Bleu cheese flavor production by submerged fermentation with on-line monitoring by gas chromatography

Eight cultures of *Penicillium roqueforti* and related species were compared in shake-flask cultures on the basis of aroma. *P. decumbens* IFO 7091 was chosen as having the aroma closest to real Bleu cheese, and was used for fermentation studies. Gas chromatography was employed to obtain a continuous record of 2-pentanone concentration in the fermentor. Methyl ketones are characteristic of Bleu cheese flavor, and a subjective correlation was observed between the aroma of the fermentor off-gas and the 2-pentanone concentration measured. Cell growth and milk fat utilization increased consistently with increasing agitation and aeration, but 2-pentanone production did not correlate with growth and was maximum at intermediate values of agitation and aeration. The maximum 2-pentanone concentration, 100 ppm, was approximately ten times the concentration in Bleu cheese on a dry solids basis. Production of Bleu cheese flavor by submerged fermentation offers the opportunity for a new commercial value-added product from butterfat.

Keywords: *Penicillium*; Roquefort; butterfat; cream; gas chromatography; 2-pentanone

Introduction

Production of Bleu or Roquefort type cheeses by traditional methods is a time-consuming process which few dairy-product manufacturers are willing to attempt. At the same time, butterfat, which is the source for the characteristic flavor of Bleu cheese, is an underutilized, underpriced commodity. To fulfill the need for value-added products from butterfat, and to satisfy the demand for Bleu cheese as a flavoring ingredient in salad dressing and other prepared foods, the development of a submerged fermentation process for Bleu cheese flavor was undertaken.

The origins of this idea can be traced in papers and patents dating back thirty years. It was previously known that odd-numbered 2-alkanones are characteristic of Bleu cheese flavor. Early studies attempted to establish the conditions for formation of these compounds by oxidation and decarboxylation of the corresponding fatty acids [2,10]. The question of whether spores or mycelium of *P. roqueforti* were more active in carrying out this transformation received considerable attention [5,7]. For rapid technical production of methyl ketones, fatty acids prepared by prehydrolysis of milk fat with purified lipase were used as the starting material [3,4,8,15]. A review of early work on the biochemistry of flavor production by *P. roqueforti* has been published [11]. More recently, papers have appeared concerning the effect of water activity on aroma production by *Trichoderma viride* [6], the production of methyl ketones by Bleu cheese slurries [9], with immobilized spores of *P. roqueforti* [12], in milk-fat-coated microcapsules [17,18], and by submerged fermentation with *Penicillium decumbens* [24]. A commercial process is in current use in Germany [20,21], but details of the fermentation process have not been disclosed.

One problem inherent in this type of work is the time lag in measuring the methyl ketone concentrations in a rapidly changing batch process. The time required to carry out a manual assay, which may involve several extractions and column separations, renders it almost useless as a means of monitoring an ongoing fermentation. When this problem is coupled with the generally non-reproducible time course of the process, the difficulty in obtaining a consistent product quality becomes obvious. Moreover, *P. roqueforti* can destroy 2-alkanones as rapidly as it can produce them [9]. Therefore, our objective in this work was to establish a gas chromatographic method for on-line monitoring of a characteristic flavor compound, and to use this method to develop a submerged fermentation process for Bleu cheese flavor production.

Materials and methods

Shake flask cultures

One strain of *Penicillium roqueforti* was isolated from a piece of Danish blue cheese from the supermarket. Three cultures were obtained from the Institute for Fermentation in Osaka, Japan. These were *P. roqueforti* IFO 4622, *P. caseicolum* IFO 5849 and *P. decumbens* IFO 7091 [24]. Four cultures were obtained from the American Type Culture Collection in Rockville, MD, USA: *P. roqueforti* strains ATCC 6989, ATCC 10110 and ATCC 42294 and *P. decumbens* ATCC 10436. The cultures were maintained on potato dextrose agar slants; 0.5 ml of spore suspension was inoculated into each 500-ml flask containing 50 ml of sterile medium consisting of 20 ml heavy cream (35% milk

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fat) and 30 ml distilled water. The wide-mouth flasks were covered with double non-gauze 16.5 cm disk milk filters (Kendall, Boston, MA, USA) secured with rubber bands and incubated at 25–27° C with shaking. Flasks were swirled daily manually to resubmerge wall growth and were sniffed for subjective aroma evaluation. A sample of authentic Bleu cheese was used for comparison. After 5–9 days, the entire contents of each flask were analyzed for total lipids and cell dry weight by extraction in a 250-ml separatory funnel with 150 ml of 2:1 chloroform/methanol. The bottom, chloroform layer containing lipids was drained into a tared 90 × 50-mm crystallizing dish and the chloroform was evaporated under a stream of nitrogen to a constant weight of residue. The upper water/methanol layer containing lipid-free cells was filtered on a pre-dried and tared 5.5-cm glass fiber filter in a Buchner funnel. The cells were dried to constant weight at 100° C.

