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Tryptophan Catabolism in *Brevibacterium linens* as a Potential Cheese Flavor Adjunct[1]

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ABSTRACT

Attempts to develop a desirable reduced fat Cheddar cheese are impeded by a propensity for flavor defects such as meaty-brothy, putrid, fecal, and unclean off-flavors in these products. Recent studies suggest aromatic amino acid catabolism of starter, adjunct, and nonstarter lactic acid bacteria significantly impact off-flavor development. The objective of this study was to delineate pathways for catabolism of tryptophan (Trp) in *Brevibacterium linens*, a cheese flavor adjunct, and to determine the potential for this organism to contribute to this defect. Growth and production of aromatic compounds from Trp by *B. linens* BL2 were compared in two incubated conditions (laboratory and a cheese-like environment). A chemically defined medium was used to determine the cellular enzymes and metabolites involved in Trp catabolism. Trp was converted to kynurenine, anthranilic acid, and three unknown compounds in laboratory conditions. The accumulation of other unknown compounds in the culture supernatant in laboratory conditions indicated that *B. linens* BL2 degraded Trp by various routes. Up to 65% of Trp was converted to anthranilic acid via the anthranilic acid pathway. To assess this potential before cheese making, the cells were incubated in cheese-like conditions (15°C, pH 5.2, no sugar source, 4% NaCl). Trp was not utilized by BL2 incubated in this condition. Enzyme studies using cell-free extracts of cells incubated in laboratory conditions and assayed at optimal and nonoptimal enzyme assay conditions revealed Trp transaminase (EC 2.6.1.27) was active before enzymes of the anthranilic acid pathway were detected. The products of Trp transaminase activity were not, however, found in the culture supernatant, indicating these intermediates were not exported nor accumulated by the cells. Enzymes assayed in nonoptimal conditions had considerably lower enzyme activities than found in laboratory incubation conditions. Based on these results, we hypothesize that these enzymes are not likely to be involved in the formation of compounds associated with off-flavors in Cheddar cheese.
(**Key words:** tryptophan catabolites, anthranilic acid, Cheddar cheese flavor)

Abbreviation key: AAA = aromatic amino acids, **ATase** = agar aminotransferase, **CDM** = chemically defined medium, **CFE** = cell-free extract, **IAA** = indole acetic acid, **MEKC** = micellar electrokinetic chromatography, **TSB** = tryptic soy broth.

INTRODUCTION

Brevibacteria are industrially important microorganisms used in manufacture of Limburger, Roquefort, Stilton, and other Trappist-type cheeses (Ades and Cone, 1969; Sharpe et al., 1977). Their high proteolytic, varied lipolytic activity, aminopeptidase activity, and diverse biochemistry extend their use as a novel flavor adjunct in the manufacture of low-fat Cheddar cheese (Weimer et al., 1997). Additionally, the ability of brevibacteria to produce volatile sulfur compounds has fueled further interest in their potential as flavor adjuncts (Dias, 1998a; Ferchichi et al., 1986; Hayashi et al., 1990; Lamberet et al., 1997; Sharpe et al., 1977; Weimer et al., 1997). However, use of adjuncts to improve one aspect may have a detrimental influence in another aspect of cheese flavor. To assess this aspect of *Brevibacterium linens*, we examined production of aromatic compounds associated with off-flavors from Cheddar cheese.

Initial attempts to use cell-free extracts (**CFE**) of brevibacteria to accelerate ripening in full-fat Cheddar cheese resulted in uncontrolled ripening and consequently unbalanced flavor development and over-pro-

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duction of off-flavors (Hayashi et al., 1990a; Law and Wigmore, 1983). Studies in cheese-like conditions showed that the methanethiol-producing capability was retained in whole cells of *B. linens* but not in CFE (Dias, 1998; Weimer et al., 1997). This indicates that the enzymes responsible for methanethiol production, if released into the cheese matrix during ripening, may not be active in producing the essential flavor compounds (Dias, 1998a; Weimer et al., 1997). Therefore, knowledge of altered cell physiology of brevibacteria in the cheese matrix and its implications on cheese flavor biochemistry is warranted.

The quality of reduced fat Cheddar cheese is compromised by flavor defects such as bitter, meaty, brothy, and off-flavors that may originate from aromatic amino acids (**AAA**) and their metabolites (Steele and Unlu, 1992). For example, metabolism of Trp, Tyr, and Phe lead to the formation of indole, skatole, tryptamine, *m*-cresol, *p*-cresol, phenol, benzoic acid, and phenethanol. The off-flavors generated by these compounds include putrid, fecal, rosy, and unclean flavors in some cheeses (Aston and Creamer, 1986; Dunn and Lindsay, 1985; Law and Sharpe, 1977; Lowrie and Lawrence, 1972; Manning, 1979; Urbach et al., 1972; Urbach, 1995). The AAA metabolisms in other bacteria are known to be either catalyzed by enzymes or to occur by spontaneous chemical degradations (Lindsay, 1991), but knowledge of these mechanisms in brevibacteria is lacking. This limits the assessment of the use for accelerated cheese ripening.

