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Modeling of sporicidal effect of hydrogen peroxide in the sterilization of low density polyethylene film inoculated with *Bacillus subtilis* spores

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ABSTRACT

The sporicidal effect of hydrogen peroxide (H_2O_2) in the sterilization of low density polyethylene film (LDPE) was evaluated using a Central Composite Design (CCD). The effects of contact time (5-19s), bath temperature (23–70 °C) and concentration of H_2O_2 (0–35%) in an immersion bath were investigated. A 16 cm^2 film surface was evenly inoculated with 100 μL of the test microorganism *Bacillus subtilis* var. *globigii* ATCC9372 spores. The effective H_2O_2 sporicidal activity was demonstrated at concentrations from 18 to 35% and in a temperature range from 46 to 70 °C, resulting in 2–7 decimal reductions of *B. subtilis* spores. A quadratic mathematical model representative of the action of H_2O_2 on the *B. subtilis* spores was developed as a function of concentration, time and temperature. Test specimens sanitized with 28% H_2O_2 at 60 °C for 8 s showed 4 decimal reductions. In the same sterilization procedure, but extending the time to 16 s, this value increased to 7 decimal reductions, demonstrating the efficiency of H_2O_2 as a function of contact time. The sterilization system tested showed satisfactory performance in the sterilization of LDPE films, being capable of reaching up to 7 decimal reductions of the bacterial spore population.

1. Introduction

The sterilization of packaging materials is one of the steps required in the aseptic packaging technology. The sterilization of the packaging can be carried out by different methods, such as physical (heat, irradiation), chemical (hydrogen peroxide, peracetic acid, etc) or a combination of the two. A combination of physical and chemical methods is, in general, more efficient, being the method most commonly used by industry (Lewis & Heppell, 2000; Richardson, Christian, & Tucker, 2007; Von Bockelmann, 1991).

Hydrogen peroxide (H_2O_2) is the chemical compound most used in the process of sterilization. Amongst other benefits, it is not toxic at residual concentrations and allows for a quick recovery treatment (Toledo, 1975; Von Bockelmann & Von Bockelmann, 1986). Of importance are the microbiological efficiency of the sterilization process and the elimination of the chemical used, which might get into the filled product as a residue. Depending on the design of the aseptic packaging equipment, different means of applying the sterilant are used: spray; vapor; roller systems; immersion bath, etc (Von Bockelmann & Von Bockelmann, 1998). Regarding the plastic polyethylene sheet coming from the roll, the sterilization is usually effected by immersion in a H_2O_2 solution varying from 10 to 35% for a few seconds at either room or higher temperatures (Cerny, 1985). For a 10% solution of H_2O_2 at 60 °C, the Q_{10} value was about 1.6. Increases in concentration from 10% to 15% and from 15% to 20% each gave an increase of about 50% in the rate constant (Swartling & Lindgren, 1968). Peracetic acid, peroctanoic acid and ethylene oxide are among the other chemical sterilization agents suitable for controlling microbial adhesion to packages in the food industry (Fluckiger, 1995).

Peracetic acid is a more powerful antimicrobial agent than H_2O_2, being rapid and efficient against a broad spectrum of microorganisms, even at low concentrations. This antimicrobial agent is also an effective sporicide at low temperatures and shows continuous action in the presence of organic materials (Baldry, 1983). In various commercially available products used in the sterilization and sanitization of industrial equipments, peracetic acid is the active compound. Similar to H_2O_2, the sporicidal activity of peracetic acid is increased by raising its concentration and temperature (Block, 1991; Leaper, 1984; Sagripanti & Bonifacino, 1996). Experiments carried out by Sagripanti and Bonifacino (1996) demonstrated the effect of temperature on the D-values of *Bacillus subtilis* var. *globigii* spores exposed to 0.03% peracetic acid.

Peroctanoic acid is a chemical product formed by an equilibrium mixture of H_2O_2, acetic acid and octanoic acid. The improved

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Nomenclature

γ	number of decimal reductions
N_0	initial number of CFU/test specimen
N	final number of CFU/test specimen
t	time (s)
T	temperature (°C)
C	concentration of H_2O_2 (%)

effectiveness of peroctanoic acid in comparison to peracetic acid is attributed to its capacity of altering the membrane of microbial cells, which may be associated with its hydrophobic characteristics (McDonnell & Russell, 1999).

