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Supercritical Fluid Application in Food and Bioprocess Technology

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1. Introduction

There are several old and new applications for the supercritical fluid (SCF) technology in bioprocessing, including the nonthermal cell inactivation (Dillow et al., 1999; Spilimbergo and Bertucco, 2003; Hong and Pyun, 2001), permeabilization (Aaltonen and Rantakyla, 1991), extraction of fermentation products (Bruno et al., 1993; Hampson and Ashby, 1999; Isenschmid et al., 1995), removal of biostatic agents and organic solvents from fermentation broth, SCF disruption of yeasts (Castor and Hong et al., 1995; Lin and Chen, 1994; Lin et al., 1992; Nakamura et al., 1994) and bacteria (Juhasz et al., 2003; Khosravi- Darani et al., 2004), destruction of industrial waste (Kim and Hong, 2001), fractionatation and purification of biopolymers (Khosravi- Darani et al., 2003), removal of chlorinated compounds from water, and treatment of lignocellulosic materials (Puri, 1983). Some products possibly produced by the SCF technology may be found in processes to obtain vitamin additives, de-alcoholized beverages, de-fat potato chips, and encapsulated liquids. For more information on the other examples, the readers are referred to the literature (King and Bott, 1993; Brunner, 2005; McHugh and Krukonis, 1994; Bertucco and Spilimbergo, 2001). Khosravi-Darani et al. have reviewed all aspects of the supercritical fluid extraction (SCE) in the downstream processing of bioscience (Khosravi-Darani and Vasheghani-Farahani, 2005).

There are also several applications for the SCF technology in food engineering including: extraction of compounds from natural products (the processing of hops, the extraction of caffeine, vanilla, beta-carotene, and vegetable oils), food sterilization, removal of undesired extractable (pesticides residues, hazardous chemicals from fish tissue, oil from dry-milled corn germ), and fractionation of cod liver oil (Bruno et al., 1993). Catalytic reactions in supercritical CO_2 have been receiving an increased attention during the last decade (Sarkari et al., 1993).

This chapter has focused on SCF special applications in the field of food biotechnology. The application of SCF is simple, inexpensive, and noninjurious to the structure and function of enzymes (Lin et al., 1992) and protein activities (Kamat et al., 1995; Zheng and Tsao, 1996; Kasche et al., 1988). The supercritical carbon dioxide (SC-CO_2) is the most commonly used

fluid. It's low critical temperature of 31.1°C and the pressure of 7.3 MPa make it an ideal medium for processing volatile products (Wells and DeSimone, 2001). The non-toxicity, non-flammability, as well as the selectivity of the process and the ease of recovery are the most important features. Most of SCFs are available in a relatively pure grade at a reasonable cost as compared with the industrial grade liquid solvents. Therefore, many subsequent downstream clean-up steps are unnecessary supercritical extraction (SCE). By replacing the SCE to avoid liquid extraction, O_2 is always very efficiently displaced from the matrix. This prevents oxidation and autoxidation reactions from becoming a problem, as they are often in liquid extraction schemes. This fact is a particular advantage in biotechnology since many important natural products and drugs are oxygen-sensitive (Teja and Eckert, 2000).

There are also some limitations in the SCF applications e. g. change of the phase equilibrium; alter of phase diagram of the solvent, difficult prediction and design of extraction conditions; necessity for addition of impurities as modifiers (called entrainers or cosolvents) to SCF in quantities up to 5(%v/v); impossible real time control by the most accurate equations of state, necessity to unfired pressure vessels; high initial capital outlay due to the high cost of compressors (Bruno et al., 1993). Other applications of the SCF in food biotechnology can be summarized as follows: removal of fat; alcohol recovery from wine (Guvenc et al., 1998); encapsulation of liquids (Heremans and Smeller, 1998), recovery of tocochromanols (vitamin E) and beta-carotene (provitamin A).

Particulate products can be also achieved by means of SCF processing e.g. concentrated powder after spraying of CO_2–liquid mixture into a spraying chamber at ambient conditions together with the substrate; also flash release of CO_2 from the liquid will result in the formation of small droplets. The prevention of oxidation processes and easier handling, dosage, and storage are among the purported advantages of this process (Brunner, 2005).

2. The effect of high pressure and temperature on food constituents

High pressure (100–1000 MPa) affects biological constituents and systems. Several physicochemical properties of water are modified, such as density, ionic dissociation, pH, and the melting point of ice. The pressure-induced unfolding, aggregation, enzyme inactivation (e.g., of ATPase) and gelation of food proteins occurred due to effect on non-covalent bonds and interactions. Chemical reactions, macromolecular trans-conformations, changes in the membrane structure and the melting point are enhanced under pressure . Several of these phenomena, are involved in the high inactivation ratio of most vegetative microbial cells: gram negative bacteria, yeasts, complex viruses, molds, and gram-positive bacteria, in this decreasing order of sensitivity to pressure. Other parameters like pressure, holding time, temperature and the composition of medium influence this resistance. The pH has little influence, but high salt or sugar concentrations and low water contents, exert very strong baro-protective effects. Many other articles have also dealt with the stability of proteins as a function of pressure (Zagrobelny and Bright, 1992; Athes et al., 1998) and particularly with that of enzymes (Degraeve and Lemay, 1997; Marie-Olive et al., 2000).

3. The recovery and purification of biological products

Vijayan et al. have broadly classified the process applications into three product segments: High-value, Low-volume (HVLV); Intermediate-value, Intermediate-volume (IVIV) and

Low-value, High-volume (LVHV) products (Vijayan et al., 1994). The production of HVHV has been found to be very favorable for the exploitation of the SFE technology. The types of foods processed include: flavor, fragrance, spice extracts, and essential oils from plants (Wang and Muttucumaru, 2002), animals, and other materials; hop extraction to produce alpha and beta acids as well as essential oils; purification and fractionation of aroma constituents. In fact, the advent of food processing as a modern industry has ushered in the use of food extractives rather than raw materials.

