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Biodegradation of furfural by *Bacillus subtilis* strain DS3

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Abstract

An aerobic bacterial strain DS3, capable of growing on furfural as sole carbon source, was isolated from actived sludge of wastewater treatment plant in a diosgenin factory after enrichment. Based on morphological physiological tests as well as 16SrDNA sequence and Biolog analyses it was identified as *Bacillus subtilis.* The study revealed that strain DS3 utilized furfural, as analyzed by high-performance liquid chromatography (HPLC). Under following conditions: pH 8.0, temperature 35°C, 150 rpm and 10% inoculum, strain DS3 showed 31.2% furfural degradation. Furthermore, DS3 strain was found to tolerate furfural concentration as high as 6000 mgl^{-1}. The ability of *Bacillus subtilis* strain DS3 to degrade furfural has been demonstrated for the first time in the present study.

Key words

Bacillus subtilis, Biodegradation, Furfural

Introduction

In diosgenin industry, furfural and its derivatives mainly contribute to organic pollutants in wastewater, due to carbohydrate ingredient in *Dioscoreae zingiberensis* which forms a raw material of diosgenin industry. Furfural is produced from hemicellulose in *Dioscoreae zingiberensis* (Sangarunlert *et al*., 2007) by acid hydrolysis of pentosan, followed by acid catalyzed dehydration of pentoses at high temperature. Pentosan is a carbohydrate generally found in bagasse, peanut shells, sorghum rod, oat hulls and corn-cobs. It is dehydrated to form furfural by hot acid. Furfural (2-furaldehyde, CAS number 98-01-1) is an important chemical in petroleum industry and used in several chemical processes. It is mainly obtained from lignocellulosic plant materials, such as oat husk or corn-cobs, by treating them with hot sulfuric acid (Almeida *et al*., 2009). It is a byproduct produced from the production and storage of fruit juices, wines, and medical solutions (Christopher *et al*., 1991). Furfural can contaminate the product streams of lignocellulose breakdown in production of sugar from biomass. This contaminant inhibits the fermentation of glucose, and thus prevents efficient formation of the product. Furfural is the most widespread simple furan in environment (Almeida *et al*., 2009). It is an aromatic aldehyde, with cycle structure, known as one of the toxic fermentation inhibitors (Okuda *et al*., 2008). They damage microbes by reducing enzymatic and biological activities, breaking down DNA and inhibiting protein and RNA synthesis(Abdulrashid *et al*., 1997). Furfural has been found to decrease the growth rate and ethanol production rate after pulse addition in both anaerobic and aerobic batch cultures during fermentation(Zhang *et al*., 2010).

Some cyclic and aromatic organic compounds such as phenols and furfural have long life and get decayed and decomposed slowly in natural environment. Report are available on physical and chemical processes, such as photodegradation by UV combined with H_2O_2, pretreatment with Fenton's reagent, which are applied for furfural removal (Borghei *et al*., 2008). They are however costly and inappropriate for large wastewater treatment plants. Bioremediation is potentially a low-cost and efficient way for removal of contaminants, especially organic compounds (Waterston *et al*., 2005).

Application of bacterial strains to utilize furfural and derivatives has been reported in recent years. They mainly focus on detoxification by removing furfural from wastewater and biomaterial hydrolysate which are rich in fibers. Simultaneously, biotransformation of furfural and derivatives to obtain metabolite is a hot issue in agricultural and food chemistry industry. Under aerobic conditions, many bacteria transform or degrade furfural

(Hong *et al*., 1981; Zhang *et al*.,2011; Loi *et al*., 2011). Furfural is transformed to furfuryl alcohol by *Saccharomyces spp*. (Boopathy *et al*., 1991). Similarly degradation of furfural under anaerobic conditions by sulfate-reducing bacteria has been reported by Koenig (1989). However, the highest furfural tolerance of anaerobic bacteria was reported to be 25mM, respectively (Koenig *et al*., 1989). In few sewage treatments plants, presence of furfural in wastewater has been found to limit the growth and practical application of other microbes in the plant (Zhao *et al*., 2005). As there is limited information about the potential of furfural degrading aerobic bacteria, more efficient biological degradation processes of furfural needs to be developed for industrial applications. In view of the above, a Gram-positive strain DS3 capable of effectively utilizing furfural as sole carbon source was isolated and identified under aerobic condition in the present study. Furfural degradation with continuous process was also examined under different initial concentration, pH and inoculum density.

