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Protein degradation during anaerobic wastewater treatment: derivation of stoichiometry

Ian R. Ramsay[1] & Pratap C. Pullammanappallil*,[1,2]
[1]*Advanced Wastewater Management Centre, The University of Queensland, Brisbane Qld 4072, Australia;* [2]*School of Environmental Science, Murdoch University, Perth WA 6150, Australia (* author for correspondence: e-mail: pratap@murdoch.edu.au)*

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Abstract

The stoichiometry of reactions that describe protein degradation in anaerobic treatment systems were investigated. A methodology was developed to describe protein degradation to organic acids using a single reaction step. The reactions for individual amino acid fermentation and their mediating organisms were reviewed. The dominant fermentation pathways were selected based on a number of assumptions. Using the amino acid content of a model protein, it was then possible to determine stoichiometric coefficients for each major organic acid product in the overall degradation of the protein. The theoretical coefficients were then compared to those determined from two experimental runs on a continuously-fed, well-mixed, laboratory-scale anaerobic wastewater treatment system. In general, the coefficients compared well thus validating the use of a single reaction step for the overall catabolic reaction of protein degradation to organic acids. Furthermore, even when the protein concentration in feed or the feed flow rate was doubled, the amino acid fermentation pathways were found to occur predominantly by only one pathway. Although the choice of Stickland reactions over uncoupled degradation provided good comparisons, an electron balance showed that only about 40% of the amino acids could have proceeded coupled to other amino acid reactions. Uncoupled degradation of the remaining amino acids must have relied on the uptake of hydrogen produced from these reactions by hydrogen-consuming methane bacteria.

Introduction

Anaerobic treatment is becoming increasingly applied to a range of industrial wastewaters, especially in the agro-processing industry, which typically produces wastewater of high concentrations of readily degradable organic material in the form of carbohydrates, protein and fats. Carbohydrate content of a process wastewater stream often accounts for the majority of the organic load. In some industries, however, protein is also a major part of the organic load. For example, the protein component of a dairy wastewater stream can account for more than forty percent of the total chemical oxygen demand (Barnett et al. 1994). Other processing industries such as abattoir, whey, cheese, casein, fish and certain vegetable processing also typically produce wastewater containing significant amounts of proteins.

Modelling of anaerobic degradation in both sewage sludge digestion and wastewater treatment has been studied extensively. However, the majority of this work has focussed on carbohydrate degradation (Costello 1989, Romli 1993, Graef and Andrews 1974). The general methodology for formulating these models relied on a stoichiometry for degradation. The stoichiometry for the hydrolysis of carbohydrates, fermentation of sugars, acetogenesis, and methanogenesis were used to determine the biomass yields, reactant consumption and production formation in the models. This information together with the kinetics of reaction was used to derive the mass balance equations. Mathematical models for protein degradation under anaerobic environments can also be formulated fol-

lowing the same procedure outlined above. However, this would require knowledge of the stoichiometry for protein degradation.

Stoichiometry of protein degradation has been proposed for the purposes of modelling by a small number of authors including Andrews and Graef (1971), Pavlostathis & Giraldo-Gomez (1991) and Ryhiner et al. (1993). Generally, an overall catabolic reaction of protein degradation to acetate, propionate, butyrate, ammonia and carbon dioxide was developed from an average formula for protein. The stoichiometry for the protein degradation was derived empirically with some intermediates not included. This type of stoichiometry will obviously have limited application. Stoichiometry based on known mechanisms and reaction pathways is an alternative approach and could have more generic and wider applicability.

As with carbohydrate degradation, complete mineralisation of proteins to methane and carbon dioxide can be conceptualised as occurring via four major serial steps: extracellular enzymatic breakdown (protein hydrolysis), fermentation of large organic molecules to organic acids (amino acid fermentation), degradation of these fermentation intermediates to acetate (acetogenesis), and methane production from acetate or hydrogen and carbon dioxide (methanogenesis). The stoichiometry for the fermentation of intermediates like propionate and butyrate to acetate and that of production of methane from acetate and hydrogen is known from carbohydrate degradation studies. However, the stoichiometry of the remaining reactions, which is predominantly the protein hydrolysis and amino acid fermentation, need to be determined. Although extensive experimentation has been undertaken into the fermentation of amino acids, the relevance of this information to a mixed-culture anaerobic environment has not been discussed in the literature.

This paper presents a methodology to derive stoichiometry for protein degradation, in particular for the degradation of proteins to organic acids based on a review of microbial degradation studies. As an illustration, the methodology is applied to a model-protein to derive stoichiometric coefficients, which are then compared to actual experimental values from a laboratory scale anaerobic wastewater treatment system.

Literature Review

This section presents a review of the literature on microbial degradation studies in both pure and mixed culture environments, from protein hydrolysis through to fermentation of amino acids. The review investigated the microorganisms likely to be present in anaerobic treatment systems, as compared to other anaerobic environments such as in the rumen. The reactions mediated by these organisms were also reviewed. This information was then used to derive the stoichiometry for protein fermentation.

