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Capsaicin Recovery from a Cell Culture Broth

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The recovery of capsaicin from a cell *Capsicum annuum* culture broth is reported. First the solids are removed by filtration, and then liquid-liquid extraction is used to isolate the capsaicin. As observed from a previous study, chloroform was a better solvent than ethyl acetate and ethyl ether, and it was used to perform the extraction of the capsaicin in a reciprocating Karr column. The extraction studies were carried out by first using a synthetic mixture of capsaicin and water and then using cell culture broth. The operating conditions obtained in the pilot plant column are used to design a commercial-size reciprocating column.

Introduction

Capsaicinoids are an important group of organic compounds closely related to the family of alkaloids, and they are responsible for the hot chili flavor, of which capsaicin is the principal component. According to Suzuky and Iwai (1984), capsaicin [*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-*trans*-enamide] has a molecular weight of 305.2 and a condensed formula: $C_{18}H_{27}O_3N$. It is an odorless white needle crystal with severe burning pungency. It has a melting point of 64.5 °C and a boiling point of 210–220 °C at a pressure of 0.01 mmHg. It is easily soluble in ethyl ether, ethyl alcohol, acetone, methyl alcohol, carbon tetrachloride, benzene, and hot alkali. It is slightly soluble in carbon disulfide, hot water, and concentrated HCl. Capsaicin is practically insoluble in cold water.

Capsaicinoids are usually obtained by solid-liquid extraction using organic solvents like hexane to extract these compounds from the chili plants. Another way to get capsaicinoids is by plant cell tissue culture in a bioreactor. Capsaicin is excreted to the aqueous medium, since it is a secondary metabolite of chili cells.

Mavituna et al. (1987) have reported a study where they used immobilized cells to produce the secondary metabolites. In their experimentation they had a glass packed extraction column operating close to the bioreactor and using sunflower oil as the extracting agent. Strangely, to our knowledge there have been no additional reports of this work.

In order to obtain pure products (beginning with an aqueous mixture from a bioreactor) most separation processes used in biotechnology have a similar sequence that Belter et al. (1988) have called RIPP which stands for *R*emotion of insoluble material *I*solation of the desired component, *P*urification of the product, and *P*olishing of the final product to have it ready for sale or distribution.

This paper will comment on the first two steps of the sequence, to recover capsaicin from an aqueous cell culture broth. Emphasis will be on the isolation step that uses liquid-liquid extraction (LLE) as the separation process. Three different solvents are tried on a bench scale, and the best solvent is taken to a pilot Karr column where an experimental design strategy is used to specify operating conditions for an industrial Karr column. Before the real cell culture broth is used, a synthetic mixture of capsaicin and water is used to save time and get accustomed to the column operation and the analytical technique.

Materials and Methods

Production of Capsaicin. The production method was very similar to that reported by Mavituna et al. (1987), but in this study a bioreactor was used with suspended plant cells of *Capsicum annum* var. *annum* which excrete the secondary metabolite capsaicin into the medium. Different sizes for the bioreactor were used: first, test tubes of 30 cm^3 to study the effect of the different parameters over the yield of capsaicin were used, later agitated beakers of 1.0 L were applied, and so on. The last bioreactor used was a cylindrical glass bottle of 20 L, where 15 L of broth was studied. A time interval of 15–25 days was spent for every batch. As is the case in many bioprocesses, the concentration of the desired compounds in the aqueous broth was very low, in this case from 2 to 10 ppm.

Differently from Mavituna's work, in this study the capsaicin recovery process was not simultaneous to the production stage, but was applied after it.

First Stage of RIPP. Capsaicinoids are extracellular compounds that accumulate in the aqueous phase during the fermentation, but the broth also contains cells and other solids that need to be removed.

Filtration was tried using a bench Büchner funnel, a Kitasato beaker, filter paper, and a vacuum pump. From these tests it was determined that Whatman No. 2 filter paper was satisfactory and that the separation was not difficult. There was no additional washing of the solids.

Solid removal at the pilot-plant scale was carried out using a basket centrifuge with the following characteristics: diameter 0.32 m; height 0.20 m; maximum load 7.0 kg; angular velocity 1800 rpm; filtering medium screen of 1–5 μM.

Second Stage of RIPP. For this step, the high solubility of capsaicin in organic solvents was applied and liquid-liquid extraction was intended as the separation process for this isolation stage.