Trp catabolism in a variety of bacteria occurs through several pathways leading to the formation of a variety of compounds (Figure 1) (Boer et al., 1988; Comai and Kosuge 1980; Frankenberger and Poth, 1988; Narumiya et al., 1979). Indole for example, may be produced from Trp by the single step catalysis of tryptophanase (EC 4.1.99.1), while skatole is formed from indole acetic acid (**IAA**). Conversion of Trp to IAA occurs via three distinct routes (Comai and Kosuge, 1980; Frankenberger and Poth, 1988; Narumiya et al., 1979): 1) the indole acetamide pathway catalyzed by Trp 2-monooxygenase (EC 1.13.12.3) and indole acetamide hydrolase (EC 3.1.1.51) (Comai and Kosuge, 1980), 2) catalyzed by tryptophan side-chain oxidase (EC 4.1.1.43) and indole acetaldehyde dehydrogenase (EC 1.2.3.7) (Narumiya et al., 1979), and 3) the indole pyruvic acid pathway which is initiated by an Trp aminotransferase (**ATase**) (EC 2.6.1.27) (Frankenberger and Poth, 1988). Other important catabolic pathways include decarboxylation of Trp to tryptamine and ring cleavage initiated by Trp 2,3-dioxygenase (EC 1.13.11.11). The latter leads to the formation of kynurenine and anthranilic acid (Narumiya et al., 1979) (Figure 1). Jollivet et al. (1992) observed that brevibacteria catabolize Trp to indole and anthranilate, but failed to define the metabolic route. Kynurenine and anthranilic acid are not associated with any known cheese flavors, and the mechanism for its production in cheese is unknown.

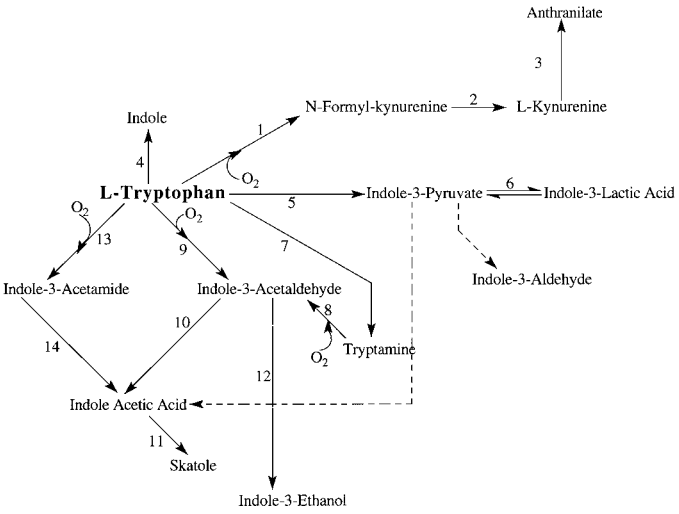

Figure 1. Metabolism of tryptophan in bacteria. 1) Trp 2,3 dioxygenase (EC 1.13.11.11); 2) kynurenine formidase (EC 3.5.1.49); 3) kynureninase (EC 3.7.1.3); 4) tryptophanase (EC 4.1.99.1); 5) Trp aminotransferase (EC 2.6.1.27); 6) indole lactate dehydrogenase (EC 1.1.1.110); 7) Trp decarboxylase (EC 4.1.1.28); 8) tryptamine oxidase (EC 1.4.3.4); 9) Trp side chain oxidase (EC 4.1.1.43); 10) indole acetaldehyde dehydrogenase (EC 1.2.1.3); 11) indole acetic acid oxidase; 13) Trp 2-monooxygenase (EC 1.13.12.3); and 14) indole acetamide hydrolase (EC 3.5.1.0). The dotted lines (———) indicate a spontaneous reaction. Adapted from http://wit.mcs.anl.gov/WIT2/; select EMP database of enzymes and metabolic pathways.

Trp catabolism in lactococci is initiated by a Trp ATase (EC 2.6.1.27) (Frankenberger and Poth, 1988; Gao et al., 1997) and is thought to be active in cheese (Yvon et al.; 1997). Brevibacteria can also initiate Trp catabolism by an ATase to form IAA (Lee and Desmazeaud, 1985), but it is not known if this enzyme is active during cheese ripening.

Enzymatic mechanisms for Trp catabolism in brevibacteria and their action in the cheese environment is important if brevibacteria are to be used as alternative flavor adjuncts in the manufacture of reduced fat cheese. Successful use of brevibacteria as whole cells in Cheddar cheese relies on a better understanding of their role in AAA metabolism in cheese, especially to avoid use of an organism that may add undesirable flavors. Therefore, the objective of this study was to investigate Trp catabolism by *Brevibacterium linens* BL2 in laboratory (no sugar, pH 6.5, 25°C, 220 rpm) and cheese-like conditions (no sugar, pH 5.2, 4% NaCl, 15°C, and static incubation).

MATERIALS AND METHODS

Bacterial Strain and Growth Media

Brevibacterium linens BL2, was obtained from the Utah State University culture collection. Stock cultures were frozen in tryptic soy broth (**TSB**) (Difco, Detroit, MI) containing 30% glycerol and kept at –70°C. Working stocks were maintained in TSB and incubated at 25°C with aeration (220 rpm). For each experiment, a 10-ml culture was subcultured twice before inoculation into 1000 ml of chemically defined medium (**CDM**) (Jensen and Hammer, 1993).