The food extractives of spices, called oleoresins, are used in the food processing industry for their appealing flavor and to improve the product quality. The selectivity of the extraction can be achieved using SC-CO_2 and the essential oils of pepper and ginger can be extracted without much contamination of non-volatile matter by the suitable selection of the extraction pressure and temperature of CO_2 (Nguyen et al., 1994). In the SCE of ginger, the oxygenated fraction can be much higher than that in steam-distilled oils. The ginger-oils in oleoresin of ginger can be extracted without any decomposition. The piperine can be extracted with insignificant loss with longer process times. The jasmine and vanilla absolutes from this process have desirable top notes with rounded flavor and are superior to those obtained by conventional process. Examples for IVIV include: the removal of caffeine from coffee and tea; the xanthins from cocoa; excess oil from fried foods and vegetable. Studies on reducing the cholesterol content of animal fats and the extraction of lipids are in progress. Separation of the polyunsaturated fatty acids (PUFA), notably eicosapentacnoic acid (EPA) and decosahexanoic acid (DHA), which are purported to have beneficial physiological activity, from a mixture of fatty acid and ethyl esters from fish oils is reported (Hammam, 1992). With respect to the SFE of LVHV products, there are some doubts as to whether it is competitive with traditional extraction methods, e.g., the oil production from vegetable seeds; the processing of grain flours to improve quality and fractionation of beef tallow. Table 1 shows some valuable extractives from natural materials in food industry.

The SFE process parameters including pressure and temperature variations have been measured by HPLC and GC/MS (Wang and Muttucumaru, 2002).

4. The inactivation of food related bacteria

The application of high pressure ranging from 100 to 1000 MPa, is one of the most promising methods for the food treatment and preservation at room temperature (Debs-Louka et al., 1999). High pressure inactivation of *E. coli* pressure resistant had been investigated in fruit juices and in low pH buffers. The results show that both parents and mutant strains become more pressure-sensitive in decreased pH and presence of organic acids. The high pressure treatment for 5–10 min under 300–600 MPa at 20–50°C allows the reduction of vegetative microbial cells by 4–5 log cycles. However, some enzymes, especially polyphenoloxidase in fruit juices, are more pressure-resistant and their inactivation needs additional approaches (Molin, 1983). The degradation of color and slight changes of flavor due to the higher content of dissolved oxygen in products are mentioned as an example of negative pressure effects (Knorr, 1995). Pasteurization of milk and the heat resistance of *Mycobacterium avium* subsp *paratuberculosis* (Lund et al., 2002) also vegetative and the latent form of other microorganisms have been reviewed (Sojka and Ludwig et al., 1997; Paidhungat et al., 2002; Raso et al., 1998). It seems evident that it was not possible to kill spores at room temperature with an extremely high operating pressure, up to 170 MPa. It was observed that there is an optimum range of temperature and pressure for stimulating

the germination of spores (Nakayama et al., 1996; Roberts and Hoover, 1996). Therefore, the coupled action of hydrostatic pressure and of specific temperature was investigated in order to activate spores and consequently to inactivate their vegetative forms in a second step with higher operating pressure (Hong et al., 1999). Ludwig et al. carefully studied the behavior of spores under different operative conditions of the high temperature and pressure and introduced a cycle-type treatment (Ludwig et al., 1994; 1997); this appeared to be more efficient than the double level pressure treatment. It was concluded that at higher temperature, faster germination is obtained, as well as a wider range of pressure is suitable to this scope. Salts, glucose, and amino acids were found to enhance the rate of germination. However, until now a complete inactivation of spores has not been achieved yet. The regression analysis of inactivation rates showed that pressurization at sub-zero temperatures (−20 and −10°C) enhanced the effects of pressure as pressurization at higher temperatures (i.e., pressurization at 190 MPa and −20°C gave the same effect as pressurization at 320 MPa and room temperature). The results imply that high pressure treatment at lower temperatures has a greater effect on food sterilization without destroying the original taste and flavor. Additional effects of sugars and salts on the inactivation of yeast are also described (Hashizume et al., 1995).

Many recent studies demonstrate that SC-CO_2 as a non-toxic and inexpensive gas can also be used for the inactivation of viruses (Fages et al., 1998) and pest control. It is a promising alternative method for the pasteurization and sterilization of foodstuff (particularly in the liquid phase), sterilization of thermosensitive substances, as well as thermally and hydrolytically sensitive polymeric materials in biomedical applications. Furthermore, application of SC-CO_2 seems to be attractive for its economical feasibility, as it needs very low pressure (lower than 20 MPa) compared to the so–called ultra high pressure treatment (200–700 MPa) (Bertucco and Vetter, 2001). A number of papers have been addressed to the inactivation of a wide range of microorganisms, bacteria, spores, and yeasts in physiological solutions by SCF (Watanabe et al., 2004; Erkmen, 2001; Spilimbergo et al., 2002; Clery-Barraud et al., 2004).

