Formation of 2-Acetyl-1-pyrroline by Several *Bacillus cereus* Strains Isolated from Cocoa Fermentation Boxes

Leo J. Romanczyk, Jr.,*,† Craig A. McClelland,† Laurie S. Post,† and W. Martin Aitken‡

M&M/MARS, 800 High Street, Hackettstown, New Jersey 07840, and Almirante Center for Cocoa Studies, Km. 2 Estrada BR 101, p/Barro Preto, Caixa Postal 55, 45630 Itajuipe, Bahia, Brazil

2-Acetyl-1-pyrroline was identified as the principal component responsible for a "popcorn, corn chip" aroma produced by several *Bacillus cereus* strains isolated from cocoa fermentation boxes in Bahia, Brazil. The analysis of direct solvent extracts and headspace volatiles consistently demonstrated the presence of 2-acetyl-1-pyrroline at temperatures well below those reported for its thermal formation. An examination of volatile compounds isolated by simultaneous distillation–solvent extraction showed five *B. cereus* strains to produce 30–75 μg/kg after a 2 day incubation on standard plate count agar. 2-Acetyl-1-pyrroline was produced by several ATCC *B. cereus* strains but not from ATCC *Bacillus mycoides* and *Bacillus thuringensis* strains nor from *Bacillus circulans*, *Bacillus licheniformis*, *Bacillus megaterium*, and *Bacillus subtilis* previously isolated from cocoa fermentation heaps. A series of ^{13}C- and ^{15}N-labeling experiments showed *B. cereus* to utilize glucose, glutamic acid, and proline for the formation of 2-acetyl-1-pyrroline. Since *B. cereus* occurs naturally in many unprocessed cereal grains, these results suggest the possibility that specific strains might be an unrecognized factor involved with 2-acetyl-1-pyrroline formation.

Keywords: *2-Acetyl-1-pyrroline; Bacillus cereus; cocoa; fermentation*

INTRODUCTION

Cocoa beans used in the manufacture of chocolate must first undergo a fermentation process, whereby the precursors of cocoa flavor are formed (Rohan and Stewart, 1967; Biehl et al., 1985). During the fermentation process, a succession of yeasts, lactic acid, acetic acid, and various spore-forming bacteria develop within the pulp surrounding the beans to produce a variety of microbially produced substances and conditions which contribute toward the development of cocoa flavor (Lehrian and Patterson, 1983). In the later stages of fermentation and subsequent drying phase, aerobic spore-forming bacteria of the genus *Bacillus* are typically present and may dominate other microbial populations (Ostovar and Keeney, 1973; Schwan et al., 1986). It has been suggested that this group of bacteria produces metabolites that contribute to cocoa flavor development (Lopez and Quesnel, 1973; Zak et al., 1972). In an effort to understand the contribution of microbial metabolites to cocoa flavor, a comprehensive study of the *Bacillus* genus was initiated. During the early course of our investigations, we observed several *Bacillus cereus* strains to produce "popcorn, corn chip" aromas characteristic of 2-acetyl-1-pyrroline.

2-Acetyl-1-pyrroline (A1P) has been isolated and identified from different varieties of rice (Buttery et al., 1983, 1986, 1988; Lin et al., 1990), the crust of wheat and rye breads (Schieberle and Grosch, 1985, 1987, 1991; Schieberle, 1989, 1990), corn tortillas (Karahadian and Johnson, 1993), *Pandanus amaryllifolius* leaves (Buttery et al., 1982; Laksanalamai and Ilangantileke, 1993), pearl millet (Seitz et al., 1993), cooked beef (Gasser and Grosch, 1988), honey (Blank and Fischer, 1989), popcorn (Schieberle, 1991), Chempedak fruit (Wong et al., 1992), urine of tigers (Brahmachary et al., 1990), and thermally treated *Theobroma grandiflorum* pulp (Fischer et al., 1993). To the best of our knowledge, the literature has not reported the formation of A1P by bacteria. The chance observation that A1P-like aromas were recognized at near ambient temperatures also suggested the possibility for a nonthermal formation pathway. We therefore present our preliminary findings on the formation of A1P by several *B. cereus* strains, efforts to elucidate the conditions for its formation, and results from a small survey for A1P in cocoa beans obtained from a variety of origins.

MATERIALS AND METHODS

Isolation and Identification of *B. cereus* Cultures. Samples were randomly taken from wooden cocoa fermentation boxes at the Almirante Center for Cocoa Studies and several adjacent farm locations within the Itajuipe region of Bahia, Brazil. Cultureswab transport systems containing Amies medium (with charcoal), Stuart's medium modified, or Cary-Blair medium (Difco Laboratories, Detroit, MI) were used to collect samples. The swabs were placed into tubes of Columbia broth (Difco Laboratories) and incubated 24–48 h at 35 °C for recovery. Samples of Columbia broth were streaked onto plate count (Standard Methods) agar (PCA) and tryptic soy agar (TSA) (Difco Laboratories) and incubated for 24 h at 35 °C. Isolated colonies were transferred to slants of TSA and stored at 4 °C. The isolates were Gram stained and aerobic spore-forming bacilli were selected for identification. The bacilli were speciated using the Vitek microbiological identification system (bioMérieux Vitek Inc., St. Louis, MO) (Odlaug et al., 1982). The identification of *B. cereus* strains was confirmed by testing for motility, hemolytic activity on trypticase soy sheep blood agar, lecithinase production, and rhizoid growth on nutrient agar (Harmon, 1982). Carbol–fuchsin stain was used to determine the presence of protein toxin crystals (Harmon, 1982).

