Acta Scientiarum
http://periodicos.uem.br/ojs
ISSN on-line: 1807-8664
https://doi.org/10.4025/actascitechnol.v48i1.74265

The Use of Supercritical CO_2 with Additives to Enhance Starch-Digesting Enzyme Activities in *Bacillus subtilis*

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ABSTRACT. Supercritical carbon dioxide (SC-CO_2) has been proposed as a sterilization technique and utilized as a breeding method to enhance the activity of enzymes produced by microorganisms by treating their vegetative cells. This study explores whether this breeding method could be applicable to bacterial spores. Spores of *Bacillus subtilis* were treated with SC-CO_2 in the presence of additives such as dimethyl sulfoxide (DMSO) or hydrogen peroxide (H_2O_2). Various concentrations of these additives were employed to screen for strains exhibiting increased starch-digesting enzyme activity. The resulting strains, D2-5 and H2-1, demonstrated improved starch-digesting enzyme activities after SC-CO_2 treatment with 2% DMSO or 0.6% H_2O_2, respectively, which were 74.7% and 67.3% higher than the wild-type strain. Eight successive subcultures of these two strains indicated their hereditary stability. The findings demonstrated that SC-CO_2, in combination with specific additives, could effectively increase the activity of enzymes produced by microorganisms when their spores were treated.

Keywords: Mutation breeding; dimethyl sulfoxide; hydrogen peroxide; spore.

Received on November 18, 2024.
Accepted on April 25, 2025.

Introduction

Supercritical carbon dioxide (SC-CO_2) refers to CO_2 in a state where the temperature and pressure exceed its critical point values (Tc = 31.05°C, Pc = 7.39 MPa), resulting in a homogeneous single phase (Soares et al., 2019). It possesses the remarkable capability to permeate solids akin to a gas, while simultaneously exhibiting solvent properties similar to a liquid, enabling it to dissolve materials (Garcia-Gonzalez et al., 2007). SC-CO_2 can readily penetrate into cells and interact with intracellular components to induce microbial inactivation (Dillow et al., 1999). Additionally, CO_2 has the potential to interact with water present within cells, forming carbonic acid, which reduces the intracellular pH level and deactivates cellular components such as enzymes (Silva et al., 2016). SC-CO_2 is environmentally benign, nontoxic, nonflammable, noncorrosive, cost-effective, and readily available (Foster et al., 2003; Gong et al., 2023; Obaidullah & Almehizia, 2023). It employs mild operating conditions, especially at lower temperatures, compared to traditional steam sterilization. Therefore, it has been proposed as an alternative pasteurization technique suitable for heat-sensitive products, such as food, medicine, and biomaterials (Brunner, 2005; Cheftel, 1995; Damar & Balaban, 2006; Foster et al., 2003; Garcia-Gonzalez et al., 2007; Silva et al., 2016; Spilimbergo & Bertucco, 2003; Zhang et al., 2006 A). Since the first work published by Kamihira et al. (1987), it has received special attention for over two decades.The biocidal effects of SC-CO_2 have been investigated on various bacteria and fungi with vegetative forms and spores (Calvo & Casas, 2018; Damar & Balaban, 2006; Efaq et al., 2017; Garcia-Gonzalez et al., 2007; Silva et al., 2016; Spilimbergo & Bertucco, 2003; Zhang et al., 2006 A). SC-CO_2 can effectively reduce many vegetative bacteria to the extent that sterilization can be achieved, but it has poor effects on bacterial spores. Spores are the most resistant form of microorganisms and are less susceptible to CO_2 compared to vegetative cells (Spilimbergo & Bertucco, 2003). Some studies have reported conflicting conclusions regarding spore inactivation by SC-CO_2, which may be due to differences in treatment conditions, systems, and types of organisms (Damar & Balaban, 2006). The inactivation ratios vary from 0.5 to 8-log for spores of different microorganisms. Total inactivation of spores by SC-CO_2 can be achieved under high temperatures with long treatment times, or by combining it with other approaches, such as an acid environment (Haas et al., 1989), micro-bubble method (Ishikawa et al., 1997), pressure cycling (Dillow et al., 1999), and adding specific additives (Hemmer et al., 2007; Seok & Kim, 2013; Shieh et al., 2009; Silva et al., 2016; White et al., 2006; Zhang et al. 2006B; Zhang et al. 2006C). Notably, when sterilization is not completely

achieved, some cells survive, and mutagenesis may occur, especially when additives are included in the SC-CO_2 treatment. Under these circumstances, besides CO_2, the additives may enter the cell with SC-CO_2 and react with intracellular substances. Previous studies have reported the use of SC-CO_2 with or without additives to increase the lipase activities of *Flavobacterium* sp. YY25 when treating vegetative bacteria (Zhang et al., 2008; Zhang et al., 2013).