In the field of industrial applications, it is worthy to quote some recent publications that have dealt with the inactivation in complex substrates and solid food (Haas et al. 1989; Gould, 2003; Arreola et al., 1991; Erkmen, 2000; 2001). All these authors tested the efficiency of the SC-CO_2 mainly in natural foods (e.g. milk, fruit juice, and eggs) in a batch system. Spilimbergo has also checked with high pressure CO_2 on red orange juice of Sicily (Spilimbergo et al., 2002). Spores of *B. coagulans, B. subtilis, B. cereus, B. licheniformis, and Geobacillus stearothermophilus* were subjected to CO_2 treatment at 30–200 MPa and 35–65°C. All of the bacterial spores except the *G. stearothermophilus* spores were easily inactivated by the heat treatment. The treatment with CO_2 and 30 MPa of pressure at 95°C for 120 min resulted in 5-log-order spore inactivation. The activation energy required for the CO_2 treatment of *G. stearothermophilus* spores was lower than the activation energy for heating or pressure treatment (Matsuda et al., 2004). Lund et al. have studied heat resistance of *Mycobacterium avium* subsp *paratuberculosis* and related problems in milk pasteurization (Lund et al., 2002). Kamihira et al. found a sterilizing effect of SC-CO_2 on various microorganisms at 20.3 MPa and 35°C (water content of 70 to 90) (Kamihira et al., 1987). Dried cells were not sterilized when treated under the same conditions. Bruna evaluated the composition changes of strawberry puree during high pressure pasteurization (Bruna et al., 1994). The inactivation effect of the native microorganisms in raw milk and raw cream is nearly the same. Fat does not influence the inactivation. The inactivation of milk enzymes

phosphohexose isomerase, gama-glutamyltransferase, and alkaline phosphatase occurs as a result of SC treatment (Sojka and Ludwig, 1997; Ishikawa et al., 1997). Blickstad et al. (1981) reported the effect of CO_2 on pork microflora and found that increasing the partial pressure of CO_2 added to the packaging atmosphere and prolonged the shelf life of the meat.
Factors, such as temperature, pressure, and moisture, contribute to a more effective treatment by increasing the diffusivity of CO_2 (Isenschmid et al., 1995; Kamarei and Arlington, 1988; Schreck and Ludwig, 1997; Smelt, 1998). Within certain limits, a longer duration of exposure to CO_2 permits better sterilization; exposure time can be decreased by increasing the temperature (Lund et al., 2002). Microbial resistance to CO_2 also depends on the type of microorganism, the phase of growth, moisture, (Lin and Chen, 1994; Kamihira et al., 1987) and the suspension medium, the last of which can inhibit the bactericidal effect of compressed CO_2, especially in some food systems rich in proteins (Ishikawa et al., 1995). Lin et al. suggested that swollen cell walls, due to the presence of water, become more CO_2 permeable (Lin et al., 1994). Finally, though the use of the CO_2 sterilization offers cost and environmental advantages, there is no guarantee that a CO_2 sterilizer will receive FDA approval. The analysis of the CO_2 sterilization shows a lower cost per cubic foot ($6) than EtO ($19) because of the shorter cycle time, lower cost per load, and lack of regulatory constraints without negative environmental and health effects.

Products	SCF	T/ °C	P/ MPa
Lecithin and Soya oil	Near critical CO_2 + deodorized propane	--	8
Progestrone, Testostrone, Chlosterol	CO_2 with or without N_2O	35-60	8-25
Glycerides of fatty acid short chain	CO_2	40	31
Cholesterol	CO_2	40-60	8-12
Pure saturated triglycerides with acyl chain length of 12-36 carbons	CO_2	40	13-30
Lipids from egg yolk	CO_2 & (methanol or ethanol)	--	--
Fatty acid-ethyl esters derived from cod river oil	CO_2	50	15
cis-5, 8,11,14,17 EPA and Cis-4, 7, 10, 16, 19 DHA from menhaden oil	CO_2	--	--
PUFA from fungus *saprolengia parasitica*	CO_2	60	35

Table 1. Some examples of supercritical fluid extraction of valuable constituents from natural materials in food industry (Nguyen et al., 1994)

The CO_2 technology showed some disadvantages including high capital cost; space needed to store the CO_2 cylinders; design and build a prototype that satisfy the temperature, pressure, humidity, and agitation requirements for the CO_2 sterilization (Schreck and Ludwig, 1997). High pressure has the advantage of retaining taste, color, and texture much better than heat treatments and also affects food constituents; proteins, lipids, and starches may undergo conformational changes. In Japan, high pressure pasteurization of acidic foods, such as fruit juices and jam, is being practiced on an industrial scale. Such "cold" processing might also be utilized in a useful way in industrial cheese-making processes of

typical hard cheeses from "raw milk". With this process it could be possible to reduce the overall microbial load adverse to making cheese without modifying the subtle chemico-physical balance of milk. As pressure-treated milk shows modified properties during further processing, such as changed rennet or acid coagulation characteristics, coagulation time, and gel firmness. Further studies should be carried out to understand whether the structural changes of milk compounds could worsen cheese-making processes (Cuoghi, 1993). Also, the effect of high pressure on microorganisms and enzymes of ripening cheeses were studied. A significant decrease of total microbial count was obtained at the pressure above 400MPa. It was found that the inactivation of microorganisms was affected more by their initial number than by the type of cheese and its maturity. *E. coli* was completely inactivated in 400 MPa pressurized cheeses irrespective of their initial count. *Enterococci* were inactivated at 400 MPa, while the pressure of 600 MPa was needed to achieve this effect in a 2-week-old cheese. Yeasts and moulds were inactivated with 200 MPa. Aminopeptidases and endopeptidases of both cheese and its extract lost the catalytic abilities at 600 MPa irrespective of the type and ripening time of cheeses (Reps et al., 1994). On the whole, the SCF sterilization has been reported as a successful approach in the sterilization of several kinds of food including; fruits, juices, vegetables, jam, meat, milk, wine, liquid whole egg, natural pigments, yoghurt, and even chocolate (Hammam, 1992).

5. The use of semi-preparative SCF chromatography for the separation and isolation of flavor and food constitutes

Many successful applications of this technique for the analysis of triglycerides in butter fat and fish oil have been described. The coupling of SFC with thin layer chromatography resulted in a powerful method for identification of trace substances such as phenolic antioxidants (Flament et al., 1994).