Materials and Methods

Medium composition : Mineral salt medium (MSM) was prepared using 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 1.0 g NH_4Cl, 0.05 g $CaCl_2$. Solid plates contained 3 g beef extract, 10 g peptone and 5 g NaCl (per litre). pH was adjusted to 7.0 and medium was sterilized by autoclaving at 121°C for 20 min. Solid media plates were prepared by adding 15 g agar into above liquid media.

Isolation and identification of strain : The activated sludge used in the present study was collected from wastewater treatment plant of a diosgenin factory, located in Shiyan City, Hubei province, China. 2g of sludge was inoculated in 250 ml flask containing 100 ml sterile water and 0.01% sodium pyrophosphate (w %) in a rotary shaker aseptically at 150 rpm, in order to smash zoogloea. 0.5ml of this mixture was put into fresh MSM containing furfural (500mg l^{-1}) as sole carbon source. The carbon source, was added through a 0.22µm filter sterilized into the mineral medium after auto claving. It was incubated at 30 °C in a rotary shaker at 150 rpm till it became turbid. This process was carried out in dark to prevent photooxidation of furfural. The steps were repeated till the liquid became clarified and degradation of furfural came to a stable level. The culture was then diluted and spread onto solid beef extract peptone plates(agar 1.5%), which was incubated at 30°C for 4-5 days. The spread–plate method was used to isolate pure culture. Colonies appearing on agar plates were purified and selected to verify their degrading capacity. Some of the most efficient isolates were selected. The present study focused on *Bacillus subtilis* genus strain DS3.

Phylogenetic analysis : Biologmicrostation, a standardized micromethod, was applied for utilization of carbon source by strain DS3. The strain was identified according to Bergey's Manual of Determinative Bacteriology. A 16S rDNA gene was amplified by polymerase chain reaction (PCR) with universal primer pair 20F (50-AGA-GTTTGATCCTGGCTCAG-30) and 1500R (50-GGTTACCTTGTTACG- ACTT-30)(Gu*et al*., 2007). The conditions for PCR were as following: 5 min of denaturation at 94°C, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 90 sec and final extension at 72°C for 10 min. After purification by agarose gel electrophoresis, PCR fragments were ligated into linearized vector pMD18-T (TaKaRa Biotechnology, Dalian, China) and transformed into *Escherichia coli* DH5a. An automatic sequencer (Applied Biosystems. model No. 3730) was used to obtain 16S rDNA sequences using sequencing primers M13-47 (50-CGCCAGGGTTTTCCCAGTC A GAC-30) and RV-M (50-GAGCGGATAACAATTT CACA CAGG-30) (Jia *et al*., 2006). Alignment of 16S rDNA sequences from GenBank was performed using ClustalX 1.8.3 with default settings. Phylogenesis was analyzed using MEGA version 5.1 software. Distances were calculated using Kimura 2-parameter distance model. Phylogenetic trees were built using Neighbor-Joining method. Dataset was bootstrapped 1000 times.

Tolerance of isolated strain to furfural : The growth of strain was determined at 600nm absorbance. For assessing the potential of strain to grow on MSM with furfural, isolated strain was inoculated in 50 ml of MSM with furfural and incubated at 30°C at 150 rpm for 4 days. Subsequently, 0.5 ml of culture was inoculated on agar plates. These were incubated at 35°C under darkness for 4 days. The concentration of furfural ranged from 500-6000 mg $^{-1}$ and the growth of strain was observed and noted.