Fermentation

P. decumbens IFO 7091 was used in all fermentations. Early fermentation runs were done in a Mouse fermentor (Queue Systems, Parkersburg, WV, USA) containing either 360 g of cream and 540 ml of distilled water or 450 g of cream and 450 ml of distilled water. The cream was either pasteurized, nonhomogenized heavy cream (35% fat) or pasteurized and homogenized light cream (13.5% fat). The medium was sterilized for 15 min at 121° C. The inoculum consisted of spores from a single slant. Temperature was 25° C, aeration was 0.04–0.15 L min^{-1} and agitation was 400 rpm. Dissolved oxygen and pH were monitored continuously. Later runs were done in a larger computer-controlled fermentor (Lab-Line, Melrose Park, IL, USA) containing 1 kg light cream (13.5% fat) and 1 L distilled water, sterilized 15 min at 121° C. Temperature was 25° C, agitation was 600 or 1000 rpm and aeration was varied from 0.03 to 0.53 L min^{-1}. Dissolved oxygen and pH were monitored continuously and recorded every hour. At the end of 8–29 days fermentation, the fermentor was sterilized 15 min at 121° C and the contents were emptied, weighed and blended. Two 33-g samples were analyzed for total lipids and cell dry weight by the method used for shake flasks.

Gas chromatography

A portable gas chromatograph, Model M200D (Microsensor Technology, Fremont, CA, USA) was installed on the fermentor exhaust line. This unit contains two complete miniature gas chromatographs, each consisting of two 4-m × 0.1-mm columns, type OV-73 (column A) or OV-1701 (column B) with column heaters and miniature silicon injector valves and thermal conductivity detectors. It was configured for continuous gas flow through the injectors. At low aeration rates, 0.03–0.04 L min^{-1}, the entire fermentor exhaust was directed through the instrument. At higher aeration rates, a portion of the fermentor exhaust gas was pumped through the instrument with a peristaltic pump. The column temperatures were 75° C and the injection time was 150 ms.

Pure compounds, 2-pentanone, 2-heptanone, 2-nonanone, 2-undecanone, 2-pentanol, 2-heptanol, 2-nonanol, and 2-undecanol (Fluka, Ronkonkoma, NY, USA) were used as standards. Run times and detector responses for air saturated with the 5- and 7-carbon compounds are shown in Table 1. Higher column temperatures would have been required to analyze the 9- and 11-carbon compounds since they did not elute before the 160-s maximum of the instrument. In early fermentation runs, although weak signals were detected for 2-heptanone in column B and for 2-pentanone and 2-heptanone in column A, the greatest response was for 2-pentanone in column B. Rigorous calibration was carried out for this signal only. From the published value of vapor pressure, 35 mm Hg [19], and the peak area in Table 1, the detector response per standard atmosphere of 2-pentanone was calculated to be 86000 atm^{-1}.

Table 1 Chromatography of standards

	Compound	Elution time (s)	Peak area
Column A[a]	2-pentanone	21.9	3380
	2-pentanol	23.4	327
	2-heptanone	64	225
	2-heptanol	78	24
Column B[a]	2-pentanone	37.6	3970
	2-pentanol	40.4	498
	2-heptanone	133	375
	2-heptanol	149	36

[a]Column A was type OV-73 and Column B was type OV-1701. Both were 4 m × 0.1 mm and were operated at 75° C and 150 ms injection time

In order to convert from atmospheres to parts-per-million in the fermentor, it was necessary to know the Henry's law constant. Although the value for 2-pentanone in water is published, it was felt that it might be different in the 50% cream medium, and it was therefore measured independently. To the sterilized, spent medium, 2-pentanone was added, 0.1 ml at a time, and the response recorded after equilibration. From the slope of the resulting curve (Figure 1), a Henry's law constant of 7.0×10^{-5} atm m^3 mol^{-1} was calculated. This was a little lower than the published value for water, 8.5×10^{-5} atm m^3 mol^{-1} [19]. This experiment was done at a low air flow rate of 0.05 L min^{-1}. To assure that the Henry's law calculation would be valid at all air