Protein hydrolysis

Proteins are natural polymers composed of amino acid units joined one to another by peptide (or amide) bonds. Proteins are hydrolysed by extracellular enzymes (called proteases) into their constituent polypeptides and amino acids. Protein hydrolysis in anaerobic environments has not been well studied except for regions in the gut of animals such as in the rumen, for example Bryant (1977) and Prins (1977). There seems to be major differences between protein degradation in the rumen and in anaerobic digesters. For instance, in the rumen, carbohydrate-fermenting bacteria degrade proteins and the fermentation of amino acids alone does not provide sufficient energy for growth. In anaerobic reactors, however, proteolytic bacteria predominantly mediate protein degradation and the processes involved are energy yielding. Most studies have shown the main proteolytic bacteria in digester sludge are gram-positive bacteria, principally from the genus Clostridia and these play a dominant role in the fermentation of amino acids as well (McInerney 1988).

Amino acid fermentation

Amino acids vary significantly in size and structure and are fermented via different pathways to a range of products depending on the type and concentration of amino acids present. These products include various organic compounds (predominantly short-chain and branched-chain organic acids), ammonia, carbon dioxide and small amounts of hydrogen and sulphur-containing compounds. Amino acid fermentation has been reviewed by numerous authors including Hippe et al. (1992), Andreesen et al. (1989), McInerney (1988), Gottschalk (1986), Cato et al. (1986), Barker (1981), Barker (1961), Sheehan (1981), Massey et al. (1976), and Kotze et al. (1969). These reviews provide

much information on biochemical and physiological aspects of amino acid fermentation by isolated species. These papers are briefly summarised here.

There are principally two ways in which amino acids can be degraded: (1) pairs of amino acids can be degraded coupled through the Stickland reaction; and (2) single amino acids can be fermented in a process that requires the presence of hydrogen-utilising bacteria. The Stickland reaction is common for amino acid decomposition. This usually involves one amino acid that acts as an electron donor (the product is shorter by one carbon atom than the original amino acid), while another acts as an electron acceptor (the product has the same number of carbon atoms as the original amino acid). Certain amino acids can serve both as an electron donor and an electron acceptor (for example Leucine). The Stickland reaction is the simplest way to ferment amino acids and provides the cell with approximately 0.5 mole ATP per mole amino acid transformed (Andreesen et al. 1989). Decomposition by pairs of amino acids in Stickland reactions occur rapidly as compared to uncoupled amino acid decomposition (Barker 1961).

Anaerobic bacteria known to ferment amino acids are listed in Table 1. Based on work by Mead (1971) and McInerney (1988), the bacteria have been classified into five groups (I-V) based on their involvement in Stickland reactions and the amino acids they typically utilised. Table 1 also describes the type of enzyme production of each bacteria, based on the information from Hippe et al. (1992), and characteristics of each bacteria group.

Group I bacteria are organisms that carry out Stickland reactions. These organisms all utilise proline and produce δ-aminovalerate, α-aminobutyrate or γ-aminobutyrate as intermediates in the fermentation process. This type of reaction has only been reported with Clostridial species. Amino acids known to be commonly involved in Stickland reactions include proline, serine, arginine, ornithine, glycine, leucine, isoleucine, valine, serine, lysine, alanine, cysteine, methionine, aspartate, threonine, phenylalanine, tyrosine, and tryptophan.

Organisms that do not carry out Stickland reactions but ferment amino acids are shown in Groups II, III, IV and V. These include mainly obligate spore-formers (Clostridial species) and some non-sporing obligate anaerobes, i.e., *Peptostreptococcus* (*Micrococcus*) spp. Other organisms that have also been found to degrade amino acids but are not shown include *Campylobacter* spp., *Acidaminococcus fermentans, Acidaminobacter hydrogenoformans, Megasphaera elsdenii, Eubacterium acidaminophilum*, and some sulphate-reducing bacteria (Zindel et al. 1988).

Group II bacteria all utilise glycine and some species also utilise arginine, histidine and lysine. Group III bacteria all utilise histidine, serine and glutamate and some species also utilise arginine, aspartate, threonine, tyrosine and tryptophan. Group IV bacteria includes only *C. putrefaciens* which uses serine and threonine. Group V bacteria include only *C. propionicum* which uses alanine, serine, threonine and cysteine. None of these bacteria produce δ-aminovalerate as found with Stickland reactions.

Under mixed-culture, mixed-amino acid conditions, uncoupled degradation of amino acids only occur if there is a shortage of amino acids that can act as electron acceptors (Nagase & Matsuo, 1982). For proteins such as casein, albumin and gelatine, this would only account for less than 10 percent of the total amino acids fermented (Nagase & Matsuo 1982). Hence, this indicates that amino acids are preferentially fermented via Stickland reactions during anaerobic treatment of protein containing wastewater.