Additional *Bacillus* Test Strains. The following *Bacillus* cultures were obtained from the American Type Culture Collection (ATCC) (Rockville, MD): *B. cereus* strains ATCC 9139, 10702, 11778, 13061, 14579, 14737, 27522, 33018, 33019, and 49064; *Bacillus mycoides* ATCC 6462; and *Bacillus*

* Author to whom correspondence should be addressed [telephone (908) 850-2284; fax (908) 850-2697].
† M&M/MARS.
‡ Almirante Center for Cocoa Studies.

0021-8561/95/1443-0469$09.00/0

thuringensis ATCC 10792. *Bacillus circulans*, *Bacillus licheniformis*, *Bacillus megaterium*, and *Bacillus subtilis* were previously isolated from cocoa fermentation heaps (Schwan et al., 1986). All cultures were stored on TSA slants at 4 °C until used.

Source of Cocoa Beans. Several 1 ton lots of Brazilian cocoa beans were fermented and sun-dried at the Almirante Center for Cocoa Studies. Samples of cocoa beans from different origins were received through commercial sources.

2-Acetyl-1-pyrroline Standard. An authentic sample of synthetic 2-acetyl-1-pyrroline was received as a generous gift from Ron C. Buttery at the Western Regional Center at Albany, CA.

Sample Aroma Recovery Procedures. Individual *Bacillus* samples were streaked onto plates containing 25 g of PCA and incubated at 35 °C for up to 4 days. At times 0, 24 h, 48 h, 3 days, and 4 days, the contents of three plates were combined with 1 L of deionized (DI) water, and the slurry was immediately subjected to a 2.5 h simultaneous steam distillation–solvent extraction (SDSE) using redistilled diethyl ether as the solvent. The distillates were dried over anhydrous Na_2SO_4 and then concentrated under a stream of nitrogen. For controls, uninoculated plates containing equal amounts of PCA were incubated at 35 °C for similar time periods.

SDSE distillates were prepared from 1 kg lots of cocoa beans roasted at 155 °C for 10 min. The roasted beans were manually dehulled and ground to a coarse powder in a Tekmar mill. A 100 g sample was subjected to SDSE under the conditions described above. SDSE distillates were similarly prepared from fermented, sun-dried, unroasted cocoa beans with hulls intact. For quantitative experiments, collidine (2,4,6-trimethylpyridine) (Aldrich Chemical Co., Milwaukee, WI) was added at the beginning of each distillation.

Cold (5 °C), redistilled diethyl ether (2 × 25 mL) was used to directly extract the contents of two PCA plates inoculated with *B. cereus* strains immediately after a 2 day incubation at 35 °C. The extracts were dried over anhydrous Na_2SO_4 and concentrated under a stream of nitrogen.

The procedure described by Vitzthum and Werkhoff (1978) was used with slight modification to collect the headspace volatile components present in three PCA plates inoculated with *B. cereus* strains immediately after a 2 day incubation at 35 °C. The first modification consisted of collecting the headspace volatiles at ambient temperature without diluting the sample in water. The second used 5 mL of redistilled diethyl ether to elute absorbed volatiles from the Tenax GC trap. The volume of solvent was reduced to approximately 25 μL by careful evaporation under a stream of nitrogen.

Precursors to 2-Acetyl-1-pyrroline. Filter-sterilized stock solutions of L-proline, L-ornithine hydrochloride, and L-glutamic acid (Aldrich Chemical Co.) were added at various concentrations to autoclaved PCA cooled to 45–50 °C. Various concentrations of amylose, amylopectin, *N*-acetyl-D-glucosamine, fructose, D-fructose 1,6-diphosphate dipotassium salt, D-glucose, D-(+)-glucosamine, D-lactose, maltose, ribose, and sucrose (Sigma Chemical Co., St. Louis, MO) were similarly prepared. These sugars were also substituted at the same level (0.1%) for glucose used in PCA. Three plates containing each additive were inoculated with *B. cereus* strain 35 and allowed to grow for 48 h at 35 °C. SDSE distillates were prepared for quantitative analysis as described below. PCA's containing 1% D-glucose (U-$^{13}C_6$, 98%+), L-glutamic acid (^{15}N, 95–99%), and L-proline (^{15}N, 98%+) (Cambridge Isotope Laboratories, Andover, MA) were individually prepared. SDSE distillates were obtained from single plates containing each labeled additive for GC-MS and GC-AED analysis as described below. Uninoculated plates containing labeled or unlabeled additives served as the controls. One additional control experiment was performed within this series of experiments. *B. cereus* strain 35 was first grown on PCA for 2 days at 35 °C, and the cells were collected by centrifugation. The cell fraction was washed with DI water, resuspended to a volume of 30 mL with DI water, and autoclaved at 121 °C for 15 min. The cell fraction was recovered by centrifugation, streaked onto PCA containing 1% glucose, and allowed to incubate at 35 °C for an additional 2 days. The SDSE distillate was prepared and examined for A1P by GC-MS as described below.

Gas Chromatography. Separations were achieved on a 60 m × 0.25 mm i.d. SPWAX-10 (Supelco Inc., Bellefonte, PA) fused silica capillary column programmed for 2 min at 70 °C, then 3 °C/min to 150 °C for 20 min, and then 25 °C/min to 250 °C for 20 min on a Hewlett-Packard Model 5890A gas chromatograph equipped with a nitrogen phosphorous detector (NPD). The operating conditions were NPD at 300 °C, injector at 250 °C, and linear velocity ($\bar{\mu}$) = 26 cm/s (helium), and 1 μL injections were split 50:1.

Linear retention indices were calculated using C_9–C_{14} *n*-paraffin standards (Alltech Associates, Deerfield, IL) as references (Majlat, 1974). Separations were achieved on 60 m × 0.25 mm i.d. SPWAX-10 and SPB5 fused silica capillary columns programmed for 2 min at 70 °C and then 3 °C/min to 200 °C on a Hewlett-Packard Model 5987A GC-MS system. GC-MS operating conditions were identical to those described below.

