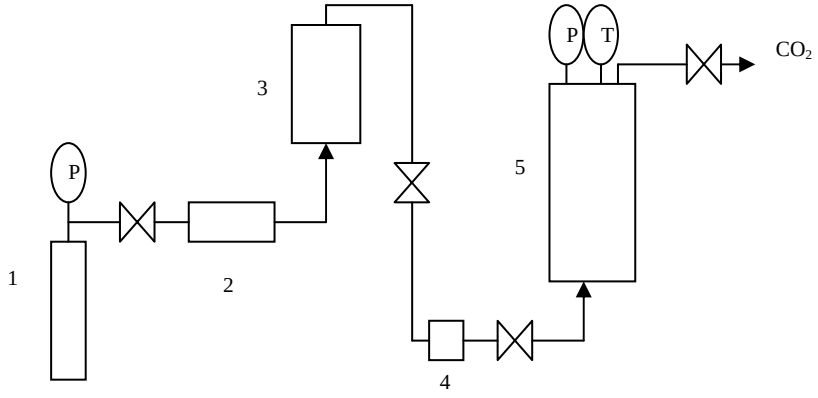

Figure 1 - Supercritical CO_2 treatment system. 1, CO_2 cylinder; 2, filter; 3, Cooling bath; 4, high-pressure plunger-pump; 5, reactor (thermostat of the pressure vessel). The labels T and P refer to the temperature probe and the pressure gauge, respectively.

Cell mutation measurement

After the treated seed liquid was poured into plate and cultivated for 48 h for the count, single colonies appeared on the agar medium plate. All the colonies from the plate of moderate colonies-number were selected individually and inoculated into the slant medium (beef extract 0.5%, peptone 1%, NaCl 0.5%, agar 2%, pH 7.5). After 48 h of culture at 30°C, they were stored at 4°C as potential mutant strains. The selected potential mutant strains as well as the wild strain were tested for lipase yield by fermentation. The positive mutation rate for each treatment was calculated as the number of the positive mutant strains divided by the number of all selected strains on the corresponding agar medium plate.

Lipase assays

At the end of fermentation, the fermentation liquid was centrifuged at 6000 g for 15 min and the clear supernatant containing extracellular lipase was obtained. The spectrophotometric method was used for rapid and routine measurement of lipase activity using pnitrophenylpalmitate (pNPP) as the substrate (Mahadik et al. 2002). The substrate solution was prepared by adding 0.1 mL of solution A (150 mg of pNPP in 50 mL of isopropanol) into 2.85 mL of solution B (2.22 g of Triton X-100 and 0.56 g of gum arabic in 500 mL of phosphate buffer, pH 9.0). The emulsion obtained remained stable for at least 2 h. The assayed mixture consisting of 2.95 mL of substrate solution and 0.05 mL of suitably diluted enzyme was incubated at 50°C for 10 min. The pNPP released was measured at 410 nm in spectrophotometer. One unit of lipase activity was defined as the amount of enzyme, which released 1.0 μmoL of pNPP in 1 min under the above assay conditions. The lipase yield of the fermentation liquid was expressed as u/mL.

Statistics

All the experiments were repeated for three to four times. The data shown in the corresponding figures were the mean values of the experiments, and it was indicated that the relative standard deviations were all within ±5%.

RESULTS AND DISCUSSION

In a previous work, 8 MPa, 35°C and 30 min was obtained as the acceptable treatment conditions of supercritical CO_2 to obtain ideal mutant strains. The mutagenicity could be intensified by adding 1.0% DMSO into the seed liquid before supercritical treatment. The corresponding positive mutation rate was 45.6%. A mutant strain with about 44.2% increase in lipase yield than the wild

strain was obtained by supercritical CO_2 and 1.0% DMSO (Zhang et al. 2008). In order to obtain a mutant strain with more productive lipase, a better substitute for DMSO was considered to be explored.

The effects of HZH on mutation in supercritical treatment

HZH is a monohydrate of hydrazine and relatively safer than anhydrous hydrazine (Satyanarayana and Bhattacharya 2004). Here, HZH was used as a potential mutagen to treat the seed liquid in supercritical CO_2 (condition: 8 MPa, 35°C, 30 min). The results in Figure 2 showed that the effects of HZH in supercritical treatment on cell were content-related. The survival of the cells decreased with the increase in HZH content and reached a minimum at 1.0%. Afterwards, the survival rate slightly increased. HZH can absorb CO_2 in the air. Apparently, in the supercritical system, higher concentration of HZH was easy to react with CO_2 into a hydrazine compound, which reduced the inactivation effects.