The following solvents were tried: chloroform; ethyl acetate; diethyl ether. According to Suzuky and Iwai (1984), none of these solvents react with capsaicin.

The effects on different extraction parameters were analyzed by a factorial experiment design. After an exhaustive analysis of the problem, we selected the following parameters: temperature; solvent to feed ratio; pH.

Extraction experiments were conducted using a Mixxor separatory cylinder apparatus (Cole-Palmer Instruments Co.). A synthetic mixture of capsaicin, water, and methanol was mixed with each solvent and then separated,

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Table I. Experimental Design for Optimization of Extraction Variables

factors	level 1	level 2
temperature (°C)	23	30
solvent/feed	1.00	1.50
pH	6.50	8.50

Experiments Performed with Each Solvent[a]

expt no.	A	B	C	D	E	F	G
1	1	1	1	1	1	1	1
2	1	1	1	2	2	2	2
3	1	2	2	1	1	2	2
4	1	2	2	2	2	1	1
5	2	1	2	1	2	1	2
6	2	1	2	2	1	2	1
7	2	2	1	1	2	2	1
8	2	2	1	2	1	1	2

[a] Experiments D to G are interactions between A, B, and C.

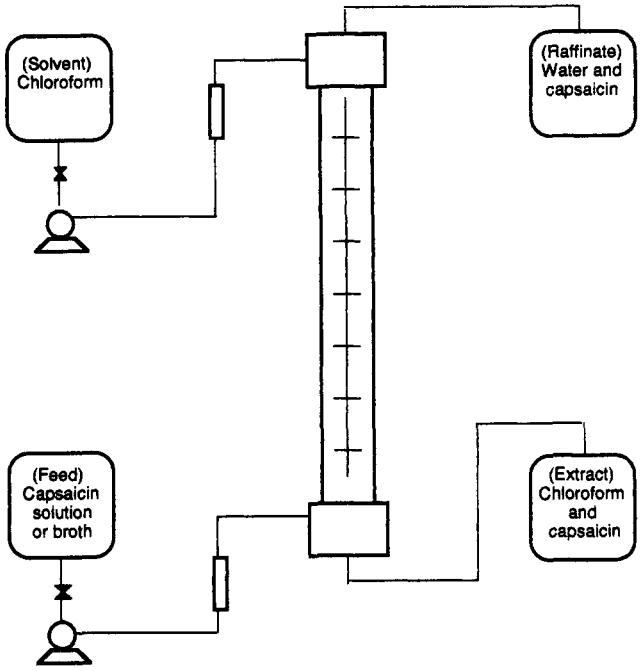

Figure 1. Extraction column and auxiliary equipment.

with different conditions of temperature, pH, and solvent to feed ratio applied according to Table I.

Analytical Determination of Capsaicin. Total capsaicin content was analyzed by high-performance liquid chromatography (HPLC), using a Econosphere C-18 analytical reverse-phase HPLC column (4.6 × 150 mm) and a CE-2212 detector at 280 nm. The mobile phase was a mixture of 70% methanol, 30% water loaded with $AgNO_3$ to get a 0.05 N solution flowing at 0.65 mL/min. The detector was connected with a PE Nelson 950 intelligent interface into an IBM personal computer. The technique has been reported by Garcia (1990).

Experimental Determination of Distribution Ratio K_d. The equilibrium distribution curve of capsaicin in water and chloroform was assessed and batch extraction experiments were also conducted using a Mixxor separatory cylinder at various temperatures to determine the efficiency of extraction. The analytical measurement of capsaicin in the organic phase allowed the calculation of 4.36 (C in ppm) as the value for the distribution ratio.

LLE Pilot Plant Studies. For this stage, a Karr reciprocating plate extraction column was used. The main portion of the column is borosilicate glass pipe. It has a