6. The application of sc-co_2 in citrus processing

- *Debittering of citrus juices*. Kimball (1987) use SC-CO_2 to extract bitter triterpenoids, such as limonin from orange juice in which the pressure increase from 2.14 to 4.28 MPa was effective on limonin reduction. A change in the final pH, vitamin C, pulp content, amino acids, and percentage of acid could not be detected.
- *Extracting and /or concentration of citrus essential oils*. Mira et al. reported that it was possible to concentrate the flavor portion of citrus oils with SC-CO_2 at 70°C and 83 MPa. These conditions were optimum forminimizing the amount of flavors lost in extraction, which also resulted in low extraction yields (Mira et al., 1999).
- *Effect on quality attributes and microorganisms*. The prevention of undesirable flavors caused by microorganisms growing under low pH conditions is important. Therefore, the SC-CO_2 treatment of orange juice had the added benefit of reducing microbial numbers.

7. The SCF application as dispersion for biocatalysis

The advantage of using enzymes in the SCF include; completion of synthesis reactions in which water is a product, the increased solubilities of hydrophobic materials, greater thermo-stability of biomolecules, readily solvent recycling, integrated biochemical reactions,

and separations. Enzymes such as alpha amylase, glucose oxidase, lipase, and catalase retained their activities in the solution of high pressure CO_2 in water. All thermal and non-thermal methods to stabilize could have their own disadvantages. So, the researchers have focused on the applicability of SC in this process (Nakamura, 1990). Soybean lipoxygenase dissolved in Tris HCl buffer (0.01M; pH 9) was irreversibly inactivated by combined pressure (up to 650 MPa) and low temperature (−15 up to 35°C) treatment. The enzyme inactivation followed a first order reaction and the phase transition of water did not change the kinetic inactivation behavior.

8. The treatment of wastes of food industries

Reports have described the use of SCFs for the treatment of lignocellulosic materials, which are the major group of wastes of food industries e.g. straw and bran of corn and cereal, leaf and pomace of sugar cane, fruitwaste, etc. SCF treatment allows further utilization of lignocellulosic materials as a resource for chemicals, pulp, and energy (Puri, 1983). Pretreatment methods have been sought to remove lignin and to permit further utilization of carbohydrates contained in lignocellulosic materials. Several SCFs (e.g. SC-methanol, SC-acetone, and SC-ammonia) an alternative to chemical pretreatments, which use strong acids or bases. Ammonia-treated lignocellulosic materials were neutralized and buffered to a pH value of 4.8 before being incubated at 50°C with a fractionated and partially purified commercial crude cellulose preparation from *Trichoderma reesei*. Aliquots taken at various times were filtered before being analyzed for sugars by HPLC. Two long-term experiments were made with diets consisting of preparations of spent hops and a sample of apple pomace, incubated in the Rumen Simulation Technique (RuSiTech). A significant increase in the total volatile fatty acids and methane production was observed when the preparations of spent hops were incubated in separate bags rather than in mixtures with other components (Cansell et al., 1997). Lignin, cellulose, and their mixture were gasified with a nickel catalyst in SC-water at 673 K and 25 MPa. The gasification efficiency was low, but increased with the amount of the catalyst when softwood lignin was included in the feedstock. One possible mechanism is the catalyst being deactivated by tarry products from the reaction between cellulose and softwood lignin. Sawdust and rice straw were gasified under the same condition (Yoshida et al., 2004).

9. The SCF application as dispersion for biocatalysis

The use of the SCF as a dispersion for biocatalysis was described in 1985 and there is now a growing trend in using the SCF as a reaction media for enzymes. The advantage of using enzymes in the SCF include; synthesis reactions in which water is a product can be driven to completion, the increased solubilities of hydrophobic materials, greater thermo-stability of biomolecules in SCF, readily solvent recycling, integrated biochemical reactions and separations. CO_2 is the most widely used SCF, however, there is a growing interest in using other SCFs (e.g., ethylene, fluoroform, ethane, sulfur hexafluoride and near critical propane).

Enzymes such as alpha amylase, glucose oxidase, lipase and catalase retained their activities in the solution of high pressure CO_2 in water. Among the enzymatic reactions in SCF, the use of lipase shows most commercial promise. A SC-CO_2/H_2O mixture may be used as a reaction medium for either hydrolytic or synthetic reactions catalyzed by lipase and other

appropriate by hydrolases (Giebauf et al., 1999). In continuous reaction of acidolysis of triolein with stearic acid, the constants of the reaction and mass transfer such as rate constant, solubility, effective diffusivity, mixing diffusivity and mass transfer coefficient depend on temperature, pressure and flow velocity (Nakamura, 1990).
Immobilized *Candida antarctica* lipase B was successfully used as catalyst to synthesize butyl butyrate from butyl vinyl ester and 1-butanol in SC-CO_2) with excellent results. A clear enhancement in the synthetic activity and selectivity was observed with the decrease in fluid density for both liquids and SC-CO_2 media (Lozano et al., 2004). Also a commercial solution of free *Candida antarctica* lipase B (Novozyme 525L) was immobilized by adsorption onto 12 different silica supports modified with specific side chains (e.g. alkyl, amino, carboxylic, nitrile, etc.). The best results were obtained for the supports modified with non-functionalized alkyl chains and when the in water activity increased from 0.33 to 0.90. Immobilized derivatives coated with ionic liquids clearly improved their synthetic activity in SC-CO_2 by up to six times with respect to the hexane medium (Lozano et al., 2007).
Pseudomonas cepacea lipase (PCL) was used to catalyze the trans-esterification reaction between 1-phenylethanol and vinyl acetate in SC-CO_2. The catalytic efficiency of enzyme enhances by increasing pressure. Moreover SC sulphur hexafluoride (SCSF6) was used as reaction medium. Results showed high stability of the enzyme in this SC medium in comparison to those achieved in SC-CO_2 (Celia et al. 2005).
Thermal stability of proteinase of *Carica papaya* was tested at atmospheric pressure, SC-CO_2, nearcritical propane and dimethyl-ether. In SC-CO_2 at 300 bar thermal activation of the enzyme was improved in the comparison to ambient pressure. Activity of the enzyme decreased in propane and dimethyl-ether (300 bar). Addition of water in the system increased activity, which was incubated in SC-CO_2 for 24 h (Habulin et al. 2005).
Isoamyl acetate was synthesized from isoamyl alcohol in SC-CO_2 by enzymatic catalysis. Among several reactants, including acetic acid and two different acetates, acetic anhydride gave best yields. An esterification extent of 100% was obtained in continuous operation using acetic anhydride (acyl donor) and Novozyme 435 (enzyme) (Romero et al. 2005). Cocoa beans had been subjected to various pod storage periods prior to fermentation were analysed for pyrazines and SCE (Sanagi et al. 1997).