Results showed that 0.5 and 1.0% HZH treatments had no significant differences in the positive mutation rates, adding up to the larger values of 58.3 and 59.6%, respectively. Pure supercritical CO_2 without HZH only provided the positive mutation rates of 27.3%. Obviously, the mutation effects were strengthened greatly by 0.5%, or 1.0% HZH in supercritical treatment. More high-quality mutant strains and a selected mutant strain of maximum lipase yield, with approximately 76.7% increase than the wild strain, were obtained with 0.5% HZH treatment (Fig. 3). It suggested that 0.5% HZH was a better mutation dose than 1.0% HZH.

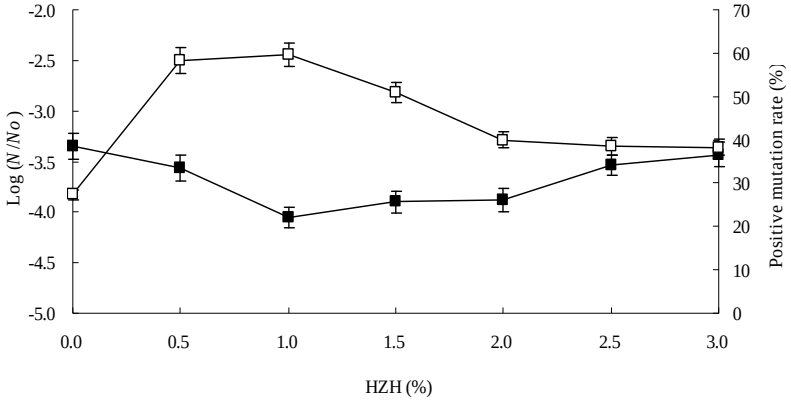

Figure 2 - Effects of HZH in supercritical CO2 on survival rate (Log (N/No)) and positive mutation rate. Legend: ■, the survival rate; □, the positive mutation rate. Conditions: pressure, 8 MPa; temperature, 35 °C; treatment time, 30 min; HZH, variable.

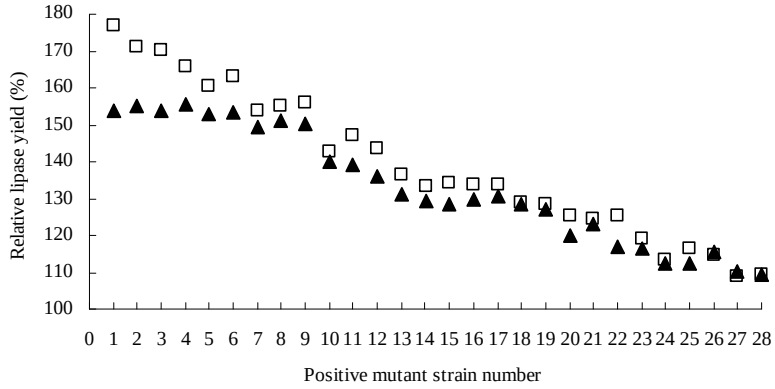

Figure 3 - Relative lipase yield of the selected positive mutant strains after 0.5% HZH and 1.0% HZH treatments, respectively. Legend: □, 0.5% HZH treatment; ▲, 1.0% HZH treatment. Conditions: pressure, 8 MPa; temperature, 35 °C; treatment time, 30 min; HZH, variable.

Although 0.5% HZH alone resulted in mutagenicity, it brought lower positive mutation rate (only 8.3%) than the case of HZH in supercritical CO_2. Perhaps supercritical CO_2 facilitated transient permeability and induced mutation function of the additive (Zhang et al. 2008). Other authors have demonstrated that the activity of some intracellular enzymes declined, or lost after supercritical treatment such as ATPase, phosphatase, peroxidase, etc (Bertoloni et al. 2006; Gui et al. 2006; Liu et al. 2008). So it was also presumed that certain relevant enzymes with lipase production might be induced, or changed by supercritical CO_2 and HZH.