Gas Chromatography–Effluent Odor Evaluations. Capillary column effluent odor evaluations were performed on a Siemens SiCHROMAT2 multidimensional gas chromatograph (Marlton, NJ) equipped with a live switching system where the effluent stream was split 1:1 at a 1/16 in., 0.5 mm i.d. glass-lined Tee (Scientific Glass Engineering, Austin, TX). One stream was directed to the FID by a 0.5 m × 0.25 mm i.d. section of SPWAX-10 fused silica capillary column. The second stream was directed to an insulated (180 °C) odor evaluation port by an identical length of capillary tubing. Helium makeup gas (20 mL/min) was added prior to the Tee piece. Chromatography conditions were identical to those described above, except that 1 μL injections were split 20:1.

Gas Chromatography–Mass Spectrometry (GC-MS). Analyses were performed on a Hewlett-Packard Model 5987A GC-MS system. Electron ionization mass spectrometry (EI-MS) of various aroma extracts was performed at 70 eV with a source temperature of 200 °C, a scan range of 35–350 amu at a rate of 1.3 scans/s. Chromatography conditions were identical to those described above.

Gas Chromatography with Atomic Emission Detection (GC-AED). A Hewlett-Packard Model 5912A atomic emission detector (AED) was coupled with an HP Model 5890 series II GC equipped with an HP 7673 autosampler, split/splitless, and on column injection port. Separations were achieved on a 60 m × 0.25 mm i.d. SPWAX-10 fused silica capillary column programmed for 2 min at 70 °C and then 3 °C/min to 250 °C for 20 min. The operating conditions were injector at 250 °C, $\bar{\mu}$ = 33 cm/s (helium), AED transfer line at 250 °C, AED cavity block at 250 °C, and 1 μL injections were split 50:1. Hydrogen, oxygen, and methane reagent gases were used as a dopant mixture to monitor ^{15}N at 420.168 nm and ^{14}N at 421.465 nm. Hydrogen and oxygen reagent gases were used as a dopant mixture to monitor ^{13}C at 341.712 nm and ^{12}C at 342.574 nm. Reagent gas pressures were preset at the beginning of each experimental run for maximum sensitivity.

Quantitation of 2-Acetyl-1-pyrroline. The quantitation of A1P was performed on SDSE distillates using collidine as the internal standard (Buttery et al., 1986). The concentration of A1P was calculated by the equation

$$\text{amount } (\mu g/kg) = \frac{(A/B)(111/121)(C)}{D}$$

where *A* and *B* represent the NPD area counts for A1P and collidine, respectively, 111/121 represents the molecular weights of A1P and collidine, respectively, *C* represents the amount of internal standard in micrograms, and *D* represents the sample weight in kilograms. The relative responses for A1P and collidine were assumed to be equivalent to their molecular weight ratio, since sufficient quantities of authentic A1P were not available to experimentally determine their relative response factors and recoveries from the sample matrices examined.

Table 1. Biochemical Profiles for *B. cereus* Strains

	strains from cocoa fermentation boxes					ATCC	ATCC	ATCC
	5	8	9	19	35	11778	14579	33019
Vitek Profile								
negative control	[−][a]	−	−	−	−	−	−	−
sucrose	[+][b]	+	+	−	−	+	+	+
tagatose	−	−	−	−	−	−	−	−
glucose	+	+	+	+	+	+	+	+
inositol	−	−	−	−	−	−	−	−
galactose	−	−	−	−	−	−	−	−
arabinose	−	−	−	−	−	−	−	−
xylose	−	−	−	−	−	−	−	−
mannitol	−	−	−	−	−	−	−	−
raffinose	−	−	−	−	−	−	−	−
salicin	−	−	−	+	+	+	−	−
amygdalin	−	−	−	−	−	−	−	−
inulin	−	−	−	−	−	−	−	−
ribose	−	+	+	+	+	−	−	−
maltose	+	+	+	+	+	+	+	+
trehalose	+	+	+	+	+	+	+	+
palatinose	−	−	−	−	−	−	−	−
sorbitol	−	−	−	−	−	−	−	−
N-acetyl-D-glucosamine	+	+	+	+	+	+	+	+
amylopectin	+	+	+	+	+	+	+	+
arabitol	−	−	−	−	−	−	−	−
tetrazolium red	−	+	+	−	−	−	−	−
potassium thiocyanate	+	+	+	+	+	+	+	+
7% NaCl	−	−	−	−	−	−	−	−
mandelic acid	+	+	+	+	+	+	+	+
oleandomycin	−	−	−	+	−	−	−	−
sodium acetate	−	+	+	−	−	−	−	−
polyamidohygrostrepin	+	+	+	+	+	+	+	+
naladixic acid	+	−	−	+	+	−	+	+
esculin	+	+	+	+	+	+	+	+
Additional Assays								
lecithinase	+	+	+	+	+	+	+	+
motility	+	+	+	+	+	+	+	+
rhizoid growth	−	−	−	−	−	−	−	−
toxin crystals	−	−	−	−	−	−	−	−
hemolysis	+	+	+	+	+	+	+	+

[a] Negative reaction. [b] Positive reaction.