Four different types of media were prepared; CDM with 5 m*M* Trp (but no Phe or Tyr), CDM containing all AAA (5 m*M* Trp, 0.275 μM Tyr, and 0.121 μM Phe), and CDM with Phe and Tyr (0.275 μM Tyr and 0.121 μM Phe), and CDM without any AAA. Each of these media was prepared in two batches, one for laboratory conditions (pH 6.5, no sugar) and the other for cheese-like conditions (no sugar pH 5.2, 4% NaCl (wt/vol), 15°C, static incubation). To prepare CDM for cheese-like conditions, the pH was adjusted by the addition of 0.16% glucono-δ-lactone, and the sterile medium was incubated at 30°C for 2 h before the addition of 4% NaCl (wt/vol). Bacteria were grown in TSB to mid-log phase (24 h), harvested, washed twice with 15 ml of saline, adjusted to a constant optical density (OD_{590nm}) in the basal medium, and used to inoculate 1000 ml of each medium to obtain an initial cell count of 10^7 cells/ml. Each medium was incubated at 25°C with aeration (220 rpm) for laboratory conditions and at 15°C without aeration for cheese-like conditions. Samples were periodically collected and analyzed for pH, cell count, Trp metabolites in the culture supernatant, and intracellular Trp catabolic enzymes. Samples of uninoculated media were also collected periodically as controls to detect nonenzymatic chemical degradations.

Viable Cell Counts

Spent and sterile media samples were collected aseptically at 12 different intervals (0, 1, 3, 6, 8, 10, 13, 15, 18, 21, 24, 27, and 33 d) in a biological safety hood (Nuaire Safety Cabinets, Model 425-400; Plymouth, MN) for identification of Trp metabolites and enzyme assays. Plate counts were done at each time interval on tryptic soy agar plates that were incubated aerobically at 25°C for 3 to 5 d.

Preparation of CFE

Cells were collected at periodic intervals (50 ml), harvested by centrifugation at 7000 $\times g$ for 15 min at 4°C, washed twice with 15 ml of 0.05 *M* potassium phosphate buffer (pH 7.2), and resuspended in 0.05 *M* potassium phosphate buffer containing 0.02 m*M* pyridoxal phosphate. CFE were prepared by sonication in a pulsed mode at 20 kHz for 5 to 15 min using a Branson-Sonifier (Cell disruptor 200; Branson Ultrasonics Co., CT). Cell lysates were centrifuged at 10,000 $\times g$ for 30 min at 4°C, and the supernatant was collected and used as CFE.

Protein Assay

The protein concentration in CFE was determined with the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) kit. Bovine serum albumin was used to obtain a standard curve, and protein was expressed as milligrams/milliliter.

Micellar Electrokinetic Capillary Electrophoresis Analysis

Culture supernatant (3 ml) was filtered through a 0.2-μm low-protein binding syringe filter (Nalgene, Rochester, NY), fractionated in a 1000 MW cut-off polyethersulfone membrane Centriprep (Pall filtron, East Hill, NY) by centrifugation at 3000 $\times g$ at 4°C for 1 h, and the filtrate was used for analysis. Capillary electrophoresis as described by Strickland et al. (1996) was done using a P/ACE 2100 automated capillary electrophoresis system fitted with System Gold software (Beckman Instruments, Inc., Fullerton, CA). Sample electrophoresis was done using 100 m*M* sodium tetraborate containing 40 m*M* SDS (pH 8.5) as the run buffer for 30 min at 15 kV, at 25°C with a 1-s pressure injection into a 75-μm i.d. × 57-cm untreated silica capillary. Sample detection was achieved at 200 nm with a detector range of 0.02 AUFS and a data collection rate of 2 Hz. The Trp metabolites that were used as standards in this study were L-tryptophan, L-kynurenine, indole-3-acetamide, indole acetic acid, indole, indole 3-aldehyde, anthranilic acid, tryptophol, skatole, and tryptamine (Sigma Chemical Co., St. Louis, MO). Compound identification was confirmed by coinjection with pure standards and by comparison of absorption spectra of unknown peaks and pure standard compounds (correlation $R^2 > 0.90$).

Isolation and Identification of the Unknown Aromatic Compounds

The unknown aromatic compounds that were identified with micellar electrokinetic chromatography (**MEKC**) were then analyzed using reverse-phase HPLC in a Beckman gradient HPLC system equipped with a 125 dual pump, a 168-diode array detector, and a personal computer-based data system controller

(Beckman System Gold version 8.1; Beckman Instruments, Fullerton, CA). The columns used were Brownlee Aquapore RP-300 (Perkin Elmer/Applied Biosystems) with 300-Å pores and 7-μm particle size. The column diameter was 2.1 mm for analytical HPLC, and 4.6 mm for preparative chromatography; column length was 10 cm. Eluant A was 0.1% (vol/vol) trifluroacetic acid, and eluant B was 0.085% trifluroacetic acid in 80% acetonitrile. The proportion of eluant B in eluant A was increased from 0 to 45% over a 45-min period. Flow rates were 0.2 ml/min for the analytical column and 1 ml/min for the preparative volume. Detection of compounds was performed at 254 nm. The unknown compounds were purified and then were lyophilized with a benchtop freeze-dryer (model 5L; VirTis, Gardiner, NY).

The unknown aromatic compounds used for mass spectrometry were dissolved in 0.1% trifluroacetic acid and analyzed by matrix-assisted laser desorption ionization time of flight on a TofSpec (LSUMC core laboratories, New Orleans, LA) mass spectrometer with external mass calibration.

Unknown compounds were also subjected to nuclear magnetic resonance spectroscopic analysis (Dept. of Chemistry, Utah State University, Logan, UT) under conditions for interpretation of both ^{1}H data and ^{13}C data. The lyophilized unknowns were dissolved in dimethyl sulfoxide or deuterated water. The areas were integrated using one of the single-hydrogen resonances as a unit reference.