In this study, spores of *Bacillus subtilis* were treated with SC-CO_2 in the presence of additives, such as dimethyl sulfoxide (DMSO) or hydrogen peroxide (H_2O_2). Strains with improved starch-digesting enzyme activity were selected from the surviving species. The aim was to demonstrate that SC-CO_2 could effectively increase the enzyme activities of microorganisms when bacterial spores were treated.

Materials and methods

Strains and preparation of spore suspension

The starch-digesting enzyme-producing strain of *B. subtilis* K2 was stored in our laboratory. The strain was maintained on agar slants with a medium composed of 0.3% beef extract, 1% peptone, 0.5% NaCl, and 2% agar at pH 7.0. Spore suspension was prepared according to Furukawa et al. (2006). The culture of *B. subtilis* K2 was grown at 37°C for 7-8 days on sporulation agar plates with the aforementioned medium. The spores were harvested by flooding the culture surface with sterile saline solution and scraping it with a sterile inoculating loop. The spores were then washed three times with fresh sterile saline, resuspended in the same sterile solution, and stored at 4°C. The suspension was diluted to approximately 10^8 colony-forming units per milliliter (CFU mL^{-1}) using the standard plating technique described below for SC-CO_2 treatment.

SC-CO_2 treatment

A stainless steel pressure vessel equipped with a temperature control system was used. The vessel's temperature was maintained by a thermostatic bath, and a pressure transducer monitored the internal pressure. A high-pressure plunger pump delivered CO_2 into the vessel. Commercially available CO_2 with 99.5% purity was used and filtered through an active carbon filter before entering the pressure vessel. Prior to each treatment, the system was cleaned with 75% ethanol and sterile distilled water. Subsequently, 30 mL of the spore suspension, along with an appropriate amount of additive, was introduced into the pressure vessel, which was then sealed. The vessel temperature was raised to the desired value, followed by pumping CO_2 into the vessel until the required pressure was achieved, at which point the treatment duration was recorded. Upon completion of the SC-CO_2 treatment, the gas valve was opened to release the CO_2, ensuring complete depressurization. The samples from the various treatments were then retrieved from the vessel.

Cell viability measurement

The enumeration of viable spores in both treated and untreated samples was conducted using the standard plating technique on solid medium with a serially diluted spore suspension in physiological saline solution. The solid medium had the same composition as the previously mentioned agar slant medium. An aliquot of 0.1 mL from each dilution was inoculated onto the medium and incubated at 37°C for 48 h. The survival rate was expressed as $\log(N/N_0)$, where N_0 represented the initial microbial count in the untreated sample, and N denoted the microbial count in the treated sample. The enumeration process was repeated three times for different samples.

Selection of strains with improved starch-digesting enzyme activities

Strains exhibiting enhanced starch-digesting enzyme activities were obtained through an initial plate screening followed by a flask test. During the plate screening, the treated and untreated spore suspensions, suitably diluted with physiological saline solution, were spread over selection agar plates. The selection medium comprised 0.2% soluble starch, 1% peptone, 0.5% beef extract, 0.5% NaCl, and 2% agar. After incubation at 37°C for 48 h, colonies with large ratios of the clearing zone diameter to colony diameter (Z/C values) were identified as potential strains. For the flask test, the selected potential strains were inoculated into 150-mL flasks containing 50 mL of growth medium and incubated anaerobically at 200 rpm and 37°C for 48 h. The growth medium consisted of 0.3% beef extract, 1% peptone, and 0.5% NaCl at pH 7.0. The fermentation supernatant served as the starch-digesting enzyme solution. The enzyme activities of the selected potential strains and the wild-type strain were evaluated under identical conditions. A potential strain was identified if its starch-digesting enzyme activity exceeded that of the wild-type strain by more than 10%.