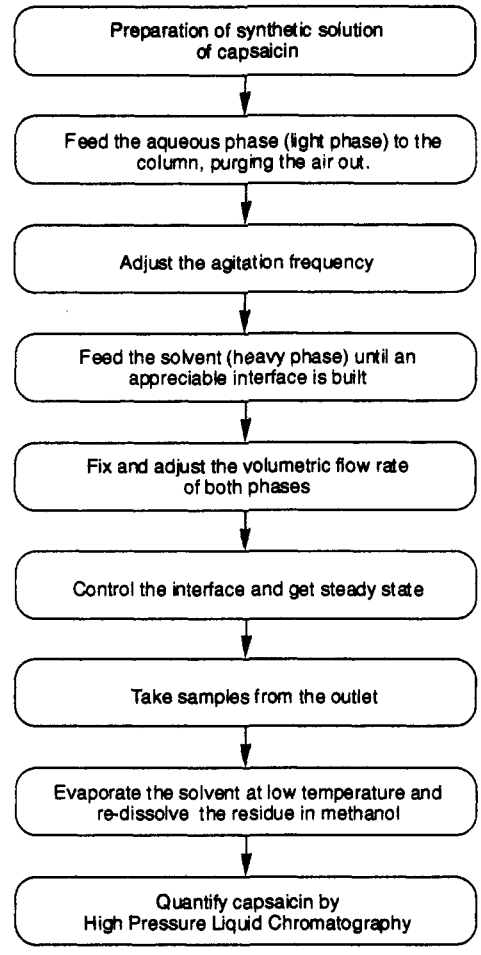

Figure 2. Flow diagram for capsaicin recovery.

total height of 5 m with an inside diameter of 2.54 cm. Inside the column there is a multiple of open-type stainless steel perforated plates mounted on a central shaft which can be reciprocated by means of a simple mechanism located above the column. Figure 1 shows the extraction column and the main auxiliary equipment.

The amplitude of reciprocation can be varied between 0 and 2.0 cm by simply adjusting the length of a crank arm. The speed of reciprocation can be varied from 0 to 400 strokes/min. The wide range of strokes per minute and amplitude provide an extra degree of freedom not present in other types of extraction columns, and thereby ensure proper agitation for all applications. The reciprocating plate column is unique in that it is so easy to take apart and put back together again that optimization of plate spacing for maximum efficiency for different applications is readily achieved.

At this stage of pilot plant the following factors were analyzed: agitation frequency, A (strokes/min); ratio of solvent to feed, R; column height, Z (m); feed concentration, C (ppm); amplitude, L (m).

An experimental design according to Taguchi methodology cited by Yuin and Willie (1987) was used to establish the optimum values for each parameter, using again the synthetic mixture of capsaicin and water. Figure 2 shows the sequence of steps followed for the experimental determination of capsaicin recovery for each test.

When the optimum values for each parameter were defined. A few tests were carried out to a real cell culture broth with the following characteristics: pH 5.88; nitrites 0.830 g/L; ammonium 0.150 g/L; carbohydrates 10.0 g/L; dry weight 2.0 g/L: capsaicin 0.022 mg/L; time of culture 20 days; density 0.958 g/cm^3.

Table II. Optimum Parameters for Capsaicin Recuperation Using a Synthetic Mixture on a Karr Column

volumetric flow rate of feed	Q_f = 24.3–26.0 L/min
volumetric flow rate of chloroform	Q_s = 13.0–13.5 L/min
solvent to feed ratio	R = 0.46–0.55
agitation frequency at flooding	A_{max} = 130 cycles/min
agitation frequency at operation	A = 80 cycles/min
amplitude	L = 2.1–2.3 cm
effective column height	Z = 366 cm
capsaicin concentration	C = 1.5–5.0 ppm

Experimental Runs with Cell Culture Broth. The goals were (1) to determine the flooding conditions and the operational range, (2) to determine the percent of recovery at operating conditions, (3) to evaluate a decrease in solvent to feed ratio, and (4) to evaluate a semicontinuous form of operation (solvent fixed inside the column and feed being recirculated).

Results and Discussion

From the extraction of capsaicin at the bench scale with the Mixxor separatory cylinder, using chloroform, ethyl acetate, and diethyl ether at several temperatures, pH values, and solvent to feed ratios, the analysis of the results on the experimental design show that the type of solvent and the ratio feed/solvent are the only significant factors, while temperature and pH are not significant. Then the last two parameters were set at ambient temperature and with the pH that has the cell culture broth.

In order to select the best solvent and to see if the type of solvent is a significant factor, a Duncan's multiple comparison was performed using among other parameters the average recuperation (of capsaicin) from each solvent, as follows: ethyl ether, 76%; ethyl acetate, 84%; chloroform, 89%.

From the Duncan's test it was found that the type of solvent was a significant factor, and then the optimum conditions for the liquid–liquid extraction were solvent, chloroform; solvent/feed ratio, 0.5; temperature, 25 °C; and pH 6.5.

Garcia (1990) has reported these studies with the bench-scale Mixxor equipment, while the studies with the pilot plant Karr column are reported by Tapia (1992).