RESULTS AND DISCUSSION

Table 1 lists the biochemical profiles obtained for five *B. cereus* strains isolated from wooden cocoa fermentation boxes in comparison to several ATCC *B. cereus* strains. All reactions were typical for *B. cereus* (Gordon, 1975; Harmon, 1982), and supplemental procedures confirmed and differentiated *B. cereus* from taxonomically similar species. On the basis of these results, the *B. cereus* strains were collectively found to be lecithinase positive, actively motile, and completely hemolytic. Complete hemolysis, motility, and absence of rhizoid growth collectively differentiated the *B. cereus* strains from *B. cereus* var. mycoides which has been previously isolated from fermented cocoa (Ostovar and Keeney, 1973). The absence of protein toxin crystals also differentiated these strains from the insect pathogen *B. thuringensis*. Microscopic examination indicated that all three species shared common features characteristic for the group I type *Bacillus* species (Priest, 1989).

During the routine transfer of bacterial cultures, we observed several *B. cereus* strains to produce aromas characteristic of A1P. This chance observation prompted us to examine the aroma in more detail, since A1P is reportedly formed by the thermal degradation of several precursors (Schieberle, 1989). Figure 1 represents typical chromatographic separations of the volatile components obtained from *B. cereus* strain 35 by three separate isolation techniques. Capillary effluent odor evaluations showed the "popcorn, corn chip" odor to be

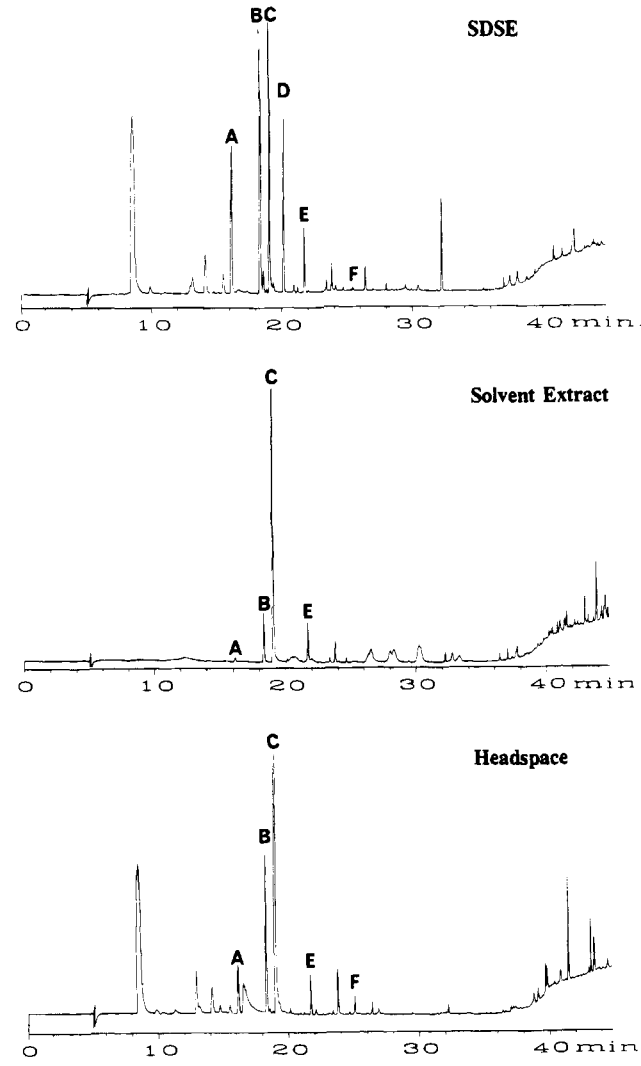

Figure 1. Gas chromatographic separations of the nitrogen specific volatile compounds obtained from *B. cereus* strain 35 on PCA by three isolation techniques. Peak identifications: (A) methylpyrazine, (B) 2,5-dimethylpyrazine, (C) 2-acetyl-1-pyrroline, (D) collidine (ISTD), (E) trimethylpyrazine, and (F) tetramethylpyrazine.

associated with a single component having a retention time of 19.0 min. The component's mass spectrum [*m*/*z* (rel intensity) 111 (18.8) [M^+], 38 (13.8), 41 (45.8), 42 (21.2), 43 (100.0), 68 (17.0), 69 (15.4), 83 (38.5)] was identical to a synthetic sample of A1P [*m*/*z* (rel intensity) 111 (26.8) [M^+], 38 (3.1), 41 (47.0), 42 (21.7), 43 (100.0), 68 (22.6), 69 (19.2), 83 (43.4)], and Kovats retention indices were used to confirm its identity [$I_k^{SPWAX-10}$ (1351) and I_k^{SPB5} (924)]. No evidence for other "cracker" aroma type compounds (2-acetyl-1,4,5,6-tetrahydropyridine, 2-acetylpyrazine, or 2-acetyl-2-thiazoline) could be detected from any of the *B. cereus* strains obtained from cocoa. However, a number of pyrazines were tentatively identified by GC-MS as part of the volatiles recovered by all three techniques. It was originally suspected that A1P might be an artifact produced by the SDSE technique, but a comprehensive examination of direct solvent extracts and dynamic headspace sampling of several *B. cereus* cultures prepared immediately after a 48 h incubation at 35 °C consistently confirmed the presence of A1P. These unexpected results raised the possibility for a nonthermal and, perhaps biological origin for A1P. Consequently, the *B. cereus* strains were examined in greater detail.

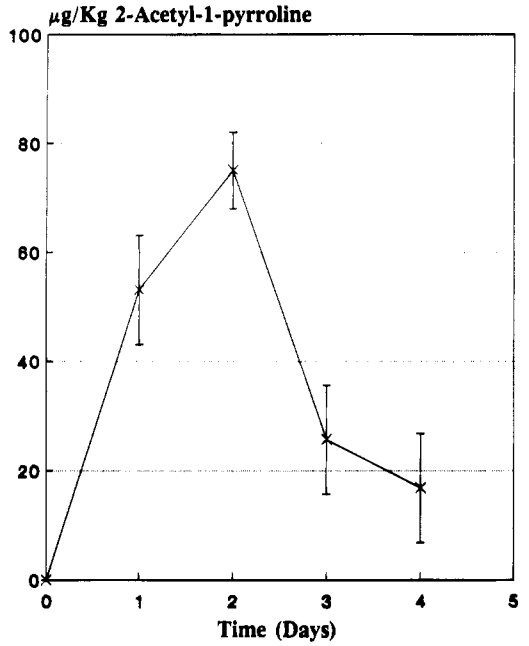

Figure 2. Relationship between the amount of 2-acetyl-1-pyrroline produced by *B. cereus* strain 35 on PCA and time. Analyses in replicate ($n = 5$) ± S.D.