Assay of starch-digesting enzyme activity

The starch-digesting enzyme activity was evaluated using the method of Yoo et al. (1987) with some modifications. Specifically, a mixture containing 5 mL of 0.125% (w/v) soluble starch in phosphate buffer (pH 6.0, 0.1 mol L^{-1}) and 0.5 mL of enzyme solution was incubated at 40°C for 5 min. To stop the reaction, 5 mL of 0.1 mol L^{-1} H_2SO_4 was added. After thorough mixing, 0.5 mL of the resulting mixture was combined with 5.0 mL of iodine solution (5 mg mL^{-1} I_2 and 50 mg mL^{-1} KI). Absorbance was then measured at 620 nm after color development. One unit of starch-digesting enzyme activity was defined as the amount of enzyme capable of degrading 1 µg of starch per minute under the assay conditions.

Results and discussion

Effect of DMSO on SC-CO_2 treatment: Survival rate curve

DMSO can react with proteins and disrupt the orderly arrangement of intercellular lipids. Here, DMSO was introduced to the SC-CO_2 treatment of the spore suspension of *B. subtilis*. The treatment conditions were set at 8 MPa, 37°C, and 30 min according to a previous study (Yan et al., 2013). Figure 1 showed the survival curve of *B. subtilis* spores at different

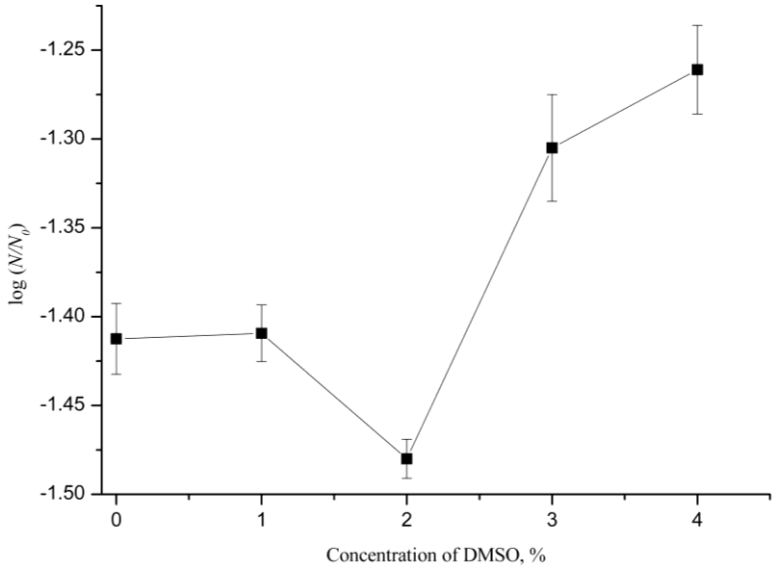

Figure 1. Effect of concentration of DMSO on survival rate of spores of *B. subtilis* K2.

concentrations of DMSO. In the first stage, the survival rate remained relatively stable when the concentration of DMSO increased from 0% to 1%. In the second stage, it decreased as the DMSO concentration increased from 1% to 2%. However, in the third stage, when the concentration of DMSO further increased to 4%, it actually increased. The survival trendline of *B. subtilis* spores was similar to that of the vegetative cells of *Flavobacterium* sp. strain (Zhang et al., 2008). It was suggested that relatively high concentrations of DMSO (> 2%) might protect cells from inactivation in SC-CO_2 (Zhang et al., 2008).

Screening strains with improved starch-digesting enzyme activities

The starch selection plate was used to initially screen potential strains as described above. Table 1 exhibited the average diameters of colonies, the average diameters of clearing zones, and the average Z/C values of the surviving strains. The average diameters of colonies increased after SC-CO_2 treatment with 0-2% DMSO but slightly decreased after treatment with 3-4% DMSO. Both the average diameters of clearing zones and the average Z/C values increased significantly after SC-CO_2 treatment with any tested concentration of DMSO. About five colonies with the largest Z/C values after each treatment were selected for further screening using the flask test and starch-digesting enzyme activity assay. Table 2 showed that approximately 44% of the selected strains (11 out of 25) exhibited increased starch-digesting enzyme activities by more than 10%. The observed increase in enzyme activity is likely due to the synergistic effects of SC-CO_2 and additives on spore structures and enzymes. SC-CO_2 can penetrate spore coats, disrupting

protective barriers and altering the internal environment of the spores. Meanwhile, DMSO may induce conformational changes in enzymes. The combination of these effects from SC-CO_2 and DMSO may promote mutagenesis, leading to enhanced activity. The strain D2-5, which survived the treatment with 2% DMSO, displayed the maximum improved enzyme activity of 74.7% with a specific starch-digesting enzyme activity of 3756 U mL^{-1}.