According to the studies reported by Garcia (1990) the second best solvent was ethyl acetate. This solvent should be kept in mind for future research, because of the possibility that chloroform could be prohibited as a solvent for this type of processes.

The best conditions from the bench-scale study were used in the pilot-scale isolation of capsaicin with the reciprocating Karr column. Still the synthetic mixture of capsaicin and water was used. In this experimental design the significant factors were the agitation frequency, the solvent to feed ratio, and the column height.

The optimum parameters for the operation of the Karr column with the synthetic mixture are shown in Table II. With these conditions the fractional volume of the dispersed phase (holdup) was about 0.12 and the recovery of capsaicin was 97–98.6%.

The results for the operation with the real cell culture broth using the Karr column show that the agitation frequency was lower than when the synthetic mixture was used; this means that the column flooded with less cycles per minute of agitation. The values of operational parameters for the real cell culture are given in Table III.

At this run a fractional holdup of 0.30 was reported, with a recovery of 99.99%. Two observations were indicated for this run: there was some turbidity at the interface, and the friction between the plates and the column was reduced.

Table III. Optimum Parameters for Capsaicin Recuperation Using Cell Culture Broth on a Karr Column

pressure	atmospheric
temperature	25 °C
effective column height	366 cm
plate spacing	5.0 cm
hole area/column area	0.55
solvent	chloroform
feed volumetric flow rate	26.0 L/h
solvent volumetric flow rate	13.0 L/h
N_t	9.15
HETS	0.40 m
agitation frequency	80 cycles/min
percentage of flooding	80%
column diameter	2.54 cm
amplitude	2.1 cm

The differences between operation with synthetic broth and operation with real cell culture broth are explained by the higher holdup of the real broth, possibly produced by the presence of other components in the broth. On the other hand, from the high value of dispersed-phase holdup we may conclude that this run was at flooding or very close to it.

Semicontinuous Operation. Inside the column 2.04 L of solvent was maintained, and 5.0 L of feed was recirculated for a certain period of time. The recovery of capsaicin in this case was 96%.

Experimental Run with Real Broth To Minimize Cost. In this case the solvent to feed ratio was lowered to 80% of the previous one, and the capsaicin recovery dropped to only 95.0% instead of the 99.99% obtained in the other run.

Design of Reciprocating Karr Columns. Although there have been many intentions to design this kind of equipment starting from basic principles, pilot plant studies continue to be a necessity. The experimentation in small columns is used for the determination of operational ranges for the superficial velocities of both phases, and the height equivalent to a theoretical stage (HETS) as a function of the reciprocative velocity.

To complete the design, the following steps recommended by Karr (1985) must be performed:

1. Calculation of the column diameter must be performed. The superficial velocities are maintained, as well as the plate spacing, hole free area, and amplitude. The new diameter is calculated from the cross-sectional area that is obtained by dividing the volumetric flow rate (m^3/s) by the superficial velocity (m/s).
2. Calculation of HETS, with eq 1, is necessary:

$$\mathrm{HETS}_2/\mathrm{HETS}_1 = (D_2/D_1)^{0.38} \quad (1)$$

3. Calculation of the reciprocative velocity, or the number of strokes per minute, with eq 2 must be performed:

$$A_2/A_1 = (D_1/D_2)^{0.14} \quad (2)$$

4. Calculation of column height, with eq 3, is necessary:

$$Z = N_t\mathrm{HETS} \quad (3)$$

The application of this design method is applied to a case study in Appendix A.

Conclusions

I. Capsaicin can be produced (until now with low yield) by suspended and immobilized culture of plant cells.

II. The first two steps of the overall separation and purification process have been evaluated by applying filtration and centrifugation for the first stage, liquid–liquid extraction with chloroform as a good solvent, and a reciprocating Karr column as the process equipment.

III. The optimal parameters were established by first using a synthetic broth, both at bench scale and with the pilot plant Karr column; then the best results and the experience were applied to the operation at the Karr column with the real cell culture broth.

IV. Now we have the basic information to design a commercial column to isolate capsaicin from cell culture broth, using chloroform as the solvent in a solvent to feed ratio of about 0.5.