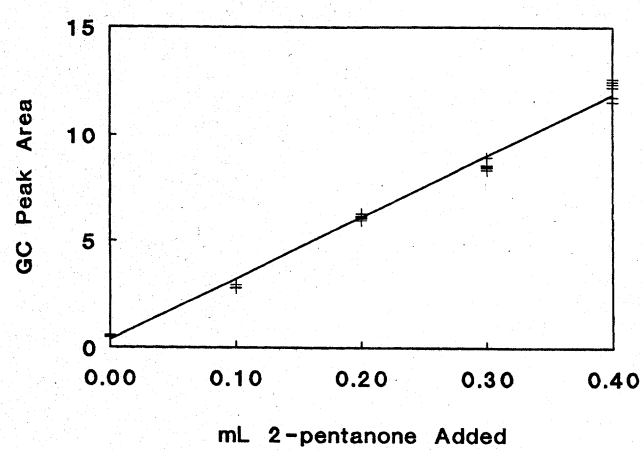

Figure 1 Calibration of gas chromatograph at 0.05 L min^{-1} air flow rate and determination of Henry's law constant for 2-pentanone in 50% light cream. Peak response was at 37.6 s, column B, 75° C, 150 ms injection time

flow rates, it was necessary to verify that the gas–liquid contact in the fermentor was sufficient to achieve saturation at higher flow rates. Because stripping of the 2-pentanone from the fermentor became significant at higher flow rates, a different technique for measuring the constant was required. From the first order, logarithmic decrease in the signal with time (Figure 2), the Henry's law constant was calculated to be 7.3×10^{-5} atm m^3 mol^{-1} at 0.37 L min^{-1} and 7.4×10^{-5} atm m^3 mol^{-1} at 0.14 L min^{-1} (data not shown). The agreement between the constants measured at different air flow rates indicates that a Henry's law calculation is valid at all flow rates in this range. The average value, 7.2×10^{-5} atm m^3 mol^{-1}, was used to calculate the parts-per-million of 2-pentanone in the fermentor from the detector response.

Results and discussion

In the first series of shake flask experiments, consisting of a total of 45 flasks, all eight cultures were compared. After 9 days, the lipids decreased from 35% of cream initially to approximately 30%, while the cell dry weight reached approximately 0.8 g (1.6% of total culture volume). These values did not vary significantly from one culture to the next. However, there was a definite difference in the aroma of the different cultures. In terms of strength of Bleu cheese aroma, the *P. roqueforti* from Danish blue cheese and ATCC 10436 and ATCC 10110 were the weakest, *P. roqueforti* IFO 4622 was slightly stronger, strains ATCC 6989 and 42294 had moderately strong aromas of Bleu cheese, and *P. decumbens* IFO 7091 and *P. caseicolum* 5849 had strong moldy cheese aromas, approximately like Bleu cheese. In a second series of shake flask experiments consisting of a total of 48 flasks, the five most promising cultures were compared. This time, *P. decumbens* IFO 7091 seemed to have the best Bleu cheese aroma, with *P. caseicolum* IFO 5849 and *P. roqueforti* ATCC 42294 nearly as good. On this basis *P. decumbens* IFO 7091 was chosen for fermentation studies.

In the fermentors the 2-pentanone concentration correlated well with the aroma of the culture. That is, the higher

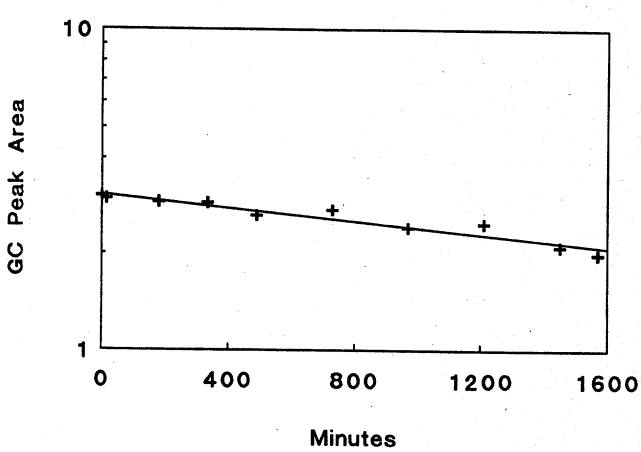

Figure 2 Calibration of gas chromatograph at 0.37 L min^{-1} air flow rate and determination of Henry's law constant for 2-pentanone in 50% light cream. Peak response was at 37.6 s, column B, 75° C, 150 ms injection time

the concentration of 2-pentanone, the more the aroma of fermentor off-gas was like Bleu cheese. In the small fermentor containing 900 ml of medium, after several days the milk fat coagulated into a solid mass floating on the surface of the liquid. Decreased aeration and increased agitation helped to keep the solid fat submerged. In the larger fermentor containing 2 L of medium, there was still some separation of solid fat from the liquid culture at 600 rpm, especially toward the end of the fermentation. At 1000 rpm the milk fat remained in suspension. The pH decreased from an initial value of approximately 6 to approximately 4.5–5.0 during the first day or two after inoculation. Then it gradually increased to a final value of approximately 5.5. Data for final cell dry weight, final total lipids and maximum 2-pentanone concentration for fermentations carried out at four different air flow rates are shown in Table 2. In two experiments, the data for 2-pentanone are missing because silicone tubing, which was discovered to be highly permeable to 2-pentanone, was employed between the fermentor and the GC. In those two experiments, most of the 2-pentanone in the fermentor exhaust escaped before it could be measured. Additional data for the experiment in which the maximum 2-pentanone concentration was 40 ppm are shown in Figure 3.