Trp Transaminase Assay

Transaminase activity was measured in a mixture containing 5 m*M* α-ketoglutarate, 1 m*M*-pyridoxal phosphate, 0.5 m*M* sodium arsenate, 0.5 m*M* EDTA, 50 m*M* sodium borate (pH 8.5) and 5 m*M* L-Trp. The reaction was initiated by the addition of 500 μl of CFE in a total reaction volume of 2 ml. The formation of indole pyruvate as a stable enol tautomer-borate complex was measured as the increase in absorbance at A_{327} at 25°C measured at T0 and T30 min (Frankenberger and Poth, 1988; Oberhansli et al., 1991). The concentration of indole pyruvate was calculated from a standard curve of indole pyruvic acid in borate buffer containing EDTA and sodium arsenate (Oberhansli et al., 1991). Controls without substrate, without cells, and without substrate and cells were also included and enzyme specific activities were expressed as units/mg of protein per milliliter per minute.

Tryptophan 2, 3-Dioxygenase and Kynurenine Formidase Assay

In the anthranilic acid pathway, Trp is first converted to formyl-kynurenine by tryptophan 2, 3-dioxygenase and then to kynurenine by kynurenine formidase (Figure 1). The lack of available commercial formyl-kynurenine required the use of enzyme inhibitors to measure these enzyme activities and assaying the accumulation of the substrate for each enzyme. Trp 2, 3-dioxygenase was inhibited using 10 m*M* potassium cyanide (**KCN**) (Iwamoto et al., 1995), while kynurenine formidase was inhibited with 30 m*M* sodium bisulfite (**$NaHSO_3$**) (Shinohara and Ishiguro, 1970). The reaction was started with the addition of 25 μl of CFE and incubated for 120 min at 37°C. The reaction was terminated by injecting the sample for MEKC analysis to analyze the aromatic intermediates. Control reactions containing the substrate, but no CFE were included. Similar reactions were also done in the presence of each inhibitor individually and all inhibitors together. Based on the production of catabolites from Trp in these reactions, specific activities were measured and reported as the sum of specific activities of both the enzymes expressed in units/mg of protein per milliliter per hour.

Kynureninase Assay

Conversion of kynurenine to anthranilic acid is catalyzed by kynureninase. The activity of intracellular kynureninase was measured in a reaction mixture (125 μl) containing 0.6 m*M* L-kynurenine, 0.04 *M* potassium phosphate (pH 7.8), and 40 μ*M* pyridoxal-5′-phosphate. The reaction was initiated by the addition of 25 μl of CFE and incubated at 25°C for 1 h (Lai et al., 1991) before analysis by MEKC. Appropriate controls containing substrate but without CFE were included. Specific activities were expressed as units per milligram of protein per milliliter per hour.

Other Trp Catabolic Enzyme Assays

Cell-free extracts of strain BL2 grown in laboratory and cheese-like conditions were assayed for tryptophanase (EC 4.1.99.1) using the spectrophotometric assays described by Sigma Chemical Co. (St. Louis, MO), and the enzyme reaction mixtures were also analyzed using MEKC analysis to detect the end product indole. Other Trp catabolic enzymes like tryptophan decarboxylase (EC 4.1.1.28) and tryptophan 2-monooxygenase activity (EC 1.13.12.3) were also evaluated as described by Hummel et al. (1986) and Hutcheson and Kosuge (1985), respectively.

Nonoptimum Enzyme Assays

To determine if the above enzymes were active in chemical conditions found in Cheddar cheese, all enzyme assays were repeated with CFE in 0.05 *M* pot-

assium citrate (pH 5.2, 4% NaCl). The reaction volumes used were the same as described for the optimum enzyme assays.

RESULTS AND DISCUSSION

Experiments were done in two incubation conditions using four different types of media. Within each growth condition (laboratory and cheese-like conditions), pH, cell viability, Trp utilization, production of Trp metabolites, and intracellular Trp catabolic enzyme activities were determined. The metabolic end products of Trp catabolism were associated with cell growth during incubation.

Cell Viability and pH

In laboratory growth conditions, the cell density reached $\geq 10^9$ cells/ml after 24 d in all media tested (Figure 2A), and the pH increased from 6.5 to 8.5. During incubation in cheese-like conditions the cell population decreased to undetectable levels after 45 d in all the media (Figure 2B). The pH remained at 5.2 during the entire incubation period.

Brevibacteria grow slowly (Jones and Keddie, 1986), and it is common for these cells to reach logarithmic growth phase 24 to 48 h after inoculation and last for 36 h. Surprisingly, in laboratory growth conditions, the logarithmic growth phase lasted 21 to 27 d in CDM (Figure 2A). Boyaval et al. (1985) isolated a strain of *B. linens* from Camembert cheese that survived during ripening because of altered cell metabolism characterized by low cellular respiration, ATP levels, and enzyme activities. Similar observations were found in Cheddar cheese made with *B. linens* BL2 (Weimer et al., 1997).