Table 2. Formation of 2-Acetyl-1-pyrroline by Various *Bacillus* Species after 48 h of Growth on PCA[a]

Bacillus species	A1P (μg/kg)	*Bacillus* species	A1P (μg/kg)
B. cereus strain 5	56 ± 6	*B. cereus* ATCC 27522	87
B. cereus strain 8	37 ± 5	*B. cereus* ATCC 33018	ND
B. cereus strain 9	35 ± 15	*B. cereus* ATCC 33019	57
B. cereus strain 19	30 ± 16	*B. cereus* ATCC 49064	ND
B. cereus strain 35	75 ± 7	*B. circulans*	ND
B. cereus ATCC 9139	ND	*B. licheniformis*	ND
B. cereus ATCC 10702	23	*B. megaterium*	ND
B. cereus ATCC 11778	ND	*B. subtilis*	ND
B. cereus ATCC 13061	ND	*B. mycoides* ATCC 6462	ND
B. cereus ATCC 14579	ND	*B. thuringensis* ATCC 10792	ND
B. cereus ATCC 14737	53	uninoculated media control	ND

[a] The amounts of A1P obtained from cocoa-derived *B. cereus* strains are reported as the average of duplicate analyses ± S.D. Single determinations were made for all the other *Bacillus* strains. ND = none detected.

The onset of A1P production by cocoa-derived *B. cereus* strains was affected by media composition. The characteristic odor of A1P could not be detected in *B. cereus*-inoculated TSA or nutrient agar but only in PCA. Maximum levels were produced within 48 h after inoculation (Figure 2). An examination of washed *B. cereus* cells taken 48 h postinoculation showed a preferential accumulation of A1P in the media and only trace levels (<2 μg/kg) within the cell fraction. Similar results were observed for all other *B. cereus* isolates obtained from cocoa. Curiously, *B. cereus* failed to produce A1P in liquid plate count medium. The production of certain bacterial metabolites on solid media, but not liquid media, has been reported (Mayr-Harting et al., 1972), although reasons for this effect are unknown.

The ability to produce A1P was a distinctive feature for *B. cereus* strains isolated from cocoa fermentation boxes (Table 2) but not from other *Bacillus* species associated with cocoa fermentation or with taxonomically similar species. By comparison, several ATCC *B. cereus* strains were also able to produce this compound.

Several model experiments were investigated to determine the possible precursors involved in the apparent metabolic formation of A1P. These experiments were based on those reported by Schieberle (1989), where thermal reactions between proline, 1-pyrroline, and several metabolites of the glycolytic pathway were shown to be responsible for A1P formation in wheat bread crust. The model experiments also accounted for obvious differences in growth media compositions. For instance, PCA contains glucose and yeast extract, whereas TSA and nutrient agar do not. On the basis of these observations, glucose, proline, and yeast extract were assumed to be the most likely precursors required for A1P formation.

Table 3. Production of 2-Acetyl-1-pyrroline by *B. cereus* Strain 35 on PCA Supplemented with Various Nitrogen-Containing Compounds[a]

additive	A1P (μg/kg)	additive	A1P (μg/kg)
5% proline	110	5% glutamate	117
1% proline	83	1% glutamate	178
5% ornithine	109	2% yeast extract	68
1% ornithine	69	3% yeast extract	38

[a] The uninoculated controls containing 1% glutamate, 1% proline, 1% ornithine, and 3% yeast extract produced no detectable amounts of A1P. The no additives control produced 75 ± 7 μg/kg A1P.

We first examined the effects produced by yeast extract additions, since Schieberle (1989) had shown yeast cells to be a principal source of precursors. However, the levels of A1P were observed to decrease as yeast extract concentrations increased (Table 3). These results indicated that other, less obvious factors were required for A1P formation.

We next examined the effects produced by proline, ornithine, and glutamic acid additions on the glutamate and proline interconversion pathway which is controlled by two key opposing enzymes. In bacteria, proline oxidase is induced by growth on proline rich media. The Δ^1-pyrroline-5-carboxylate reductase is repressed under these conditions and is derepressed under conditions of low proline availability (Bender, 1985). It was suspected that the modulation of amino acids cycled through this pathway would provide clues for the nitrogen specific precursors required for A1P formation. The results obtained from these series of experiments (Table 3) showed increases in A1P concentrations when high levels of proline, ornithine, and glutamate were present. A much greater effect was observed for the lower level of glutamate addition, suggesting that specific levels could metabolically attenuate A1P formation through its presumed conversion to proline. Additionally, A1P was not detected in any of the uninoculated controls. These results indicated that A1P was not produced as an artifact from reactions with PCA or by sample preparation.