9.1 SCFs: puissant media for the modification of biopolymers

The use of SCFs media for polymer modification has been demonstrated (Yalpani 1993). Treatment of chitosan mixtures with glucose or malto-oligosaccharides in SC-CO_2 afforded the corresponding water soluble imine-linked, branched chitosan derivatives with high degrees of conversion. Treatment of starch, maltodextrins, cellulose acetate, poly(vinyl alcohol) and paper in SC-CO_2 and O_2 (19:1 v/v) led to the corresponding oxidized materials.

9.2 Gasification of straw

Bioconversion of lignocellulosics consists of substrate pretreatment by high pressure steam (for fractionation into cellulose, hemicellulose and lignin components), enzymatic hydrolyze, followed by fermentation of the liberated sugars to ethanol. The various technoeconomic models developed by network members were used to identify probable process schemes and determine technical "bottlenecks" (Saddler 1992).

9.3 Waste treatment

Waste treatment is one of the most important and urgent problems in environmental management around the world. SC-water oxidation has attracted attention for the treatment of industrial waste, especially toxic and refractory waste. In a study, SC-water oxidation with H_2O_2 was applied as the oxidant to the treatment of a model municipal solid waste containing proteins, fats, vitamins, fiber, and inorganic minerals. The effects of temperature, oxidant concentration, and reaction time on the decomposition of solid waste were investigated in a batch reactor with hydrogen peroxide over the temperature range of 673-823 K. (Mizuno et al. 2000). SC-water is very reactive, corrosive, and miscible with air and oxygen. An industrial process was describes the use of SC water to treat aqueous solutions containing organic compounds (Haas et al. 1989). The operation of a process based on SCF technology was described to treat waste of recombinant fermentation (Krishna et al.1986).

9.4 Particle formation

The rapid expansion of SCF is a promising new technology for particle formation and distribution of biodegradable polymeric (Debenedetti et al. 1993). Because of the extreme fragility of organic aerogels attempts are made to develop inorganic aerogels. Such microcellular polymers foams can be obtained directly by polymerization in a near critical diluent and SC drying in the same reactor vessel. In polymer industry, polymerization is stopped by adding a termination agent. The polymer solution was contacted with superheated steam to remove unreacted monomer and polymerization solvent (de solvent process). SC-CO_2 extraction can be alternative for the de-solvent process of polymer solutions. In fact SC-CO_2 can reduce the drying process due to its capability of complete recovery by depressurizing. In addition, SC-CO_2 can dissolve the typical polymerization solvents, *n*-hexane or toluene at higher pressures. The design of the de-solvent process requires quantitative information on the distribution of organic solvent between the polymer solution and the SC-CO_2 phase (Inomata et al. 1999).

9.4.1 Preparation of liposome

Liposomes are non-toxic (mostly) and effective in encapsulation (Mortazavi et al. 2007) and controlled release in food industry (Mozafari and Khosravi-Darani 2008). Manufacturing of liposome by SCF covers three separate methods including: (i) phospholipids solvation in a near critical fluid, mixture with a protein containing buffered solution (ii) decompression of solvated phospholipids prior to injection to solution, (iii) the critical fluid decompression technique in which phospholipids are first hydrated in an aqueous buffer, mixed with SCF, with the mixture being then submitted to decompression. Several parameters can improve the characteristics of the liposomes prepared with SCF ethane. Optimization studies would be necessary to examine whether liposomes of higher quality can be made using SCF technology. Also, other SCF should be tested (Frederiksen et al. 1997).

9.5 Production of different morphologies of biocompatible polymers

SC antisolvent method has great potential for processing of pharmaceuticals (Mosqueira et al. 1981; Steckel et al. 1997) and labile compounds such as proteins (Debenedetti et al 1993; Winters et al. 1999; Yeo et al. 1994; Yeo et al. 1993) and to obtain various morphologies of biopolymers (Bleich et al. 1996; Debenedetti et al. 1993; Dixon and Johnstone 1993;

Reverchon 1999; Subramanian et al. 1997), such as microspheres (Falk et al. 1997) threads, fibers, networks (Dixon and Johnstone 1993), sponges, foams, and films. One of the advantages of using SCF in polymer processing is the possibility of producing different solid shapes and structures at low temperature with a minimum amount of residual organic solvents. Also the process is environmentally safe and economic (Elvassore et al. 2001). A basic description of these techniques is reported by Bertucco and Pallado (2000).
The conformation of monomeric enzyme trypsin has been reported in SC-CO_2 (Zagrobelny and Bright 1992). To follow in situ conformation of trypsin (as a function of CO_2 density), steady state fluorescence spectroscopy was used. Zagrobelny showed that protein denaturation can occur during the fluid compression step and that the native trypsin is only slightly more stable (1.2 kcal/mol) than the unfolded form.

9.6 Purification of natural active copolymers

Conventional purification methods are not specific and must be repeated or combined for highly purification. Although, (immuno) affinity-based procedures are rapid and specific; but they are expensive, and reagents from biological origin are needed. Also the interactions involved between the product and the support are often strong and imply the use of rather denaturing reagents (either for the product or the support) to attain an efficient desorption yield (Lemay 2002). SCE has introduced as a more suitable method for purification of natural products. This technique helps to remove trace impurities in the synthetic active biocopolymers from maleic anhydride and pinene (Jarzebski and Malinowski 1995).