The degree of sterilization required for packaging materials is associated with the potential number of microorganisms that exist on the contact surface of the packaged food (Elliott, Evancho, & Zink, 1992; Von Bockelmann & Von Bockelmann, 1986). In plastic packaging materials, this contamination varies from 0.004 to 10 CFU/100 cm^2 (Leaper, 1984; Von Bockelmann & Von Bockelmann, 1986). Due to the low level of contamination typically found on packaging materials, and assuming that all the microorganisms are bacterial spores, the degree of sterilization used in packages of low acid products varies from 4 to 6 cycles of logarithm reductions (Cerny, 1985; Elliott et al., 1992; Von Bockelmann & Von Bockelmann, 1986). This spore reduction magnitude is equal to the protection level offered by the thermal treatment used in the sterilization of low acid products.

The objective of this research was to evaluate the effectiveness of H_2O_2 on the sterilization of LDPE film as a function of concentration, temperature and contact time, in order to get data to generate a mathematical model for the sterilization of plastic films.

2. Materials and methods

2.1. Packaging material and test specimens

The packaging materials tested were low density polyethylene films (Plastunion Ind. de Plásticos Ltda, Caieiras-SP-Brazil) containing titanium dioxide (TiO_2) pigments, produced by extrusion in rolls with a width of 300 mm and thickness of 60 μm.

The test specimen consisted of a 40 × 40 mm film randomly sampled from the roll. Prior to the experimental treatments, the test specimens were sanitized by immersion in 70% ethanol for 20 min. They were then individually transferred, under aseptic conditions, onto absorbent paper in Petri dishes and oven dried at 37 °C for 12 h

2.2. Hydrogen peroxide solutions

Milli-Q water sterilized at 121 °C for 15 min was used as the control in the experiments, as well as for dilution of the H_2O_2 solutions to the concentrations determined by the complete factorial design. The H_2O_2 solutions were prepared immediately before use from a sterilized stock solution of 35% H_2O_2 (Asepticper®, Peroxidos do Brasil Ltda, São Paulo-SP-Brazil), and the concentrations were determined by permanganometry (Solvay, 2005).

2.3. Test microorganism

Bacillus subtilis var. *globigii* ATCC9372 spores were the test microorganism inoculated onto the LDPE film. The bacterial cultures were kindly donated by the Culture Bank of the Adolfo Lutz Institute, São Paulo, SP, Brazil. Spore suspensions were prepared as described by Abreu (2001). A culture of *B. subtilis* was grown in nutrient broth (Difco, São Paulo-SP-Brazil), in a 250 mL Erlenmeyer flask at 37 °C with shaking (70 rpm) for 18 h. Aliquots of the fresh *B. subtilis* culture were spread onto a sporulation medium distributed in 50 Petri dishes, and incubated for 6–8 days at 37 °C. The sporulation medium consisted of nutrient agar supplemented with 500 mg/L Bacto-dextrose (Difco, São Paulo-SP-Brazil) and 3 mg/L manganese sulfate (Merck, Jacarepaguá-RJ-Brazil). Sporulation was verified by inspection of the growth under a phase contrast microscope. Spores were washed off the agar surface with cold sterile deionized water (10 mL/plate). The spore suspension was placed in a bath containing a water-ice mixture and treated in a sonicator (FS-28, Fisher, Pittsburgh, PA) for three periods of 2 min, with 20 min rest periods. The treated suspensions were washed six times by centrifugation (8000 rpm for 20 min, at 4 °C) using sterile deionized water. The pellet was re-suspended in cold sterile peptone water (Difco, São Paulo-SP-Brazil) and centrifuged at 8000 rpm for 20 min, at 4 °C. The spore pellet was re-suspended in 0.1% sodium chloride (Merck, Jacarepaguá-RJ -Brazil) to obtain 10^7 spores/mL, and stored at 4 °C.

2.4. Inoculation of the test specimen

The test specimens (40 × 40 mm) were inoculated with a population of 10^7 spores/mL. The inoculation was performed in a laminar flow hood using a micropipette, applying 20 droplets from a total spore suspension volume of 100 μl, on to a 20 × 20 mm square in the central area of the test specimen. These LDPE spore-inoculated samples, placed on sterile filter papers in Petri dishes, were dried at 37 °C for 12 h.