HZH was proved an alternative additive of DMSO in the mutation treatment by supercritical CO_2 due to its more powerful mutagenicity. The chemical properties of HZH derive from hydrazine (Satyanarayana and Bhattacharya 2004). Hydrazine can react with the pyrimidines in DNA to form N^4-aminocytosine. The amination of cytosine may be mutagenic for bacteria. The mutations from hydrazine are probably produced by direct mis-pairing at replication rather than by error-prone repair (Kimball 1977). Here, five successive cultures of the selected mutant strain were further performed under the same fermentation conditions. All the cultures showed almost the same lipase yield, indicating the stability of the mutant genotype.

The effects of HZH on pH drop in the seed liquid

During the treatment of microorganisms by supercritical CO_2 in an aqueous medium, the dissolution of CO_2 into the liquid phase is a natural process that leads to a pH decrease in the medium. Therefore, pH drop had been considered to be one key in the supercritical inactivation of microorganisms (Kim et al. 2007; Bortoluzzi et al. 2011). Here, a pH meter was employed to measure the pH value of the seed liquid before and after the treatment by HZH and supercritical CO_2. The results of pH drop (Fig. 4) showed three stages: the stable first stage, the second stage of a rapid fall, and the third stage of a slight rise.

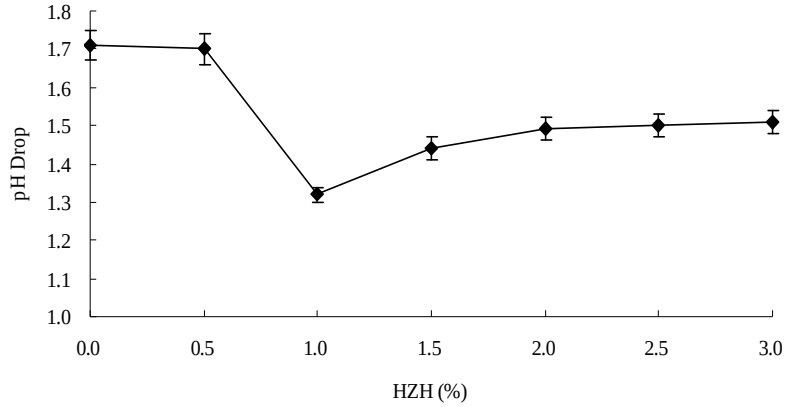

Figure 4 - Effects of HZH in supercritical CO2 on pH drop of the seed liquid. Conditions: pressure, 8 MPa; temperature, 35 °C; treatment time, 30 min; HZH, variable.

HZH is a polar solvent and can offer an alkaline environment for the seed liquid because of the highly alkaline nature of hydrazine (Satyanarayana and Bhattacharya 2004). The 0.5% HZH alone resulted increase in the pH in the seed liquid (data not shown). But 0.5% HZH in the supercritical CO_2 had almost the same pH drop as without HZH. The change of pH drop reflected a balance between the dissolution of CO_2 and the reaction of HZH with CO_2 in an aqueous medium. Low dose of HZH (0.5%) had hardly any effect on the medium environment. Apparently the mutagenic function was derived from the generated compound of HZH and CO_2.

Supercritical CO_2 can cause the chemical modification of the lipid double-layer of cell membrane, thus increasing dramatically its permeability, which contributes to interaction with the intracellular materials (Spilimbergo et al. 2007). Higher doses of HZH (1.0%, or above) had

less pH drop than pure supercritical CO_2. They produced more hydrazine molecules to neutralize the acidification of CO_2. It was presumed that HZH surrounding the cells affected the action of supercritical CO_2 and then the potential mutagenesis path from the modification of membrane was blocked, which reduced the mutation effects.

CONCLUSIONS

The effects of HZH in supercritical treatment on the survival rate and positive mutation rate of the tested strain were related to its concentration. The analysis on pH drop of the treated seed liquid provided some possible explanations for the co-mutagenesis mechanisms. An expected positive mutant strain with higher lipase yield (about 29.0 u/mL) was obtained by the synergic action of HZH (0.5%) and supercritical CO_2 (8 MPa, 35°C) on the seed liquid for 30 min. As far as mutation was concerned, HZH was a promising additive in supercritical treatment and supercritical CO_2 was a potential co-mutagen as well.

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