To substantiate proline and glutamic acid as nitrogen sources required for A1P formation by *B. cereus*, two labeling experiments were performed. In the first, 1% [^{15}N]proline was added to PCA inoculated with *B. cereus* strain 35 and the second used 1% [^{15}N]glutamic acid. Capillary GC-AED clearly showed the formation of [^{15}N]-A1P from either amino acid. The isotopic peak distribution (Table 4) indicated a 2- and 3-fold increase in the abundance for the M^+ ion (m/z 111 → 112) produced from the [^{15}N]glutamic acid- and [^{15}N]proline-labeled experiments, respectively. Unfortunately, an insufficient amount of A1P was recovered from either experiment to acquire $M^+ + 1$ (m/z 113) and $M^+ + 2$ (m/z 114) data for comparison to calculated isotopic abundances. Capillary GC-AED analysis of the uninoculated

Table 4. Normalized Isotope Abundances for 2-Acetyl-1-pyrroline Formed from (A) [^{15}N]Glutamic Acid, (B) [^{15}N]Proline, (C) [U-$^{13}C_6$]Glucose, (D) 2-Acetyl-1-pyrroline Produced by *B. cereus*, and (E) Synthetic 2-Acetyl-1-pyrroline

	isotope distribution (%)				
m/z	A[a]	B[a]	C[b]	D[a]	E[a]
111	100.0	100.0	37.1	100.0	100.0
112	12.2	22.2	15.4	7.5	6.7
113	ND[c]	ND	100.0	0.1	0.4
114	ND	ND	9.5	ND	ND
115	ND	ND	7.8	ND	ND
116	ND	ND	4.9	ND	ND
117	ND	ND	5.0	ND	ND

[a] Normalized against *m/z* 111. [b] Normalized against *m/z* 113. [c] Not detected.

Table 5. Production of 2-Acetyl-1-pyrroline by *B. cereus* Strain 35 on (A) PCA Supplemented with Various Carbon Sources and (B) Carbon Source Substitutions for Glucose in PCA[a]

A. Carbon Source Additions to PCA		B. Carbon Source Substitutions in PCA	
additive	A1P (μg/kg)	substitute	A1P (μg/kg)
2.0% glucose	18	*N*-acetyl-D-glucosamine	14
1.0% glucose	458	amylose	18
0.5% glucose	232	amylopectin	76
0.2% glucose	145	fructose	ND
1.0% amylose	514	fructose-1,6-diphosphate	ND
1.0% amylopectin	35	D-glucosamine	ND
1.0% D-lactose	70	D-lactose	ND
1.0% maltose	47	maltose	29
1.0% ribose	90	ribose	ND
1.0% sucrose	35	sucrose	ND

[a] The amount of glucose in standard PCA (0.060%), amylose (0.038%), and amylopectin (0.008%) was determined by the Boehringer Mannheim glucose test kit (Boehringer Mannheim Biochemicals Division, Indianapolis, IN). The uninoculated control containing 1% glucose produced no detectable amounts of A1P. The control containing 1% glucose and autoclaved *B. cereus* cells produced <2 μg/kg A1P. The no additives control produced 75 ± 7 μg/kg A1P. ND = not detected.

controls showed no evidence for [^{15}N]A1P from the [^{15}N]-glutamate experiment and only trace levels from the [^{15}N]proline experiment. Collectively, these results established glutamic acid and proline as two nitrogen sources required for the apparent metabolic formation of A1P by *B. cereus*. The potential precursor ornithine was not verified in this study, since a suitably labeled standard was not available. The possibility for other nitrogen sources could not be excluded, since these particular experiments only showed the incorporation of ^{15}N within A1P.

The effects produced by carbon source additions to the *B. cereus* system were also examined (Table 5A). High amounts of A1P were formed in the presence of amylose and glucose. An increase in the glucose level led to a proportional increase in the amount of A1P, except at the 2% level. At this level, poor microbial growth and the appearance of "fecal-like" aromas were observed. The amounts of A1P produced from other carbon source additions were variable and probably the result of catabolite repression, that is, the addition of a carbohydrate to PCA already containing glucose could cause a repression of microbial enzymes that would normally metabolize the added carbohydrate in the absence of glucose. A shift in a particular metabolic pathway could occur to cause an attenuation or depression in the amount of A1P. To minimize any possible effects caused by catabolite repression, various carbon sources were substituted for glucose to determine their individual effects on A1P formation. The results (Table 5B) showed A1P formation to be dependent upon the particular carbon source. For instance, A1P was formed in the presence of *N*-acetyl-D-glucosamine, amylopectin, amylose, and maltose. No A1P could be detected in the presence of fructose, fructose-1,6-diphosphate, glucosamine, lactose, ribose, and sucrose. In those cases where A1P was detected, the amounts were low. This might be the result of nonoptimum carbon source concentrations, the effects of non-glucose PCA components, or the poor growth characteristics associated with a particular carbohydrate. In general, *B. cereus* grown in the presence of these carbohydrates was not as lush as that observed from PCA containing glucose. Additionally, low levels of glucose were detected in PCA ingredients which might have affected these results. Clearly, more rigorous experimental work is necessary to determine the effects of individual and mixed carbohyrate concentrations on A1P formation. An examination of the uninoculated controls showed no evidence for A1P, again indicating that its formation was not an artifact. Additionally, less than 2 μg/kg was detected in the SDSE distillate prepared from PCA containing 1% glucose and an autoclaved *B. cereus* cell fraction. This particular result demonstrated the inability of thermally treated *B. cereus* cells to produce A1P within a medium mixture, where viable *B. cereus* produced 458 μg/kg A1P.

To reveal the role of glucose in A1P formation, 1% [U-$^{13}C_6$]glucose was added to PCA inoculated with *B. cereus* strain 35 and the SDSE distillate examined by capillary GC-AED and GC-MS. The results clearly showed the formation of [^{13}C]A1P which was not detected in the uninoculated control. The isotopic peak distribution (Table 4) showed a shift of 2 amu higher for the M^+ ion (*m/z* 111 → 113), indicating two carbons from glucose were incorporated within A1P. A shift of 2 amu higher for the ion fragment (*m/z* 43 → 45) suggested that both carbons were incorporated as the acetyl group. However, the presence of significant amounts of other M^+ isotopic peaks (*m/z* 114–117) and various ion fragments (*m/z* 83 → 85–88 and *m/z* 68, 69 → 70–73) suggested the incorporation of additional carbons within A1P, presumably through a series of biochemical pathways extant within *B. cereus*. Obviously, a more detailed microbiological and biochemical examination of the *B. cereus* system would be required to unequivocally substantiate this observation.