2.5. Treatments

The inoculated test specimens were immersed in the H_2O_2 solutions, following the time, temperature and concentration determined by the experimental design. The transfer of the test specimens to the sterilizing solutions was carried out in the laminar flow hood, using sterile forceps. For each treatment, 300 mL of H_2O_2 solution at the desired concentration were prepared using filtered sterile water under aseptic conditions. The H_2O_2 concentrations followed the experimental design.

2.6. Spore recovery and quantification

The untreated test specimens (3 units) and the test specimens for each H_2O_2 sterilization treatment (3 units), were placed individually in 200 mL glass bottles containing 20 mL of spore recovery solution. The recovery solution consisted of 18 mL of a sterile 0.5% sodium thiosulfate solution (Alasri, Valderde, Roques, & Michel, 1992) and 2 mL of a 15.000 U catalase solution (Sigma–Aldrich), sterilized by microfiltration (Luck, 1971). In order to remove the spores from the LDPE film, the glass bottles containing the test specimens were vigorously vortexed for 1 min in a clockwise direction, and then for a further 1 min in a counterclockwise direction. Aliquots of 1 mL of the solution and its serial dilutions in sterile water were plated on the surface of Plate Count Agar culture medium. The cultures were incubated at 35 °C for 48 h and the growing bacterial colonies counted.

2.7. Experimental design

A sequence of experiments carried out according to Central Composite Design (CCD) methodology was used in this work (Rodrigues & Iemma, 2005). Three independent variables were

considered: the contact time of the sample with the H_2O_2 solution (s), bath temperature of the H_2O_2 solution (°C) and the concentration of the H_2O_2 solution (%). The measurable response (dependent variable) was a function of the number of decimal reductions of the spores (γ), as follows:

$$\gamma = -\log\frac{N}{N_0} \quad (1)$$

Where: γ = number of decimal reductions; N_0 = initial number of CFU/test specimen; N = final number of CFU/test specimen;

A 2^3 complete factorial design was applied with 2 levels (−1; +1), 4 central points (0) and 6 axial points (−α; +α), giving a total of 18 assays. The objective was to determine the effects of the variables and to obtain indications concerning the efficiency of the chosen values for each variable, in order to establish the most suitable sterilization conditions for the system tested. The design and the matrix of the complete factorial design are shown in Tables 1 and 2.

2.8. Statistical analysis

The data were statistically analyzed using the software Statistica advanced version 7.0 (StatSoft, Inc., Tulsa, OK-USA), in order to obtain the response surface fitted by regression.

One dependent (decimal reduction) and three independent variables (time-s, temperature-°C and H_2O_2 concentration-%) were used, with one block and three replications. The confidence interval applied was 95%.

The variables, dependent and independent, were marked in the software and the statistical analyses were run. The estimated effects, R^2 and pure error were observed and the equation that provided an estimate of the response corresponding to the number of decimal reductions was obtained.

The response surface and response profiles were generated for an easier comprehension of the best conditions for spore killing by the sterilization agent (hydrogen peroxide).

3. Results and discussion

Table 2 shows the decimal reduction values (γ) reached by the sterilization system tested. The response surface curves are shown in Fig. 1.

More than 4 decimal reductions were reached using 18% H_2O_2 at 70 °C. Clear observations of the effects of time and temperature were not possible at high H_2O_2 concentrations, because the highest number of decimal reductions of around 7, was rapidly reached. The experimental determination of a supposedly more efficient decimal reduction of the spores by increasing the H_2O_2 concentration was not possible, due to the limitation of the spore population in the inoculated test specimens (10^7).

The effect of each experimental variable and the coefficient of the regression model were estimated in the statistical analyses ($p < 0.05$), as shown in Table 3. The regression coefficient (R^2) was 0.9513 for the complete model and 0.9349 for the fitted model.