Biodegradation experiment of furfural : 1ml of culture in the late exponential phase was added to 250 ml flask with 50 ml MSM containing different concentrations of furfural. The cultures were incubated in a rotary shaker(150rpm) at 35°C. A duplicate culture without strain was used as control. All the culture flasks were covered with black papers and kept in dark to prevent photo degradation (Borghei *et al*., 2008). Each experiment was performed in triplicate.

pH of the medium was adjusted with 5M HCl or 10M KOH in order to obtain a pH range between 5.0 and 10.0. Various volumes of furfural were added into 50 ml medium, after filtration and sterilization by a 0.22 µm filter, to obtain the desired furfural concentration between 500 and 4000 mgl^{-1}. Different volumes of inoculum were added directly to 50 ml medium to obtain desired inoculum concentration density between 2% and 10%.The culture was centrifuged for 15 min at 4000 rpm to separate the cells from it.

Chemical analytical methods : Bacterial growth was detected through medium turbidity by spectrophotometer (UNIC-7200) at 600 nm. All the samples were then filtered through a 0.22 µm Millipore membrane filter and analyzed by HPLC, equipped with Hypersil ODS C_{18}5µm silica column (250×4.6 mm. i.d.) (Sepax Technologies, USA). Separation was carried out at room

temperature. The mobile phase was acetonitrile/water (5:95 v/v), and the flow rate was 1 ml min^{-1}. The injection volume was 20 µl and ran for 18min. Detection was carried out using a UV detector at wavelength 284 nm (Ferrer *et al*., 2002). The concentration of furfural was calculated on the basis of peak area from calibration curve. Each analysis was performed in triplicate.

Data analysis : The data represent standard deviation values of two experimental repetitions where in triplicate samples were processed for every study.

Results and Discussion

After continuous enrichment and screening procedure for more than 40 days, DS3 strain was isolated and inoculated in MSM with furfural concentration ranging from 500-6000 mg, to the capacity of furfural degradation. The results showed that strain that was able to degrade furfural under aerobic condition was designated as DS3. It was Gram-positive and catalase-positive bacterium. The microscopic examination showed that strain DS3 was bacilliform. After 24 hr of incubation on agar plates, colonies of strain DS3 were white or pale yellow, opaque, flat, round and 2-5 mm in diameter. When incubation lasted for more than 48 hr, the surface of the colonies became dry and rough, with irregular appearance. The fold was seen at middle of single colony with increase in incubation time. Scanning electron microscopy, revealed that individual cell was rod-shaped and about 2-3 µm long (Fig. 1). Liquefaction of gelatin reaction of strain DS3 was positive, so was the liquefaction of gelatin, starch hydrolysis, Voges-Proskauer test, hydrolysis of casein and sugar fermentation test, whereas citrate unilization reaction and maltose fementatian tests were negative. These results indicated that strain DS3 resembled a member of *Bacillus subtilis.*

16S rDNA sequence of strain DS3 was 1437bp and was deposited in Genbank and released with Accession No. KJ641588. Fig. 2 shows the phylogenetic relationship between strain DS3 and other types of strains available in the Genbank database. Combined with the result of Biolog analysis, it was noted that strain DS3 was similar to *Bacillus subtilis.* Phylogenetic tree indicated that the strain had close relationship with *Bacillus subtilis,* which was also supported by the morphological and physiological properties.DS3 strain was identified as *Bacillus subtilis.* However, bacteria of genus *Bacillus subtilis* have not yet been reported to degrade furfural.

The study showed that strain DS3 utilized furfural as sole carbon source. As shown in Fig. 3, DS3 strain in the presence of 500-4000 mgl^{-1} furfural showed better growth than 6000 mgl^{-1} furfural in as a consequence of possible inhibition by high concentration of furfural. Growth distinction among initial furfural concentration from 500 mgl^{-1} to 4000 mgl^{-1} was slight. Meanwhile, the growth of strain DS3 decreased obviously while furfural concentration was at a higher level on account of substrate