10. Supercritical fluid extraction in bioprocess technology

Recent investigations on the applications of SCE from post fermentation biomass or *in situ* extraction of inhibitory fermentation products as a promising method for increasing yield are reviewed (Khosravi-Darani and Vasheghani-Farahani 2005). Although SC-CO_2 is unfriendly and toxic, for some living cells, which precludes direct fermentation in dense CO_2, it does not rule out other useful applications for *in situ* extraction of inhibitory fermentation products and fractional extraction of biomass constituents due to the potential of system modification by physical parameters and addition of co-solvents to selectively extract compounds of varying polarity, volatility and hydrophilicity with no contamination.

10.1 Advantages and disadvantages of SCE especially for the biotechnology industries

The advantages of utilizing SCE have been well documented (Schultz et al., 1991). The application of SCF is simple, inexpensive, non- injurious to the structure and function of some enzymes (Lin et al., 1992) and protein activities (Juhasz et al., 2003; Kamat et al., 1995; Zheng and Tsao, 1996). Nowadays, SCE is a well-known unit operation, with some industrial as well as many lab and pilot scale applications. Introduction of SC-CO_2 to fermentation broth decreases the overall viscosity, facilitates the handling of the broth and enhances mass transfer from the liquid to the SC-phase. Randolph has summarized special advantages of SCE, especially for the biotechnology industries (1990):

- High diffusivity reduces mass transfer limitations from porous solid matrices
- Low surface tension allows penetration and wetting of pores to extract from cell
- selectivity of extraction due to sensitivity of solubility to changes in P and T

- Manipulating crystal size of solid compounds produced from SCFs by change in P and T
- Separating of compounds that cannot be distilled, owing to their thermal instability.
- Increased enhancement factors (ratio of actual solubility to ideal gas solubility)
- Low reactivity and toxicity of SC-CO_2 or ethane, and their gaseous state

The main disadvantages of SCE processes include low solubility of biomolecules in SCF and high capital costs. Furthermore, insufficient data exist on the physical properties of many bio-molecules, making prediction of phase behavior difficult. The addition of co-solvents may obviate the advantage of minimal solvent residues in the final product.

10.2 Supercritical extraction (SFE) from biomass

10.2.1 Post fermentation extraction of products

There are only a few reports using SFE on bacterial cell. SCFs are found to be useful in extracting desired materials from animal tissues, cells, and organs (Kamarei and Arlington, 1988). By varying the choice of SCF, experimental conditions, and biological source materials, one may obtain lipids, proteins, nucleotides, saccharides, and other desirable components or remove undesirable components (Kamarei and Arlington, 1988). Processing of lipid natural products by SCF has been reviewed (King, 2004). SCF can be applied for obtaining aromatic and lipid components from plant tissues (Kamarei and Arlington, 1988), lignin conversion (Avedesian, 1986), carotenoids extraction from carrots (Bath et al., 1995), tomato paste waste (Baysal et al., 2000) and microalgae (Mendes et al., 1995). The CO_2 extraction process is selective in the presence of chlorophyll a.

Moreover, there are some reports which describe the SFE of bacterial (Gharaibeh and Voorhees, 1996) and fungal lipids (Cygnarowicz et al., 1992) for use in the classification of them by fatty acid profiles. A simple two–step process was developed to extract and purify medium chain length polyhydroxyalkanoates (MCL-PHA) from bacterial cells (*Pseudomonas resinovorans*) grown on lard and tallow (Hampson and Ashby, 1999). The process consists of SCE of the lyophilized cells with CO_2 to remove lipid impurities, followed by chloroform extraction of the cells to recover the MCL–PHA. SFE conditions were varied as to T 40 – 100°C, P (13.78 – 62.05 MPa), and CO_2 flow rate (0.5 – 1.5 L/min, expanded gas). The results show that the two- step process saves time, uses much less organic solvent, and produces a purer MCL-PHA biopolymer than previous extraction and purification methods. Khosravi-Darani et al. (2003) have reported the equilibrium solubility of poly(hydroxybutyrate) (PHB) in SC-CO_2. The effects of the main parameters such as P, T, and solvent density on solubility were determined at different T (35 – 75°C) and P (12.2 – 35.5) MPa. Hejazi et al. (2003) reported the effects of process variables such as exposure time, P, T, volume of methanol as a modifier, and culture history on PHB recovery from suspended *R. eutropha* in buffer solution. In another report, Khosravi-Darani et al. extended this work to obtain maximum recovery with minimum energy consumption (2004). In this work PHB recovery was examined using a combination of supercritical disruption and chemical (salt and alkaline) pretreatments. Bacterial cells, treated in growth phase, exhibited less resistance to disruption than nutrient limited cells in the stationary phase. It was also found that the wet cells could be utilized to recover PHB, but purity of the product was lower than that obtained from freeze-dried cells. Pretreatment with a minimum of 0.4% wt NaOH was necessary to enable complete disruption with two repetitions of P release. Salt pretreatment was less effective; however, disruption was improved by the application of alkaline shock.

The use of SCE of biologically active compounds (chaetoglobosin A, mycolutein, luteoreticulin, 7,8–dihydro–7,8–epoxy–1–hydroxy–3–hydroxymethylxanthone–8–carboxylic

acid methyl ester, sydowinin B and elaiophylin) from the biomass has been compared with organic solvents extraction (methanol and dichloromethane). The extraction strength of SC-CO_2 alone appeared to be lower than that of dichloromethane. All the components of interest that were extractable with dichloromethane and methanol were also extractable with methanol-modified CO_2 (Cocks et al., 1995). A technique for the SC-CO_2 extraction of the fungal metabolite ergostrol in its free (non-conjugated) form was developed and applied to samples of flour moldy bread and mushrooms. The overall method showed an 83% recovery of free ergostrol for spiked bread flour (Young and Games, 1993).