Based on the fitted regression coefficients of the model shown in Table 3, Eqn (2) was obtained, which provided an estimate of the response corresponding to the number of decimal reductions, as follows:

$$\gamma = 17.0829 - 0.5360 \times t + 0.0065 \times t^2 - 0.5431 \times T + 0.0043 \times T^2 - 0.4685 \times C + 0.0071 \times C^2 + 0.0092 \times t \times T + 0.0071 \times T \times C \quad (2)$$

Where: γ = number of decimal reductions;
t = time (s);
T = temperature (°C);
C = concentration of H_2O_2 (%).

As can be seen in Fig. 1, the optimum decimal reduction point was reached at a concentration of approximately 35% H_2O_2 in a temperature range from 46 to 70 °C. With more drastic concentrations and temperatures, the surface quickly reached its highest peak for the decimal reduction values, probably because of the higher temperature of the H_2O_2 solution, which showed a greater sporicidal effect (Block, 1991; Fluckiger, 1995; McDonnell & Russell, 1999; Toledo, Escher, & Ayres, 1973; Von Bockelmann, & Von Bockelmann 1986).

When some of the optimum values observed in Fig. 1 were inserted into the regression Eqn (2) derived in this study, the decimal reduction of the *B. subtilis* spores started at 4.5 (35% H_2O_2, 46 °C and 5 s) and went up to 14 (35% H_2O_2, 70 °C and 19 s). However, these drastic conditions may cause undesired alterations to the film properties, as well as resulting in a residual amount of H_2O_2 difficult to remove (Abreu & Faria, 2004).

In the present study, the effect of the variable contact time was not significant ($p < 0.05$), as can be seen in Fig. 2. Also, an increase in time did not result in an elevation of the decimal reductions in assays 9 and 10 (Table 2). However, the increase in time from 8 s to 16 s in assays 3 and 4, maintaining the other variables constant, resulted in an increase in the decimal reductions from 1 to 2. According to spore inactivation studies found in the literature, an increase in spore decimal reductions is obtained by increasing the time (Abreu & Faria, 2004; Leaper, 1984; Sagripanti & Bonifacino, 1996; Toledo et al., 1973). Therefore, the variable of contact time with H_2O_2 was considered in the fitted model.

Table 1
Variables of the 2^3 complete factorial design.

Levels	−1.68(−α)	−1	0	1	1.68 (+α)
Time (s)	5	8	12	16	19
Temperature (°C)	23	32	46	60	70
H_2O_2 (%)	0	7	18	28	35

Table 2
Influence of the treatments with H_2O_2 in the decontamination of test specimens, inoculated with 100 μl of *B. subtilis* spores as a function of the number of decimal reductions, according to the 2^3 complete factorial design.

Assays	t (s)	T (°C)	H_2O_2 (%)	γ
1	8 (−1)	32 (−1)	7 (−1)	1.39
2	16 (+1)	32 (−1)	7 (−1)	0.70
3	8 (−1)	60 (+1)	7 (−1)	1.52
4	16 (+1)	60 (+1)	7 (−1)	2.01
5	8 (−1)	32 (−1)	28 (+1)	0.70
6	16 (+1)	32 (−1)	28 (+1)	0.62
7	8 (−1)	60 (+1)	28 (+1)	4.13
8	16 (+1)	60 (+1)	28 (+1)	7.01
9	5 (−α)	46 (0)	18 (0)	0.64
10	19 (+α)	46 (0)	18 (0)	0.64
11	12 (0)	23 (-α)	18 (0)	1.04
12	12 (0)	70 (+α)	18 (0)	4.44
13	12 (0)	46 (0)	0 (−α)	0.06
14	12 (0)	46 (0)	35 (+α)	4.76
15	12 (0)	46 (0)	18 (0)	0.17
16	12 (0)	46 (0)	18 (0)	0.38
17	12 (0)	46 (0)	18 (0)	0.92
18	12 (0)	46 (0)	18 (0)	0.42

t (s) = contact time.
T (°C) = temperature of the H_2O_2.
H_2O_2 (%) = concentration.
γ = decimal reductions of the spores.
N_0 = initial population of *B. subtilis* spores.
N = final population of *B. subtilis* spores.