Trp Utilization and Production of Trp Metabolites

In laboratory growth conditions, Trp catabolism by cells incubated in CDM with Trp did not begin until d 15 (Figure 3A). This time point is associated with mid-log phase growth. Culture supernatants accumulated kynurenine, anthranilic acid, and three unknown aromatic compounds in the growth medium as Trp concentration decreased (Figure 3A), indicating that the anthranilic acid pathway (Trp 2, 3-dioxygenase, kynurenine formidase, and kynureninase) may be used for catabolism of Trp. These compounds were not detected in CDM without Trp. Mcfarlane and Mcfarlane (1995) suggested that Trp breakdown, as observed in this study, may occur in aerobic organisms by the action of mono- and dioxygenases via incorporation of molecular oxygen. Lee and Desmazeaud (1985) demonstrated that

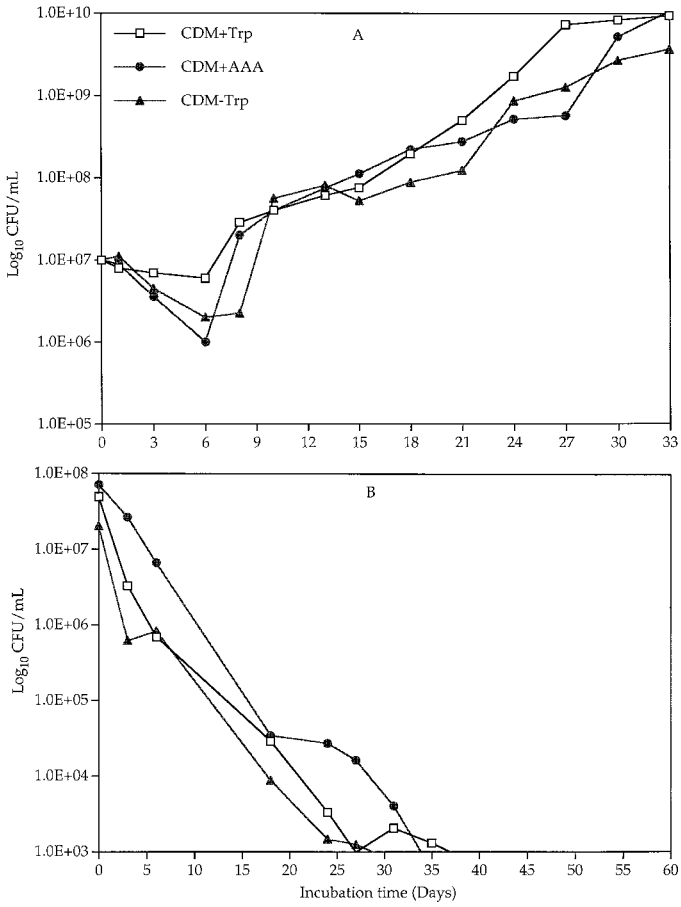

Figure 2. Cell population of *Brevibacterium linens* BL2 (cfu/ml) grown in chemically defined median (CDM) (Jensen and Hammer, 1993) under laboratory conditions (no sugar, pH 6.5, 25°C, 220 rpm) (panel A) and grown in CDM (Jensen and Hammer, 1993) under cheese-like growth conditions (pH 5.2, 4% NaCl, 15°C, static incubation) (panel B). Symbol (■) denotes Trp transaminase activity. Optimum assay conditions: pH 8.5, 25°C for 30 min; non-optimal assay conditions: pH 5.2, 4% NaCl, 25°C for 30 min. Symbol (●) denotes cumulative Trp 2-3 dioxygenase and kynurenine formidase activities. Optimum assay conditions: pH 8.5, 37°C for 2 h; non-optimal assay conditions: pH 5.2, 4% NaCl, 37°C for 2 h. Symbol (○) denotes kynurenine activity (using Trp as substrate). Optimum assay conditions: pH 8.5, 37°C for 2 h; non-optimal assay conditions: pH 5.2, 4% NaCl, 37°C for 2 h. Symbol (△) kynureninase activity (using kynurenine as substrate). Optimum assay conditions: pH 7.8, 25°C for 1 h; non-optimal assay conditions: pH 5.2, 4% NaCl, 25°C for 1 h.

Trp is catabolized by an ATase in brevibacteria, but the products of this enzyme activity, IAA and skatole, were not detected, suggesting ATase activity is not an important mechanism in this growth condition or that subsequent enzymes may not be present to mediate further conversion to nonaromatic compounds that were not observed with this technique.

Formyl-kynurenine is the first intermediate formed from Trp in the anthranilic acid pathway. In this study, kynurenine and anthranilic acid were the first interme-

diates to be detected in culture supernatant of cells grown in laboratory conditions (Figure 3A). The appearance of these compounds was associated with a stoichemetric decrease in Trp. Some reports suggest that cell wall impermeability prevents formyl-kynurenine from accumulating in the supernatant (Behrman, 1962). This may account for the lack of formyl-kynurenine in the culture supernatants or it may have been metabolized to other compounds.