Citric acid has successfully been separated from fermentation broth by a novel and unique purification process, which is characterized by organic solvent extraction and precipitation with compressed CO_2 as a poor solvent. Compressed CO_2 was then dissolved in acetone solution of crude citric acid to remove the residual impurities as precipitates using the anti-solvent effect of CO_2. Citric acid crystals could be obtained by the anti-solvent crystallization with CO_2 (Shishikura et al., 1992). Dry mouldy bran resulting from solid state fermentation of *Gibberella fujikuroi* were subjected to SCE. The extraction of the sterol by SCE was found to improve with the use of ethanol as entrainer. The solid material retained the gibberellic acid activity without any loss (Kumar et al., 1991). The solubility of cholesterol in SCFs have also been studied and the solubility is correlated by using equation of states (Hartono et al., 2001).

Extraction of ethanol from aqueous phase of a yeast fermentation broth has been described and a lower energy cost as compared to distillation has been reported (De Filippi and Moses, 1983). Shimshick reported the extraction of carboxylic acids from dilute aqueous media with SC-CO_2. The specific advantage of this application is the pH decrease of the aqueous phase, which results in a higher concentration of the free acids. This shift is necessary for effective extraction of the carboxylic acids (Shimshick, 1981). SC-CO_2 extraction has been reported to be more suitable for extraction of non-polar compounds with molecular weights less than 400. Griseofulvin is an antifungal antibiotic having a molecular weight of 353, making it amenable to SC-CO_2 extraction. The optimized conditions for SCE of griseofulvin from dried media after solid state fermentation were obtained (Saykhedkar and Singhal, 2004). Furthermore, SCF has been developed mainly for unit operation to recover intracellular enzymes, recombinant-DNA proteins and nucleic acids from microbial cell cultures (Khosravi-Darani, 2005, Castor and Hong, 1995).

10.2.2 In situ extraction from the biomass of microbial fermentation

In situ product removal is the fast removal of product from a producing cell thereby preventing its subsequent interference with cellular or medium components. Freeman and coworkers indicated future directions including application in situ extraction to a wider range of products and the developed methodologies, applicable under sterile conditions in the immediate vicinity of the producing cells (Freeman et al., 1993). End-product inhibition occurs in many fermentation processes and *in situ* removal of them typically enhances product formation rates, yields, and specificity (Christen et al., 1990; Gyamerah and Glover, 1996; Qureshi et al., 1998). Techniques that have been employed for *in situ* removal of fermentation products include liquid-liquid extractive fermentation (Adrian et al., 2000), use of selective membranes (Chang et al., 1992), cell recycling (Roca and Olsson, 2003), adsorption (Millitzer et al., 2002), microcapsule application (Stark et al., 2003) and vacuum fermentation (Qureshi et al., 1998). However, the intimate contact of an organic phase with the broth implies that the organic components of this phase may be present in the aqueous

phase at saturation levels. The disadvantage of liquid-liquid extraction is the residual of toxic solvent, which presents significant separation, purification, and environmental challenges (Job et al., 1989). Also membrane fermentation and adsorption vacuum fermentation are not cost-effective. Guvenc et al. (1998) demonstrated the feasibility of ethanol extraction from a post–fermentation broth using SC-CO_2. However, application of SC-CO_2 for *in situ* extractive fermentation has been limited by its inhibitory effect on the metabolism of a variety of yeasts and bacteria (Isenschmid et al., 1995; Van Eijs et al., 1988). This toxicity is attributed, in part, to the acidic pH (Toews et al., 1995) that results from the increased solubility of CO_2 at high partial Ps (Knutson et al., 1999). By buffering the medium and carefully controlling the compression and expansion conditions, the survival rate of cells increases. Van Eijs et al. developed an extraction procedure in which the *Lactobacillus plantarum* cell death was minimized (Van Eijs et al., 1988).

The impact of dense gases and SCF (N_2, CO_2, and ethane) on the carbohydrate consumption and ethanol formation by *Clostridium thermocellum* has been reported. Non–growing cells capable of metabolism were incubated at 60°C with cellobiose as a substrate in the presence of the three pressurized fluids. The rate and extent of ethanol production were similar in cell suspensions maintained at atmospheric and 6.9 MPa P under nitrogen (conventional method). Ethane at 6.9 MPa reduced the extent of ethanol production by less than 20% relative to the atmospheric control, whereas CO_2 at the same P reduced ethanol formation. The results suggest that pressurized hydrocarbons have benefits over SC-CO_2 for the *in situ* recovery of volatile microbial products (Knutson et al., 1999).

In situ extraction of acetone, butanol and ethanol from synthetic media, simulating the downstream processing of a *Clostridium acetobutylicum* fermentation broth has been described (Van Eijs et al., 1988). It was also observed that extraction yield is a close function of the extraction time. Also increased P helps to achieve higher yields (Guvenc et al., 1998).

The extractive fermentation of 2-phenylethyl alcohol, the rose aroma, coupling fermentation with *Kluyveromyces marxianus* and SC-CO_2 extraction has been reported (Fabre et al., 1999). Similar results show enhancement of 2-phenylethanol productivity by *Saccharomyces cerevisiae* in two-phase fed batch fermentation using solvent immobilization (Serp et al., 2003). Stark and coworkers reported the extractive bioconversion of 2-phenylethanol by *Saccharomyces cerevisiae* (2002). It has further been reported that furfural, a growth inhibitory byproduct, was successfully removed during fermentation of *clostridium* on sugars by introducing liquefied CO_2 at room T and 5.9 MPa (Sako et al., 1992).