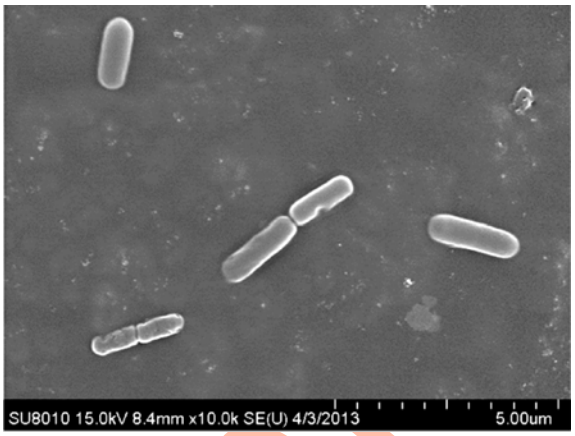

Fig. 1 : Scanning electron microscopic photograph of strain DS3

inhibition (Haddadi *et al*., 2013). The substrate inhibition led to prolongation of growth lag phase of DS3 strain. It was reported that furfural at concentrations ranging from 1 mM to12 mM can also be used as a germicide(Boopathy et al., 2009) by inhibiting the growth and fermentation of microorganisms (Agbogbo and Wenger, 2007). Furfural can severely affect the rate of ethanol production and final conversion at concentration of 3-15 mM, thus creating an unwanted limitation in ethanol production processes (Gutiérrez*et al*., 2006). Boopathy *et al*. (2009) reported that *Methanococcus* sp. strain B was not inhibited by furfural at 15 mM to 20 mM concentration, while 25 mM to 30 mM furfural concentration inhibited growth of *Methanococcus* sp. strain B. In the same sampling site, another aerobic bacterium was found to have same capability to degrade furfural, which showed tolerance to 4000 mg l^{-1}furfural (Zheng *et al*., 2014). In the present study, strain DS3 exhibited much more excellent furfural tolerance than the reported degradation strains(Boopathy*et al*., 2009).

The effect of pH on furfural degradation is shown in Fig. 4. Biodegradation of furfural occurred mainly on 3-5 days of incubation. Furfural degradation by DS3 was investigated in this study, while medium pH ranged from 5.0-10.0 and the initial furfural concentration was 1000 mgl^{-1}. Obviously pH 8.0 was optimal (with 29.68% removal). Low removal of furfural was noted at pH 5.0 and 10.0 due to growth inhibition of strain DS3 as a consequence of enzyme that catalyzed furfural degradation. However, the pH of actual diosgenin wastewater in biochemical system, from which the strain was obtained, was about 1.0-2.0. The strain thrived in such acidic condition, but showed better degradation at pH 8.0. Degradation of furfural by strain DS3 can be achieved at a wide pH range, and a weak alkaline or neutral aqueous condition is preferable, which is of great significance in practical bioremediation, as an acid environment may lead to corrosion of equipment in wastewater treatment.It is well known that genus *Bacillus* can tolerate a wide range of pH in water (Kubo *et al*., 2001).