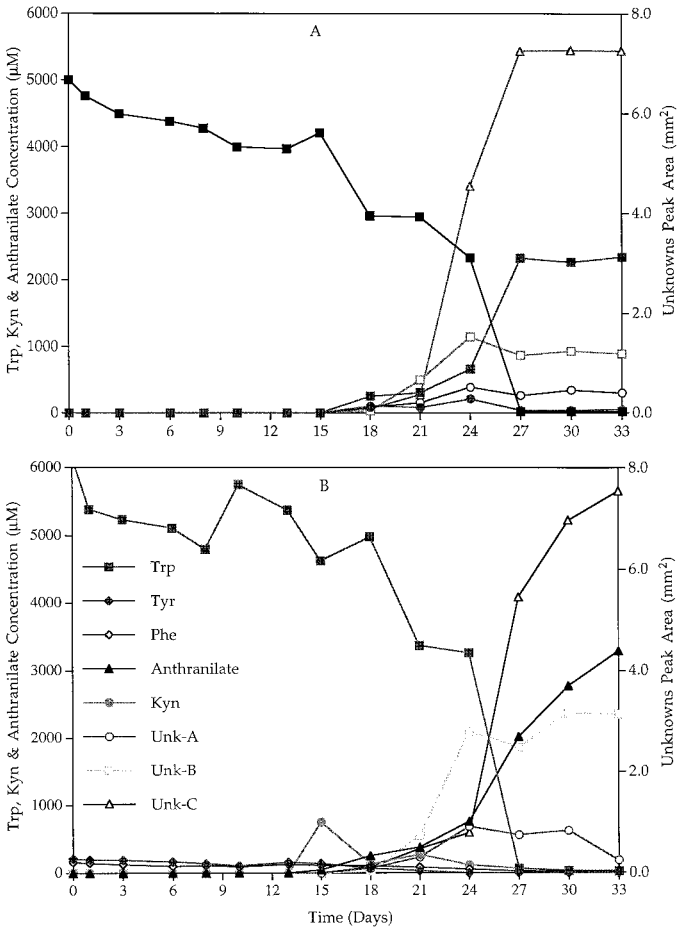

Figure 3. Trp catabolite accumulation in spent media from *Brevibacterium linens* BL2 grown in CDM (Jensen and Hammer, 1993) with Trp (panel A), and in chemically defined medium (Jensen and Hammer, 1993) with all aromatic AA (panel B) incubated in laboratory growth conditions (no sugar, pH 6.5, 25°C, 220 rpm). The samples were fractionated through a 1000 MW cut-off polyethersulfone membrane Centriprep (Pall Filtron) by centrifugation at 3,000 × *g* at 4°C for 1 h before the analysis. Symbol (■) denotes Trp transaminase activity. Optimum assay conditions: pH 8.5, 25°C for 30 min; non-optimal assay conditions: pH 5.2, 4% NaCl, 25°C for 30 min. Symbol (●) denotes cumulative Trp 2-3 dioxygenase and kynurenine formidase activities. Optimum assay conditions: pH 8.5, 37°C for 2 h; non-optimal assay conditions: pH 5.2, 4% NaCl, 37°C for 2 h. Symbol (○) denotes kynurenine activity (using Trp as substrate). Optimum assay conditions: pH 8.5, 37°C for 2 h; non-optimal assay conditions: pH 5.2, 4% NaCl, 37°C for 2 h. Symbol (△) kynureninase activity (using kynurenine as substrate). Optimum assay conditions: pH 7.8, 25°C for 1 h; non-optimal assay conditions: pH 5.2, 4% NaCl, 25°C for 1 h.

After kynurenine and anthranilic acid production, three other unknown aromatic compounds accumulated in the culture supernatant during incubation (Figure 3A). The concentrations of anthranilic acid and the unknown aromatic compounds increased until Trp was completely depleted from the medium, suggesting they were derived from Trp. Approximately 47% of the added Trp was converted to anthranilic acid (Figure 3A), while 53% of the total Trp was converted to the aromatic unknown compounds, suggesting that *B. linens* BL2 can simultaneously degrade Trp by various routes (Figure 3A). The levels of kynurenine remained constant in the culture supernatant until d 27, after which it decreased to undetectable amounts (<picomolar concentration).

In CDM with all three AAA incubated at laboratory conditions, a similar pattern as CDM with Trp alone was observed, with the exception that Trp utilization was delayed from d 15 to 21 (Figure 3B). Trp was also catabolized to kynurenine, anthranilic acid, and three unknown compounds in CDM with all the AAA. Interestingly, a greater amount of anthranilic acid was produced (~65% of Trp) than that in CDM with Trp only. Trp catabolites were not produced in CDM with Tyr and Phe, nor in CDM without AAA, indicating that Trp is the source of these compounds.

Trp was not utilized during cheese-like incubation conditions. Presumably, the mechanism for production of these compounds is the anthranilic acid pathway. To verify this hypothesis, intracellular enzyme assays were done.

Identification of the Unknown Aromatic Compounds

Initially, the unknowns were evaluated by coinjection with 20 different commercially available aromatic amino acid catabolites. None of the compounds coeluted with known standards. During the electrophoresis, the UV spectrum of each of the unknowns was determined. None of the UV absorption spectra of unknown aromatic peaks matched with the known pure standard compounds. In further efforts to determine the identity, the unknowns were isolated and sent for mass determination. Mass spectrometry found one aromatic compound to be 162 Da, while the others were not detected. Lastly, the nuclear magnetic resonance spectrum for each was attempted. We were unable to identify any of the unknown aromatic compounds, despite these efforts.

Enzyme Assays

Preliminary studies examined the initial catalytic reactions for the degradation of Trp (Figure 1) after

growth in CDM with Trp. All enzyme assays were done at their respective optimal assay conditions and again in nonoptimal assay conditions (pH 5.2, 4% NaCl). The temperature and time for incubation used were the same as in the optimum assay conditions. Cells grown in laboratory and cheese-like conditions did not contain tryptophanase, tryptophan decarboxylase, or tryptophan 2-monooxygenase activity. However, Trp transaminase and Trp 2, 3-dioxygenase were detected in both enzyme assay conditions. Enzyme assays and capillary electrophoresis studies indicated that the enzymes from two pathways were present, suggesting Trp can be metabolized via two routes, following either the Trp transaminase pathway or the anthranilic acid pathway. Each route was investigated further by incubating *B. linens* BL2 cells in CDM with and without the addition of Trp to the medium.