Selection of biocompatible solvents is critical when designing bio-processing applications for the *in situ* biphasic extraction of metabolic end-products. The prediction of the biocompatibility of supercritical and compressed solvents is more complicated than that of liquid solvents, because their properties can change significantly with P and T. The activity of the anaerobic thermophilic bacterium, *Clostridium thermocellum*, was studied when the organism was incubated in the presence of compressed nitrogen, ethane, and propane at 333 K and multiple pressure (Jason et al., 2000)

10.2.3 Fractionation of cellular biomass

SC and near critical fluids are used to fractionate biomass materials such as microbial cells in two steps. In the first step, the biomass is exposed to elevated pressure SC or near critical fluid to bring about disruption of the biomass to liberate structural biomass constituents. In the second step, the disrupted biomass is subjected to a multiplicity of SC or near critical fluid extraction steps, with different solvation conditions used for each fraction. Thus,

fractionation of the biomass to obtain one or more compounds is effected (Castor and Hong, 1995). Different solvation properties are obtained using different Ts, Ps and/or modifier concentrations. Industrial applications are designed to take benefit of the very high selectivity of SCFs with attractive costs related to continuous operation: polymer fractionation, aroma production from fermented and distilled beverages, polyunsaturated fatty acids, active compounds from fermentation broth, pollution abatement on aqueous streams, etc (Perrut, 2000). SC and near critical CO_2 have been used to fractionate cellular biomass isolated from soil, air, water, swamps, hot springs, sea water, animal or plant (Castor et al., 1998).

A SCE procedure and a chromatographic separation/detection method were developed for the detection of earth-based microorganisms. The analytical results demonstrated the feasibility of using the reported techniques to detect the chemical signature of life in barren desert sand samples (Lang et al., 2002).

Another interesting application of SCF in biotechnology is detecting the presence of a microorganism in an environmental sample. In this strategy, after exposure of sample to SCF nucleic acid will be isolated from the microorganism and detecting the presence of a particular sequence of nucleic acid by hybridization and PCR method, the contamination will be identified (Nivens and Applegate, 1996).

11. Conclusion

Application of supercritical is a promising alternative method for the pasteurization and sterilization of foodstuff, thermo sensitive substances, as well as thermally and hydrolytically sensitive polymeric materials, e.g. polymeric particles for drug delivery or implants. Furthermore, application of SC-CO_2 seems to be attractive for its economical feasibility, as it needs very low pressure (lower than 20 MPa) compared to the so–called ultra high pressure treatment (200–700 MPa). Another special applications of SCFs in food processing include the decaffeination of green coffee beans, the production of hops extracts, the recovery of aromas and flavors from herbs and spices, the extraction and fractionation of edible oils and the removal of contaminants. These applications are now extended to new areas like formulation or specific chemical reactions, due to lightening environmental regulations; concern over the use of chemical solvents in food manufacturing; increased demand for higher quality products; increased cost of energy.

In the future, two areas of SCF applications in food industry are forecast for growth; the treatment of industrial wastes and the high value added products. So new application will developed e.g. novel processes for the disruption of microorganisms of therapeutic interest, the production of liposomes with implication to the cosmetic and pharmaceutical industries, and even a process to destroy and remove viruses effectively. The emergence of such a process brings real excitement and suggests that in the field of pharmaceutical and bioprocess industries, commercial applications may find their way to its implementation in the next decade.

Future trends in industrial development of SCFs include; legal issues which require banning organic solvents, quality consideration (raw material decontamination) for instance, pests from tropical products; the extraction of residues and toxins from food materials; as well as the deodorization and removal of fat, cholesterol, caffeine.

From the results of a number of extractions reported in literature can be concluded that by application of SC-CO_2 selective, extraction of several compounds from fermentation broth is

possible. Non polar compounds can be extracted at low energy costs by this procedure. The process is cost effective due to carrying out at fermentation temperature. If whole fermentation broth put in contact with SC-CO_2, may inactivate the microorganisms. These results offer the opportunity of in situ extraction of fermentation products with SC or sub critical (liquid) CO_2. Use of SCF for both the disruption and extraction simplifies the procedure, and minimizes equipment and labor needs, time, contamination and loss of yield. In fact, the entire process can be readily automated. The use of super or near critical fluids allows for easy removal of the solvent by depressurization. The use of SCF allows the control of extraction condition by variation of temperature, pressure or modifier solvents.
The finding that fermentation conditions influence the resistance of microbial cells to disruption should be further investigated. Studies of disruption kinetics and of the influence of cell morphology on kinetics of disruption are needed, and not information is available on disruption of mycelial organisms. The effects of thermal deactivation on cell properties and pre-incubation temperature on cell resistance to heat shock have received less attention. Further work is therefore required to characterize this interaction and relate it to changes in cell and broth properties.

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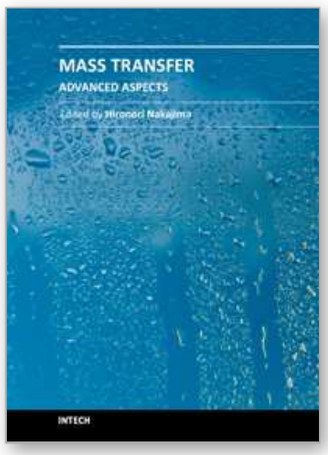

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Our knowledge of mass transfer processes has been extended and applied to various fields of science and engineering including industrial and manufacturing processes in recent years. Since mass transfer is a primordial phenomenon, it plays a key role in the scientific researches and fields of mechanical, energy, environmental, materials, bio, and chemical engineering. In this book, energetic authors provide present advances in scientific findings and technologies, and develop new theoretical models concerning mass transfer. This book brings valuable references for researchers and engineers working in the variety of mass transfer sciences and related fields. Since the constitutive topics cover the advances in broad research areas, the topics will be mutually stimulus and informative to the researchers and engineers in different areas.

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