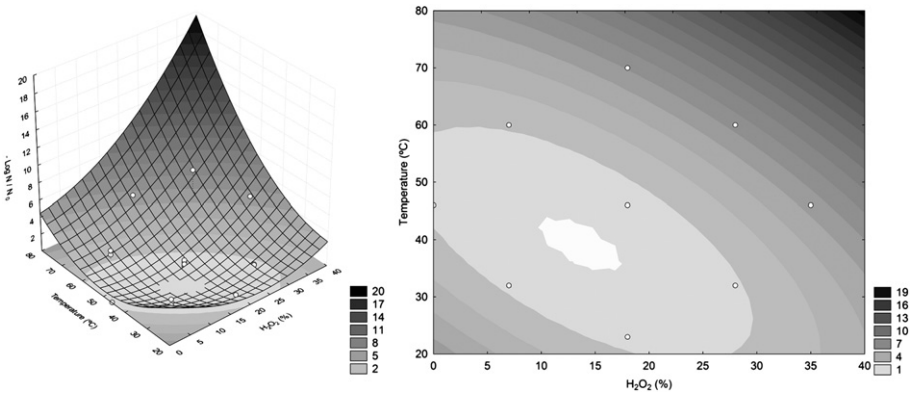

Fig. 1. Response surface showing the decimal reduction of *B. subtilis* as a function of temperature and hydrogen peroxide concentration in the sterilization of low density polyethylene film.

Table 3
Regression coefficients for the model obtained from the 2^3 design for the H_2O_2 treatments.

Variables		Effects	Standard error	Regression coefficients	p	Fitted regression coefficients
Mean		0.4202	3.9142	18.9454	0.0013	17,0829
Time (s)	(L)	0.3529	0.3278	−0.6912	0.0680	−0.5360
	(Q)	0.2092	0.0104	0.0065	0.5488	0.0065
Temperature (°C)	(L)	2.3968*	0.1017	−0.5431	0.0007	−0.5431
	(Q)	1.6802*	0.0009	0.0043	0.0016	0.0043
H_2O_2 (%)	(L)	2.1607*	0.1121	−0.5737	0.0009	−0.4685
	(Q)	1.5546*	0.0016	0.0071	0.0027	0.0071
Interaction t (s) and T (°C)		1.0350*	0.0040	0.0092	0.0498	0.0092
Interaction t (s) and H_2O_2 (%)		0.7359	0.0053	0.0088	0.1392	n.d.
Interaction T (°C) and H_2O_2 (%)		2.0751*	0.0015	0.0071	0.0017	0.0071

(L) linear and (Q) quadratic.
*significant at $p \leq 0.05$.
n.d. not determined.

The maximum H_2O_2 concentration applied in the calculation of the decimal reductions must be 35% and the minimum contact time must be 5s. Extrapolation over 35% may lead to incoherent results. The optimum times applied were from 12 to 16s, with the H_2O_2 concentration above 25% (Fig. 3).

There were difficulties and limitations in comparing the data obtained in this study with that available in the literature, since few of the reported experiments analyzed a surface contaminated with dry inoculum and for short periods of time. Also most were carried out under moist conditions.

Another experimental parameter that may induce variations in the results is the surface to which the inoculum is applied. The spores can adhere more or less efficiently to certain surfaces, due to interactions of a hydrophilic or hydrophobic nature, being easier or harder to remove (Gangi, Leonard, Rodriguez, & Margolin, 1997; Ronner, Husmark, & Henrikisson, 1990). Ronner et al. (1990) determined that the *Bacillus cereus* spores were highly hydrophobic whereas the spores of *B. subtilis* ATCC6633 were more hydrophilic than hydrophobic. Plastic materials are typically hydrophobic surfaces (Reuter, 1993). The authors also observed that, on both surfaces, the spores had a greater adherence capacity than the vegetative *Bacillus* cells.

The most suitable study found in the literature for comparison with the present work was carried out by Han, Schornick, and