Transaminase Activity in Laboratory Growth Conditions

Brevibacterium linens BL2 was inoculated and sampled immediately to analyze the initial enzyme activity. Trp transaminase activity in cells from all media was highest on d 0, decreased progressively during incubation, and was absent after 8 d (Figure 4). The initial activity could be the effect of residual transaminase carried over in cells from growth in TSB broth containing a high concentration of AAA from pancreatic digest of casein and papaic digest of soybean meal.

Transaminase activity was also detected when assayed in nonoptimum conditions (0.05 *M* potassium citrate, pH 5.2, 4% NaCl). Activities were detectable during the first 6 d of incubation (Figure 4). Although ATase activity was detected, products from this enzyme were not observed to accumulate in the culture supernatant, suggesting an alternative pathway exists to degrade Trp. Other Trp catabolites that contribute to off-flavor in cheese, like indole and skatole, were not detected in the culture supernatant, suggesting that the enzymes needed for their subsequent conversion were not active in these conditions. After ATase activity ceased, activities of enzymes in the anthranilic acid pathway were detected in cells grown in CDM with Trp (Figure 4) and CDM with all three AAA (Figure 4). These enzyme activities were not detected in cells grown in CDM without Trp (Figure 4).

Although Trp was not catabolized when incubated in cheese-like conditions, reports of the ability of some cheese starter bacteria to retain ATase activity prompted us to investigate those observations in brevibacteria. The CFE obtained from cells incubated in cheese-like conditions were assayed for ATase activity in optimal and nonoptimal assay conditions. In both assay conditions, ATase activity was detected only on d 1, suggesting it was residual activity from growth in TSB (data not shown). The Trp concentration did not decrease during the entire study, indicating that this pathway for Trp catabolism was not active in cheese-like growth conditions and that residual enzyme activity from previous growth will decrease at the pH and salt content of Cheddar cheese.

Enzymes in the Anthranilic Acid Pathway

Accumulation of kynurenine via Trp 2, 3-dioxygenase produces N-formyl-kynurenine, which is subsequently converted to kynurenine by kynurenine formidase. Because pure N-formyl-kynurenine was not available, it was not used as a substrate. Therefore, enzyme assays using CFE measured the amount of kynurenine formed with Trp as the substrate and inhibitors added. The specific activity was the result of both enzymes reported as the sum of two enzyme activities (Trp 2, 3-dioxygenase and kynurenine formidase). Also, enzyme inhibitors were used to inhibit Trp 2, 3-dioxygenase enzyme activity with KCN, and kynurenine formidase activity with $NaHSO_3$ in CFE from cells grown in laboratory conditions with CDM with Trp and CDM with all three AAA.

The addition of KCN (inhibition of Trp 2, 3-dioxygenase) or $NaHSO_3$ (inhibition of kynurenine formidase) individually or in combination resulted in no production of kynurenine or anthranilic acid, confirming complete inhibition of this pathway when initiated with Trp. In the absence of either inhibitor, both kynurenine and anthranilic acid were detected, confirming that Trp was metabolized by the anthranilic acid pathway.

Combined activity in optimum assay conditions was detected from d 8, increased between d 10 to 18, and then decreased to negligible levels (Figure 4). In nonoptimal enzyme assay conditions, enzyme activities were reduced by more than 50% in all CFE tested (Figure 4). In these assays, anthranilic acid was also detected, suggesting that kynurenine produced by action of Trp 2, 3-dioxygenase and kynurenine formidase was further converted by kynureninase (Figure 4) to anthranilic acid.

Kynureninase was active in CFE obtained from cells incubated in laboratory growth conditions (CDM with Trp and CDM with all three AAA) and tested in optimal enzyme assay conditions. Activities varied over culture incubation time (Figure 4). The enzyme activity decreased by 50 to 65% in nonoptimal enzyme assay conditions compared with the optimum conditions (Figure 4). Negligible activities were observed in CFE prepared from cells incubated in CDM with Phe and Tyr (Figure 4), suggesting kynureninase was not produced in the