Collectively, these results were remarkably similar to those described by Schieberle (1989), except that our findings pointed to a possible biological origin for A1P. The detection of A1P at temperatures well below those involved in its thermal generation, coupled with the selective ability of certain *B. cereus* strains to produce this compound, lend additional validity for a biological formation pathway. Although the precise mechanism for A1P formation by *B. cereus* has yet to be determined, our results may offer insight to its origin, especially in those cases where the possible role of microorganisms is suspected (cf. Blank and Fischer, 1989; Brahmachary et al., 1990; Seitz et al., 1993; Fischer et al., 1993) or similar biochemical pathways might be implicated (cf. Buttery et al., 1982; Laksanalami and Ilangantileke, 1993). Certainly, the natural occurrence of *B. cereus* in many unprocessed cereal grains (Priest, 1989), in-

cluding rice (Gilbert, 1983), raise intriguing questions on whether or not they play an unrecognized role in A1P formation. Similarly, the examination of other *Bacillus* species present in starter cultures used in panary fermentations (Margalith, 1981; Robinson et al., 1958) might provide additional clues for A1P formation in different breads.

Since numerous *Bacillus* species are part of the natural microbial population involved with cocoa fermentation, it seemed possible that under suitable fermentation conditions, A1P might be produced. To investigate this possibility, a small survey was performed on a series of fermented and dried cocoa beans obtained from various growing regions (Brazil, Ecuador, Ghana, Indonesia, Ivory Coast, Malaysia, Nigeria, and Venezuela). The results failed to detect the presence of A1P from any sample. Even if present at the fermentation stage, the general instability of A1P (Buttery et al., 1986) and variations in cocoa-drying procedures (Wood and Lass, 1985) would make it unlikely for A1P to survive storage and roasting conditions to contribute to cocoa flavor development.

ACKNOWLEDGMENT

We are grateful to Valdomiro Santana Moreira of Fazenda Esperanza, Henrique de Araújo Andrade of Fazenda Santa Rita, and Aidil de Guimarães of Fazenda Santa Carolina for permission to obtain cultures of their respective cocoa fermentaries within the Município of Itajuipe, Bahia, Brazil, and to Anya Timofeyeva at Rutgers University, NJ, and Dave Manley at M&M/MARS, Hackettstown, NJ, for assistance in the preparation of numerous isolates.

LITERATURE CITED

Bender, A. A. *Amino Acid Metabolism*, 2nd ed.; John Wiley & Sons: New York, 1985; Chapter 6.

Biehl, B.; Brunner, E.; Passern, D.; Quesnel, V. C.; Adomako, D. Acidification Proteolysis and Flavor Potential in Fermenting Cocoa Beans. *J. Sci. Food Agric.* **1985**, *36*, 583–598.

Blank, I.; Fischer, K.-H. Intensive Neutral Odorants of Linden Honey. Differences from Honeys of Other Botanical Origin. *Z. Lebensm.-Unters.-Forsch.* **1989**, *189*, 426–433.

Brahmachary, R. L.; Sarkar, M. P.; Dutta, J. The Aroma of Rice and Tiger. *Nature* **1990**, *344*, 26.

Buttery, R. C.; Juliano, B. O.; Ling, L. C. Identification of Rice Aroma Compound 2-Acetyl-1-pyrroline in Pandan Leaves. *Chem. Ind. (London)* **1982**, *23*, 478.

Buttery, R. C.; Ling, L. C.; Juliano, B. O.; Turnbaugh, J. G. Cooked Rice Aroma and 2-Acetyl-1-pyrroline. *J. Agric. Food Chem.* **1983**, *31*, 823–826.

Buttery, R. C.; Ling, L. C.; Mon, T. R. Quantitative Analysis of 2-Acetyl-1-pyrroline in Rice. *J. Agric. Food Chem.* **1986**, *34*, 112–114.

Buttery, R. C.; Turnbaugh, J. G.; Ling, L. C. Contribution of Volatiles to Rice Aroma. *J. Agric. Food Chem.* **1988**, *36*, 1006–1009.

Fischer, N.; Hammerschmidt, F.-J.; Brunke, E. J. Presented at the 207th National Meeting of the American Chemical Society, Chicago, IL, August 1993; Paper AGFD 9.

Gasser, U.; Grosch, W. Identification of Volatile Flavor Compounds with High Aroma Values from Cooked Beef. *Z. Lebensm.-Unters.-Forsch.* **1988**, *186*, 489–494.

Gilbert, R. J. In *Food Microbiology: Advances and Prospects*; Roberts, T. A., Skinner, F. A., Eds.; Academic: New York, 1983; pp 47–63.

Gordon, R. E. *Agricultural Handbook Stock Number 202-275-2091*; U.S. Dept. of Agric.: Washington, DC, 1975.

Harmon, S. M. New Method for Differentiating Members of the *Bacillus cereus* Group: Collaborative Study. *J. Assoc. Off. Anal. Chem.* **1982**, *65*, 1134–1139.

Karahadian, C.; Johnson, K. A. Analysis of the Headspace Volatiles and Sensory Characteristics of Fresh Corn Tortillas Made from Fresh Masa Dough and Spray Dried Masa Flour. *J. Agric. Food Chem.* **1993**, *41*, 479–499.