Several conclusions can be drawn from the data in Table 2. The cell dry weight consistently increased with increasing aeration and/or agitation. This is to be expected for an aerobic organism and is also supported by the fact that the dissolved oxygen dropped to nearly zero within a day or two of inoculation and stayed there throughout the fermentation. Total lipids did not decrease from their initial value at low aeration rates, but utilization of the butterfat was observed at 0.50 and 0.53 L min^{-1}. At 0.14 L min^{-1} and 600 rpm, the total lipids remained unchanged, but they decreased at 0.15 L min^{-1} and 1000 rpm. This is also in keeping with a mass-transfer-limited oxygen supply. The 2-pentanone concentration was highest at 0.14 L min^{-1} and 600 rpm. Increasing the aeration and agitation to 0.50 L min^{-1} and 1000 rpm nearly eliminated 2-pentanone production. Thus, flavor production did not correlate with growth or fat utilization. The highest concentrations of 2-pentanone observed during the fermentations, 40–100 ppm, were approximately the same as those reported for dry Bleu cheese produced by traditional methods [11]. However, the fermentation product was about 90% water. Therefore, on a dry basis, the concentration of 2-pentanone was about 10 times higher than in authentic Bleu cheese. Although they were not measured, it is likely that the concentrations of other flavor compounds were similarly high. The amounts of 2-heptanone and 2-nonanone in samples of authentic Bleu cheese are from two to ten times as much as that of 2-pentanone [11]. However, the higher volatility of 2-pentanone suggests that it may be equally important for flavor and aroma.

Because of its volatility at elevated temperatures, approximately 50% of the 2-pentanone content of the fermentor was lost during the final sterilization. To avoid the loss of volatile flavor compounds as well as cooking of the product which causes browning and off flavors, the batch sterilization procedure used here should be replaced with a

Table 2 Two-liter fermentations of *Penicillium decumbens* IFO-7091 in 50% light cream at 25° C

Air flow rate (L min^{-1})	Agitation (rpm)	Duration (days)	2-Pentanone (ppm, maximum)	Cell dry wt (%, final)	Total lipids (% of cream, final)[a]
0.03	600	29	40	0.98	13.1
0.06	600	9	—[b]	1.2	13.5
0.14	600	11	100	1.6	13.6
0.15	1000	8	6	2.5	9.8
0.53	600	14	—[b]	2.2	8.7
0.5	1000	10	1	2.4	7.7

[a]Fresh cream 13.5% total lipids
[b]Results invalidated by the use of silicone tubing which is permeable to 2-pentanone

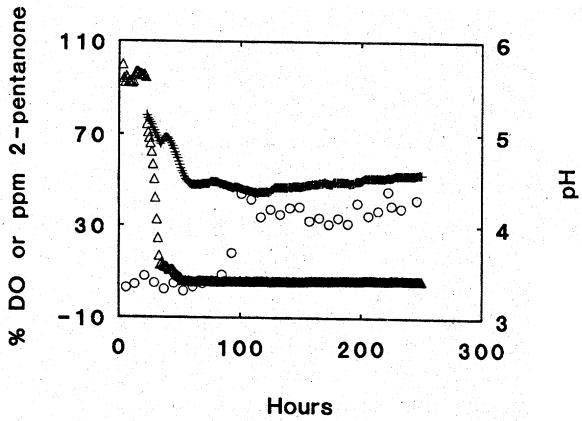

Figure 3 Data for fermentation of 50% light cream by *P. decumbens* IFO 7091 and production of 2-pentanone at 25° C, 600 rpm and 0.03 L min^{-1} air flow rate. +, pH; △, percent dissolved oxygen (% DO); ○, ppm

high-temperature, short-time, continuous sterilization. It has been reported that the flavor quality of a similar product was actually improved by heat treatment at 130° C for 4 s [15].

Many species of *Penicillium* produce mycotoxins [13]. Production of mycophenolic acid [22], roquefortine, eremofortin C [1,14] and PR toxin [16,23] by strains of *P. roqueforti* has been reported. Although it is generally accepted that the concentrations normally found in Bleu cheese, combined with the relatively small amounts normally consumed, render cheese produced by traditional methods safe, it is possible that improved growth conditions in submerged fermentation could result in much higher and perhaps dangerous concentrations. In spite of this potential difficulty, this process offers the promising possibility of a value-added product from butterfat.

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