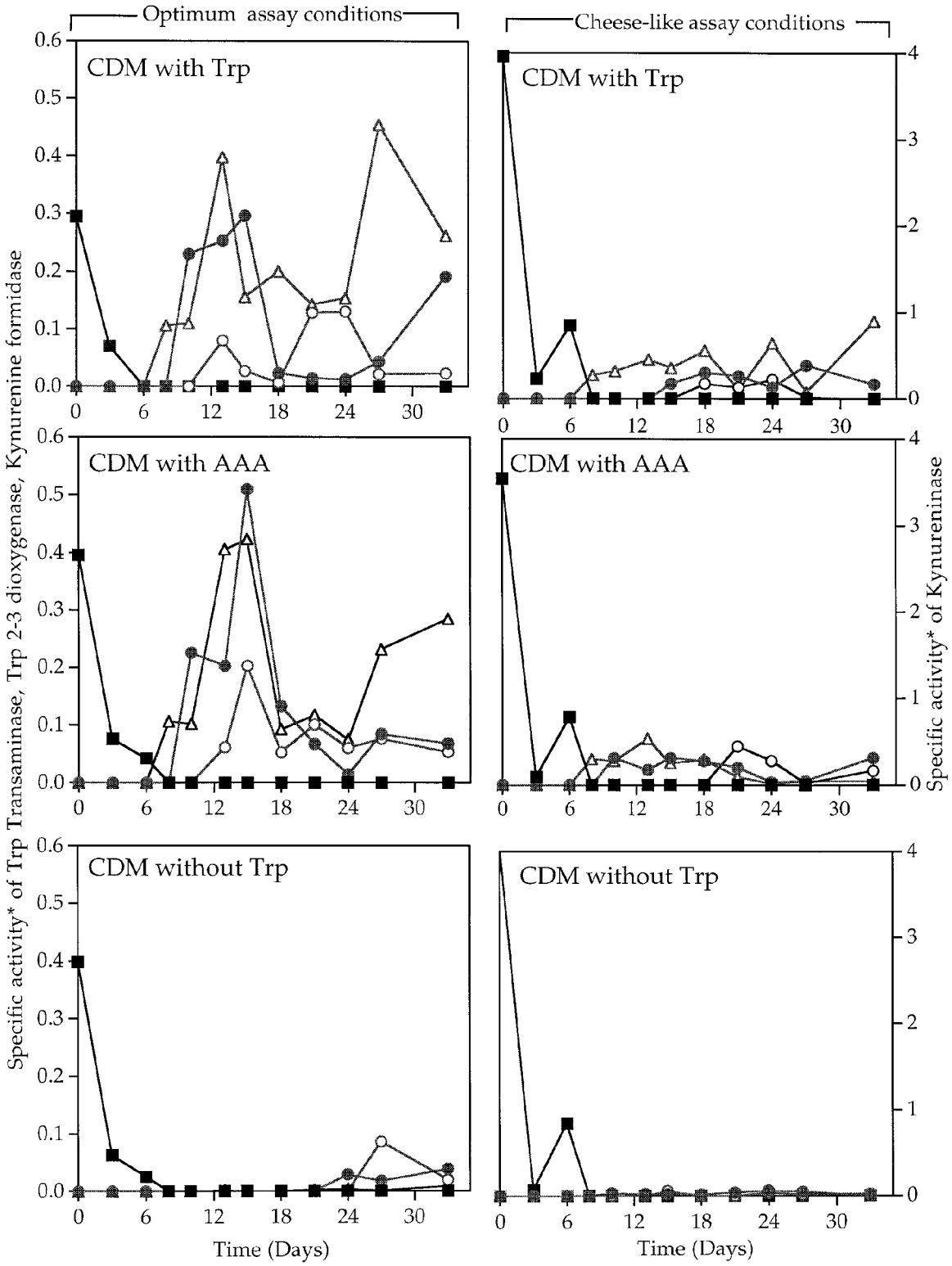

Figure 4. Enzyme specific activity (μmoles/mg of protein/ml) profiles of *Brevibacterium linens* BL2 cells grown in media and assayed in enzyme optimal assay conditions, and cheese-like conditions assay conditions (potassium citrate buffer pH 5.2, 4% NaCl). Symbol (■) denotes Trp transaminase activity. Optimum assay conditions: pH 8.5, 25°C for 30 min; non-optimal assay conditions: pH 5.2, 4% NaCl, 25°C for 30 min. Symbol (●) denotes cumulative Trp 2-3 dioxygenase and kynurenine formidase activities. Optimum assay conditions: pH 8.5, 37°C for 2 h; non-optimal assay conditions: pH 5.2, 4% NaCl, 37°C for 2 h. Symbol (○) denotes kynurenine activity (using Trp as substrate). Optimum assay conditions: pH 8.5, 37°C for 2 h; non-optimal assay conditions: pH 5.2, 4% NaCl, 37°C for 2 h. Symbol (△) kynureninase activity (using kynurenine as substrate). Optimum assay conditions: pH 7.8, 25°C for 1 h; non-optimal assay conditions: pH 5.2, 4% NaCl, 25°C for 1 h.

absence of Trp in CDM. Specific activities of kynureninase studied as a separate assay using kynurenine as a substrate were much greater compared with those detected when Trp was used as a substrate in both optimal and nonoptimal assay conditions (Figure 4). Despite this, kynureninase activity was minimal in nonoptimal assay conditions. From these observations, we hypothesized that the enzymes of the anthranilic acid pathway present in *B. linens* BL2 follow a cascade of reactions to produce kynurenine and anthranilic acid. However, these compounds are not associated with off-flavors in cheese.

CONCLUSIONS

The aim of this study was to delineate pathways for catabolism of Trp in *B. linens* BL2 and determine the potential of this culture to produce aromatic compounds associated with off-flavors in Cheddar cheese. Trp was degraded via the anthranilic acid pathway in laboratory growth conditions. This was inhibited at the pH and salt concentration found in Cheddar cheese. Additionally, Trp was degraded by other mechanisms leading to unknown aromatic catabolites that we could not identify. *B. linens* BL2 incubated in cheese-like conditions did not utilize Trp, nor produce aromatic metabolites associated with cheese flavor, indicating that the contribution of this organism to the production of Trp catabolites was negligible. Therefore, it is unlikely that *B. linens* BL2 will produce compounds associated with off-flavors in cheese.

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