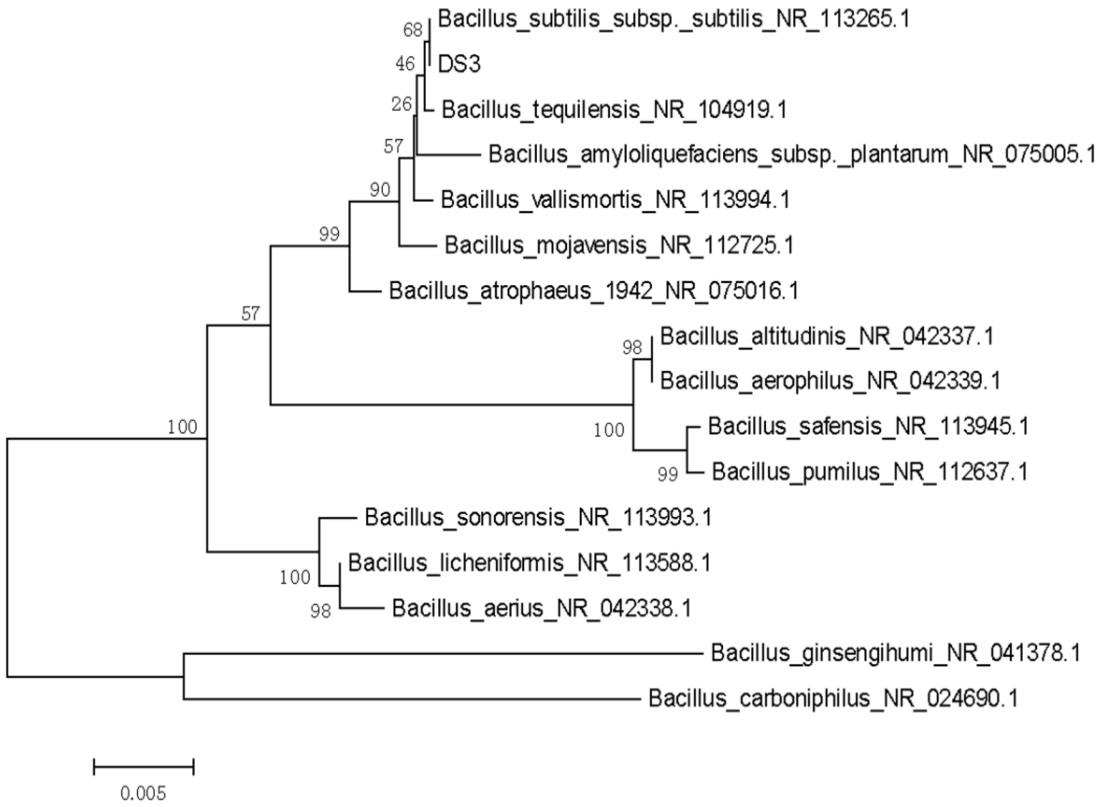

Fig. 2 : Phylogenetic analysis of DS3 isolate and related species by Neighbor Joining method. Bootstrap values (%) are indicated at nodes. The scale bars represent 0.005 substitution/site. The GenBank accession number for each microorganism used in the analysis is shown in parentheses after species name

Fig. 5 shows that degradation of furfural by strain DS3 at different concentration ranged from 500 mgl^{-1} to 4000 mgl^{-1}. Strain DS3 was capable of growing up to 6000 mgl^{-1} furfural but did not show good growth at initial concentration condition(in Fig. 3). When 500 mgl^{-1} furfural was applied, furfural removal was low in the beginning. As shown in Fig. 5, the lag phase was extended at higher furfural concentration. 20%-30% furfural was degraded on 3-5 days, and the removal became stable on 6-7days. Strain DS3 did not mineralize the substrate completely regardless of initial furfural concentration. Incomplete furfural degradation, in case of various substrate concentrations, was probably due to accumulation of possible toxic metabolites.

As shown in Fig. 6, degradation of furfural by strain DS3 at different inoculum density ranged between 2% to 10%. Obviously, furfural degradation rate varied in direct ratio with the inoculum density of strain DS3. Strain DS3 showed best degradation ability when the inoculum density was 10%. A large amount of strains participated in the reaction to degrade furfural. In general, mass metabolites discharged into the media may affect the activity and application of strains (Lu *et al.*, 2014).But it did not appear in the present study. When inoculum density was 10%, metabolites were not enough to inhibit growth and degradation of strain DS3. The strain showed 31.2%removal at optimal inoculum density of 10% on 7th day. The removal ratio may get promoted if inoculum density increased more than 10% in the further study, but it would cost more due to high inoculum density, and lead to lack of nutrition for microbes.

Bacillus subtilis has been widely employed in wastewater treatment, mainly focusing on heavy-metal ion absorption such as cadmium and uranium. It has also been used for removal of organic compounds like, naphthalene and polycyclic aromatic hydrocarbons (PAH) in soil (Susan *et al.*, 1993). It exists extensively in the natural environment and reproduces rapidly. This strain is not sensitive to environment, which is beneficial for application in wastewater treatment and chemical industry.

The hypothetical catabolic pathway, based on substrate specificity of furfural-degrading culture, has been proposed (Koenig *et al.*, 1990). It states that furfural is firstly oxidized to 2-furoic acid, utilizing NAD^+ or dichlorophenol-indophenol plus