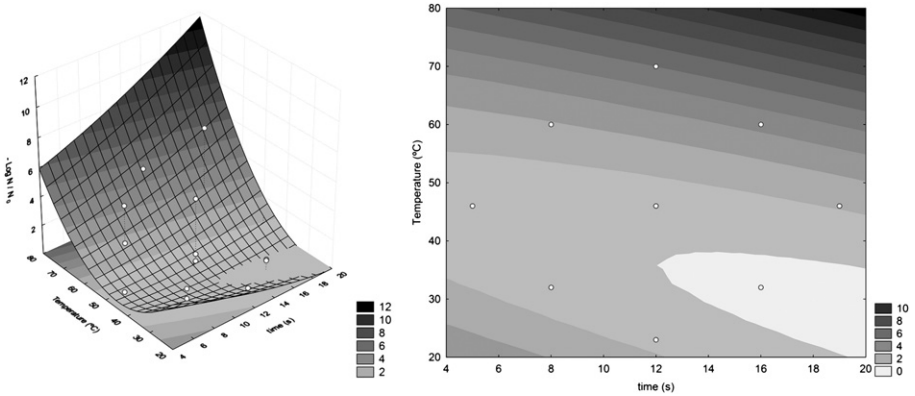

Fig. 2. Response surface showing the decimal reductions of *B. subtilis* as a function of time and temperature in the sterilization of low density polyethylene film.

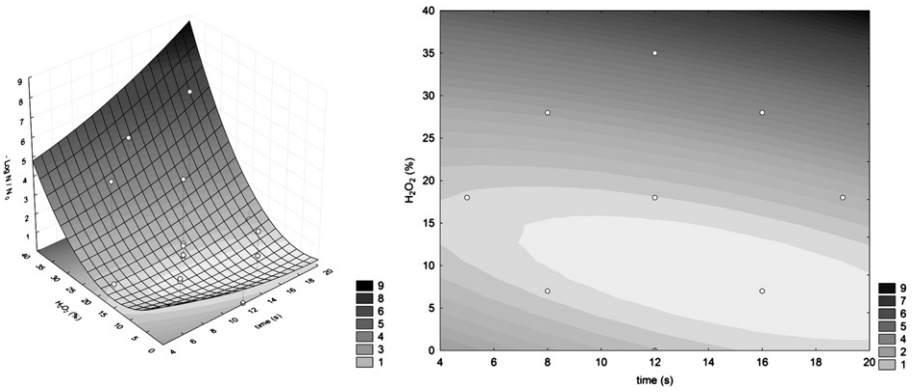

Fig. 3. Response surface showing the decimal reductions of *B. subtilis* as a function of hydrogen peroxide concentration and time in the sterilization of low density polyethylene film.

Table 4
Comparison of the study carried out by Han et al. (1980), with the quadratic model developed in the present experiment.

Values for the parameters from Han et al. (1980)				Values for the parameters found in the present work			
t (s)	T (°C)	H_2O_2 (%)	γ	t (s)	T (°C)	H_2O_2 (%)	γ
12	46	35	2.9	12	46	35	4.8
8	60	28	4.8	8	60	28	4.1
12	70	18	5.1	12	70	18	4.4

γ = number of decimal reductions.

Loncin (1980), who inoculated aluminum surfaces with spores of the same species of *B. subtilis* var. *globigii* ATCC9372. The treatments consisted of sterilization with 0.6%–30% H_2O_2 solutions from 0 to 80 s. Despite the experimental differences, a comparison was possible by inserting the values from the study carried out by Han et al. (1980) in regression Eqn (2), obtained in this study. The differences in intensity of the interaction between the surface material and the spores can be seen in Table 4.

Considering the differences in the experimental conditions, except for the data 46 °C and 12 s, it can be said that the results obtained by the present model are coherent with those obtained by Han et al. (1980). In that condition, there was a significant variation in the results (2.9 versus 4.8 decimal reductions) showing a better situation for spores destruction in this study. This can be explained by the commercial solution of H_2O_2 (Asepticper®, Peroxidos do Brasil Ltda, São Paulo-SP-Brazil). The presence of anionic surfactant in the stock solution of H_2O_2 formulation probably increases the powerful of the sterilizing agent, reaching more decimal reductions.

4. Conclusions

The model obtained based on the response surface, showed the number of decimal reductions of *B. subtilis* spores to increase with sterilization treatments using H_2O_2 concentrations above 18% and temperatures higher than 60 °C. Under such conditions at least 4 decimal reductions were achieved in the spore populations, using time intervals of from 12 to 16 s.

The data reported herein demonstrated that the sterilization system evaluated was efficient in sterilizing low density polyethylene film, with up to 7 decimal reductions of the *B. subtilis* population. Such results can be used to design systems for the sterilization of plastic packaging material used for the aseptic filling of foods.

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