Laksanalamai, V.; Ilangantileke, S. Comparison of Aroma Compound (2-Acetyl-1-pyrroline) in Leaves from Pandan (*Pandan amaryllifolius*) and Thai Fragrant Rice (Khao Dawk Mali-105). *Cereal Chem.* **1993**, *70*, 381–384.

Lehrian, D. W.; Patterson, G. R. In *Biotechnology*; Reed, G., Ed.; Verlag Chemie: Weinheim, 1983; Vol. 5, Chapter 12.

Lin, C. F.; Hsieh, T. C. Y.; Hoff, B. J. Identification and Quantification of the Popcorn-like Aroma in Louisiana Aromatic Della Rice (*Oryza sativa*, L.). *J. Food Sci.* **1990**, *55*, 1466–1467, 1469.

Lopez, A. S.; Quesnel, V. C. Volatile Fatty Acid Production in Cocoa Fermentation and the Effect on Chocolate Flavor. *J. Sci. Food Agric.* **1973**, *24*, 319–326.

Margalith, P. K. *Flavor Microbiology*; Charles C. Thomas: Springfield, IL, 1981; Chapter 6.

Majlat, P. Calculation and Application of the Retention Indexes in Programmed-Temperature Gas Chromatography. *J. Chromatogr.* **1974**, *91*, 89–103.

Mayr-Harting, A.; Hedges, A. J.; Berkeley, R. C. W. In *Methods of Microbiology*; Norris, J. R., Ribbons, D. W., Eds.; Academic: New York, 1972; Vol. 7A, Chapter 7.

Odlang, T. E.; Jarzynski, V.; Caputo, R. A.; Mascoli, C. C. Evaluation of an Automated System for Rapid Identification of *Bacillus* Biological Indicators and Other *Bacillus* Species. *J. Parenter. Sci. Technol.* **1982**, *36*, 47–54.

Ostovar, K.; Keeney, P. G. Isolation and Characterization of Microorganisms Involved in the Fermentation of Trinidad's Cocoa Beans. *J. Food Sci.* **1973**, *38*, 611–617.

Priest, F. G. In *Bacillus*; Harwood, C. R., Ed.; Plenum: New York, 1989; Chapter 3.

Robinson, R. J.; Lord, T. H.; Johnson, J. A.; Miller, B. S. The Aeorbic Microbiological Population of Pre-Ferments and the Use of Selected Bacteria for Flavor Production. *Cereal Chem.* **1958**, *35*, 295–305.

Rohan, T. A.; Stewart, T. The Precursors of Chocolate Aroma: Production of Reducing Sugars During Fermentation of Cocoa Beans. *J. Food Sci.* **1967**, *32*, 399–404.

Schieberle, P. In *Thermal Generation of Aromas*; Parliment, T. H., McGorrin, R. J., Ho, C.-T., Eds.; ACS Symposium Series 409; American Chemical Society: Washington, DC, 1989; pp 268–275.

Schieberle, P. The Role of Free Amino Acids Present in Yeast as Precursors of the Odorants 2-Acetyl-1-pyrroline and 2-Acetyl tetrahydropyridine in Wheat Bread Crust. *Z. Lebensm.-Unters.-Forsch.* **1990**, *191*, 206–209.

Schieberle, P. Primary Odorants in Popcorn. *J. Agric. Food Chem.* **1991**, *39*, 1141–1144.

Schieberle, P.; Grosch, W. Identification of the Volatile Flavor Compounds of Wheat Bread Crust Comparison of Rye Bread Crust. *Z. Lebensm.-Unters.-Forsch.* **1985**, *180*, 474–478.

Schieberle, P.; Grosch, W. Quantitative Analysis of Aroma Compounds in Wheat and Rye Bread Crusts Using a Stable Isotope Dilution Assay. *J. Agric. Food Chem.* **1987**, *35*, 252–257.

Schieberle, P.; Grosch, W. Potent Odorants of the Wheat Bread Crumb. Differences from the Crust and the Effect of a Longer Dough Fermentation. *Z. Lebensm.-Unters.-Forsch.* **1991**, *192*, 130–135.

Schwan, R. F.; Vanetti, M. C. D.; Silva, D. O.; Lopez, A.; DeMoraes, C. A. Characterization and Distribution of Aerobic, Spore-Forming Bacteria from Cocoa Fermentations in Bahia. *J. Food Sci.* **1986**, *51*, 1583–1584.

Seitz, L. M.; Wright, R. L.; Waniska, R. D.; Rooney, L. W. Contribution of 2-Acetyl-1-pyrroline to Odors from Wetted Ground Pearl Millet. *J. Agric. Food Chem.* **1993**, *41*, 955–958.

Vitzthum, O. G.; Werkhoff, P. In *Analysis of Foods and Beverages Headspace Techniques*; Charalambous, G., Ed.;

Academic: New York, 1978; pp 115–133.

Wong, K. C.; Lim, C. L.; Wong, L. L. Volatile Flavor Constituents of Chempedak (*Artocarpus polyphema* Pers.) Fruit and Jackfruit (*Artocarpus heterophyllas* Lam.) from Malaysia. *Flavour Fragrance J.* **1992**, *7*, 307–311.

Wood, G. A. R.; Lass, R. A. *Cocoa*, 4th ed.; Longman: London, 1985; Chapter 13.

Zak, D. L.; Ostovar, K.; Keeney, P. G. Implication of *Bacillus subtilis* in the Synthesis of Tetramethylpyrazine During Fermentation of Cocoa Beans. *J. Food Sci.* **1972**, *37*, 967–970.

Received for review June 21, 1994. Revised manuscript received November 7, 1994. Accepted November 16, 1994.[⊗]

JF940331T

[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1995.