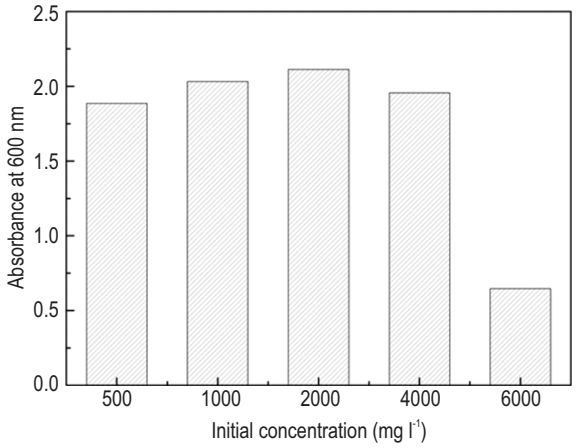

Fig. 3 : Effect of furfural concentration on growth of DS3

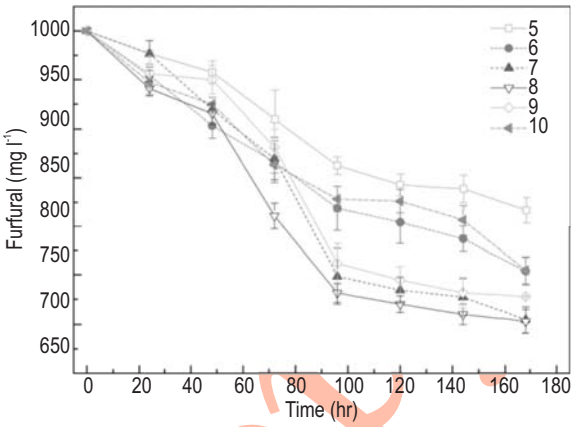

Fig. 4 : Effect of pH on biodegradation of furfural

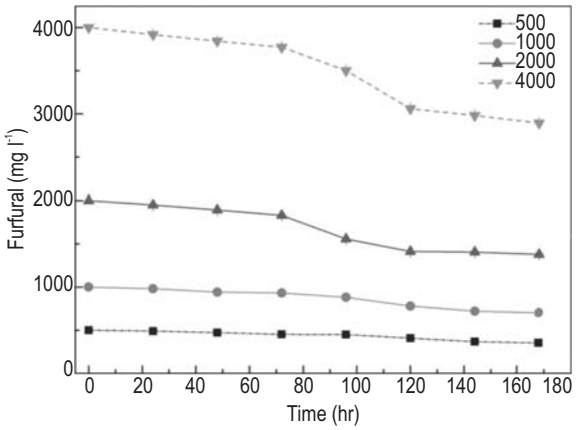

Fig. 5 : Effect of initial concentration on biodegradation of furfural

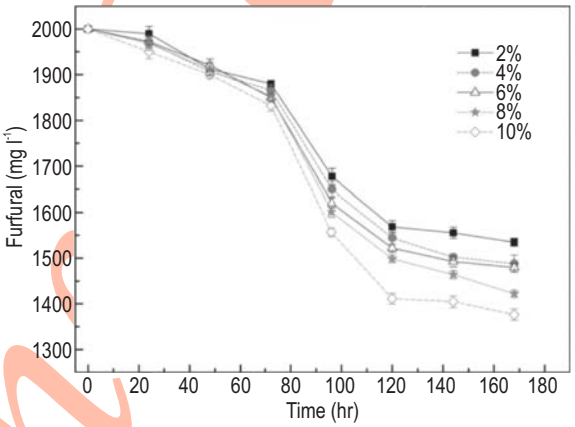

Fig. 6 : Effect of inoculums density on biodegradation of furfural

phenazine methosulfate as electron acceptors by enzyme from *Pseudomonas putida* Fu1, belongs to *Saccharomycetes*. Meanwhile, *Bacillus cereus* participates in degradation of organic compounds by several enzymes such as protease, lipase and amylase, produced extracellularly *(*Susan *et al*., 1993*)*. The reaction of enzyme leads to the effects of several factors above (pH, initial concentration and inoculums density) on furfural biodegradation of aerobic bacterium strain DS3 possibly.

Bacillus subtilis strain DS3 capable of growing on furfural as sole carbon source was isolated from actived sludge showed more efficiency than that reported before (Fallico *et al*., 2008; Boopathy, 2009). Under optimum condition of pH 8.0, 35°C , 150rpm and inoculum of 10%, the strain showed furfural degradation ability of 31.2%. Which indicated that bacterium isolates had great potential for biological detoxification.

Acknowledgments

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