Table 1. Plate screening results after SC-CO_2 treatment with DMSO.

Concentration of DMSO (%)	Average diameter of colony (mm)	Average diameter of clearing zone (mm)	Average Z/C value
untreated	5.9	17.6	3.0
0	6.8	25.0	3.7
1	7.2	24.8	3.4
2	7.6	24.7	3.2
3	5.9	23.3	4.0
4	5.5	24.0	4.4

Table 2. Starch-digesting enzyme activities of the selected strains after SC-CO_2 treatment with DMSO.

Strain	Concentration of DMSO (%)	Enzyme activity (U mL^{-1})	Increase in enzyme activity (%)
the wild stain	–	2150	–
D0-1	0	2221	3.3
D0-2	0	2138	-0.6
D0-3	0	2344	9.0
D0-4	0	2253	4.8
D0-5	0	2373	10.4
D1-1	1	2310	7.4
D1-2	1	2526	17.5
D1-3	1	2280	6.1
D1-4	1	2555	18.8
D1-5	1	2400	11.6
D2-1	2	2433	13.2
D2-2	2	2896	34.7
D2-3	2	2225	3.5
D2-4	2	2701	25.6
D2-5	2	3756	74.7
D3-1	3	2070	-3.7
D3-2	3	2469	14.8
D3-3	3	2343	9.0
D3-4	3	2250	4.7
D3-5	3	2130	-0.9
D4-1	4	2234	3.9
D4-2	4	2057	-4.3
D4-3	4	2465	14.7
D4-4	4	2340	8.8
D4-5	4	3093	43.9

Effect of H_2O_2 on SC-CO_2 treatment: Survival rate curve

H_2O_2, a potent oxidizing agent commonly used in medical disinfection, was employed as an additive to treat the spore suspension of *B. subtilis*. Figure 2 depicted the survival curve of B. subtilis spores at varying H_2O_2 concentrations. The curve exhibited two distinct stages: an initial stable phase followed by a subsequent stage marked by rapid inactivation. As the H_2O_2 concentration increased from 0 to 0.6%, the survival rate of *B. subtilis* spores remained relatively stable. However, with an increase in H_2O_2 concentration from 0.6% to 1.2%, there was a sharp decline in the survival rate. Similarly, it was easier to inactivate spores of *Bacillus pumilus* (Zhang et al., 2006B), *Bacillus atrophaeus* (Zhang et al., 2006C), and *Geobacillus stearothermophilus* (Hemmer et al., 2007) using relatively high concentrations of H_2O_2. This might be because higher concentrations of H_2O_2 enhanced the penetration of SC-CO_2 across cell walls and through cytoplasmic membranes to the spore core (Shieh et al., 2009; Zhang et al., 2007).