V. Other variations have been evaluated such as semicontinuous operation and a lower solvent to feed ratio.

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Nomenclature

A = agitation frequency, cycles/min
A_c = cross-sectional area of extraction column, m^2
C = concentration, ppm
D = diameter of column, m
HETS = height equivalent to a theoretical stage, m
L = amplitude of one stroke, m
N_t = number of theoretical stages, dimensionless
Q_f = volumetric flow rate of dispersed phase, m^3/s
Q_s = volumetric flow rate of solvent, m^3/s
R = solvent to feed ratio, dimensionless
U = superficial velocity (based on empty column), m/s
V = volume, m^3
Z = effective height of column, m

Subscripts

1 = pilot plant column
2 = industrial column

Appendix A

Case Study. Design a Karr liquid–liquid extraction column to isolate from plant cell tissue culture and bioseparation the equivalent to 20 000 kg/year of oleoresin with 2.5 wt %. Consider: (A) that the extraction column will work the equivalent time of 2 months (60 days = 1440 h = 5 184 000 s) and (B) that the composition of capsaicin in the cell culture broth is 5 ppm = 0.000 005 weight fraction.

Solution.

(a) The mass of pure capsaicin to be produced is

$$\left(20000\ \frac{\text{kg of oleoresin}}{\text{year}}\right)\left(0.025\ \frac{\text{kg of capsaicin}}{\text{kg of oleoresin}}\right) = 500\ \frac{\text{kg of capsaicin}}{\text{year}}$$

(b) The mass of cell culture broth with 5 ppm of capsaicin needed to produce 500 kg of capsaicin per year is

$$\text{kg of broth} = \frac{500\ \text{kg of capsaicin}}{0.000006\ \frac{\text{kg of capsaicin}}{\text{kg of broth}}} = 100000000\ \text{kg of broth}$$

(c) The volume of broth is given by the following, assuming that the density of broth is equal to that of water:

$$V = \frac{10000000\ \text{kg of broth}}{1000\ \frac{\text{kg of broth}}{\text{m}^3\ \text{of broth}}} = 100000\ \text{m}^3\ \text{of broth}$$

(d) The volumetric flow rate of broth feed to the extraction column (for a working time of 5 184 000 s) is

$$Q_{f_2} = \frac{100000\ \text{m}^3\ \text{of broth}}{5184000\ \text{s}} = 0.0193\ \frac{\text{m}^3}{\text{s}}$$

(e) Step 1. Calculation of column diameter, with equal superficial velocity in both (pilot and commercial) columns is performed as follows.

The broth superficial velocity in pilot plant column is given by

$$U_{f\text{-}1} = Q_{f\text{-}1}/A_{c\text{-}1}$$

From Table II

$$Q_{f\text{-}1} = 26\ \text{L/h} = 0.0000072\ \text{m}^3/\text{s}$$

$$A_{c\text{-}1} = 0.0254^2(3.1616/4) = 0.000506\ \text{m}^2$$

then

$$U_{f\text{-}1} = \frac{Q_{f\text{-}1}}{A_{c\text{-}1}} = \frac{0.0000072}{0.0005067} = 0.01425\ \frac{\text{m}}{\text{s}}$$

For the commercial column with ($U_{f\text{-}1} = U_{f\text{-}2}$)

$$A_{c\text{-}2} = \frac{Q_{f\text{-}2}}{U_{f\text{-}2}} = \frac{0.0193\ \text{m}^3/\text{s}}{0.01425\ \text{m/s}} = 1.354\ \text{m}^2$$

then the column diameter will be $D_{c\text{-}2} = 1.31\ \text{m}^2$.

(f) Step 2. Calculation of HETS for the commercial column is performed as follows.

Taking $\text{HETS}_1 = 0.40$ m from Table II, and using eq 1,

$$\text{HETS}_2 = \text{HETS}_1\left(\frac{D_2}{D_1}\right)^{0.38} = 0.40\left(\frac{1.31}{0.0254}\right)^{0.38} = 1.79\ \text{m}$$

(g) Step 3. Calculation of reciprocant velocity is performed as follows.

Taking $A_1 = 80$ cycles/min from Table II and applying eq 2,

$$A_2 = A_1\left(\frac{D_1}{D_2}\right)^{0.14} = 80\left(\frac{0.0254}{1.31}\right)^{0.14} = 46\ \text{cycles/min}$$

(h) Step 4. Calculation of column height is performed as follows.

Application of eq 3 with $N_t = 9.15$ from Table II gives

$$Z_2 = N_t\text{HETS}_2 = 9.15 \times 1.79 = 16.4\ \text{m}$$

(i) Summary. The commercial Karr column needs diameter 1.31 m, height 16.4 m, and agitation 46 cycles/min.

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