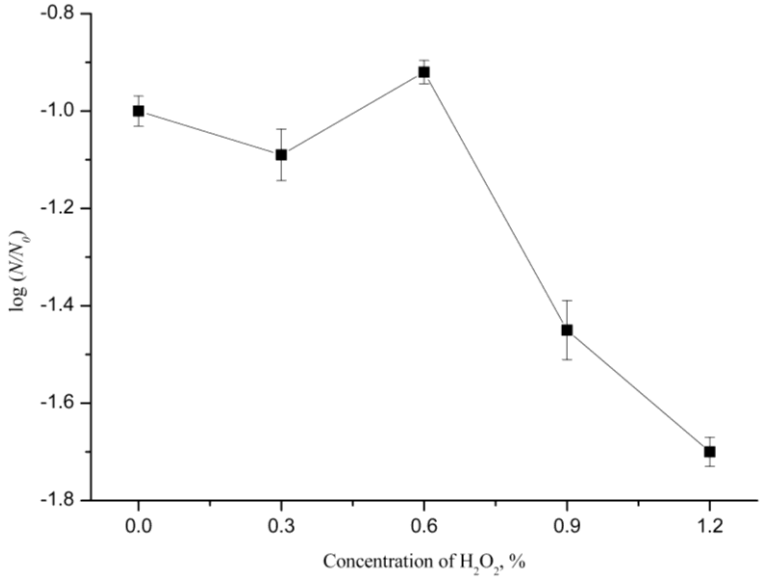

Figure 2. Effect of concentration of H_2O_2 on survival rate of spores of *B. subtilis* K2.

Screening strains with improved starch-digesting enzyme activities

Table 3 exhibited the average diameters of colonies, the average diameters of clearing zones, and the average Z/C values of the surviving strains on starch selection plates. The results were nearly opposite to those obtained with DMSO. The average diameters of colonies decreased after SC-CO_2 treatment with 0-0.3% H_2O_2 and increased after treatment with 0.6-1.2% H_2O_2.

All average diameters of clearing zones and Z/C values decreased compared to those of the untreated strains. Therefore, only a few colonies with increased Z/C values were selected for further starch-digesting enzyme activity assays. Table 4 showed that four out of nine selected strains exhibited increased starch-digesting enzyme activities by more than 10%. The strain H2-1 displayed the maximum improved enzyme activity of 67.3% with a specific starch-digesting enzyme activity of 3597 U mL^{-1}.

Table 3. Plate screening results after SC-CO_2 treatment with H_2O_2.

Concentration of H_2O_2 (%)	Average diameter of colony (mm)	Average diameter of clearing zone (mm)	Average Z/C value
untreated	5.9	17.6	3.0
0	5.3	15.5	2.9
0.3	5.1	13.9	2.7
0.6	6.4	17.3	2.7
0.9	6.5	16.2	2.5
1.2	6.2	15.6	2.5

Table 4. Starch-digesting enzyme activities of the selected strains after SC-CO_2 treatment with H_2O_2.

Strain	Concentration of H_2O_2 (%)	Starch-digesting enzyme activity (U mL^{-1})	Increase in enzyme activity (%)
the wild stain	–	2150	–
H0-1	0	2238	4.1
H0-2	0	2313	7.6
H1-1	0.3	2539	18.1
H2-1	0.6	3597	67.3
H2-2	0.6	1709	-20.5
H3-1	0.9	1232	-42.7
H3-2	0.9	2767	28.7
D4-1	1.2	2162	0.6
D4-2	1.2	2481	15.4

Hereditary stability of strains D2-5 and H2-1

The strains D2-5 and H2-1 were studied for their stability in starch-digesting enzyme production over eight generations under the same fermentation conditions. The results, presented in Table 5, showed that the

enzyme activity of D2-5 ranged from 3678 to 3756 U mL^{-1} and that of H2-1 ranged from 3531 to 3597 U mL^{-1} over eight generations, indicating only minor changes in enzyme activities. This demonstrated that D2-5 and H2-1 were stable isolates.

Table 5. Starch-digesting enzyme activity of strains D2-5 and H2-1 for eight generations.

Passage times	1	2	3	4	5	6	7	8
Enzyme activity of D2-5 (U mL^{-1})	3756	3741	3698	3710	3681	3696	3709	3678
Enzyme activity of H2-1 (U mL^{-1})	3597	3550	3576	3580	3542	3531	3559	3548

Conclusion

SC-CO_2 treatment with additives (DMSO or H_2O_2) effectively enhanced the starch-digesting enzyme activity of *B. subtilis*. After treating the spores of *B. subtilis* with SC-CO_2 and DMSO or H_2O_2, two stable isolates, D2-5 and H2-1, with high starch-digesting enzyme activities were obtained. The specific enzyme activities of strains D2-5 and H2-1 were 3756 and 3597 U mL^{-1}, which were 74.7% and 67.3% higher than that of the wild strain, respectively. This study first demonstrated the potential of SC-CO_2 combined with additives to enhance enzyme activities in microbial spores, offering a novel approach for microbial breeding. Future work should explore the application of this technique to other microbial species and enzymes and investigate the underlying mechanisms in greater detail.

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