Stoichiometric equations for the fermentation of different amino acids are summarised in Table 2. These equations cover most common pathways described in the literature but do not include the pathways of some specialist bacteria such as *C. propionicum*. In a number of cases an amino acid is known to degrade by more than one pathway. All reactions have therefore been described as either Stickland or non-Stickland reactions. Five amino acids involved in Stickland reactions can act as either electron donors or electron acceptors. For these amino acids, the reactions have been labelled in Table 2 as either oxidation or reduction.

Illustration: Degradation of casein

This section details the application of a methodology, based on the information above, to derive the stoichiometry for the degradation of a protein during anaerobic treatment. For the purposes of this illustration, a single protein, casein, will be used. Casein would be found predominantly in the wastewater of the dairy-related industries.

Table 1. Classification of anaerobic bacteria which degrade amino acids

	Group Sspecies	Enzyme production*	Amino acids utilised	Characteristics
I	*C. bifermentans*	proteo/saccharolytic	proline, serine, arginine, glycine,	organism that carry out Stickland
	C. sordellii	proteo/saccharolytic	leucine, isoleucine, valine,	reaction;
	C. botulinum types A, B, F	proteo/saccharolytic	ornithine, lysine, alanine,	proline utilised by all species;
	C. caloritolerans	–	cysteine, methionine, aspartate,	δ-aminovalerate,
	C. sporogenes	proteo/saccharolytic	threonine, phenylalanine,	α-aminobutyrate and
	C. cochlearium – one strain.	specialist	tyrosine, tryptophan, and	γ-aminobutyrate produced.
	C. difficile	saccharolytic	glutamate	
	C. putrificum	proteo/saccharolytic		
	C. sticklandii	specialist		
	C. ghoni	proteolytic		
	C. mangenotii	proteolytic		
	C. scatologenes	saccharolytic		
	C. lituseburense	proteo/saccharolytic		
	C. aerofoetidum	–		
	C. butyricum	saccharolytic		
	C. caproicum	–		
	C. carnofoetidum	–		
	C. indolicum	–		
	C. mitelmanii	–		
	C. saprotoxicum	–		
	C. valerianicum	–		
II	*C. botulinum types C*	proteo/saccharolytic	glycine, arginie, histidine and	glycine used by all species;
	C. histolyticum	proteolytic	lysine	δ-aminovalerate not produced,
	C. cochlearium – one strain	specialist		
	C. subterminale	proteolytic		
	C. botulinum types G	–		
	P. anaerobius	–		
	P. variabilis	–		
	P. micros	–		
III	*C. cochlearium – one strain.*	Specialist	glutamate, serine, histidine,	δ-aminovalerate not produced;
	C. tetani	Proteolytic	arginine, aspartate, threonine,	histidine, serine and glutamate
	C. tetanomorphum	Saccharolytic	tyrosine, tryptophan and	used by all species.
	C. lentoputrescens	–	cysteine	
	C. limosum	proteolytic		
	C. malenomenatum	specialist		
	C. microsporum	–		
	C. perfringens	proteo/saccharolytic		
	C. butyricum	saccharolytic		
	P. asaccharolyticus	–		
	P. prevotii	–		
	P. activus	–		
IV	*C. putrefaciens*	proteolytic	serine and threonine	δ-aminovalerate not produced
V	*C. propionicum*	specialist	alanine, serine, threonine, cysteine and methionine	δ-aminovalerate not produced

Sources: Hippe et al. (1992)*; McInerney (1988); Elsden & Hilton (1976, 1978 and 1979); Mead (1971); Barker (1961); Nisman (1954); *C – Clostridium*; *P – Peptostreptococcus*; Specialists – neither proteolytic nor saccharolytic species but specialised organisms that use only one or a few substrates.

Step 1. Assumptions

A number of assumptions have been made in the development of the stoichiometry. First, it was assumed that the hydrolysis of protein is much slower than the subsequent fermentation of amino acids. Second, it was assumed that the amino acid fermentation pathways remain constant and occur predominantly by only one pathway, regardless of hydraulic and organic loading conditions. If amino acids do not accumulate in the system, in the case that the former assumption is true, then amino acid fermentation pathways are unlikely to change. Based on these assumptions, the processes of protein hydrolysis and amino acid fermentation could then be represented by a single overall

Table 2. Stoichiometry for amino acid fermentation (catabolic reactions only)

No.	Reaction	Type	References	
1	$C_6H_{13}O_2N$ (Leu) + 2 H_2O → $C_5H_{10}O_2$ (3-methylbutyrate) + NH_3 + CO_2 + $2H_2$ + ATP	Stickland	Oxidation	Elsden & Hilton (1978); Mead (1971)
2	$C_6H_{13}O_2N$ (Leu) + H_2 → $C_6H_{12}O_2$ (4-methylvalerate) + NH_3	Stickland	Reduction	Elsden & Hilton (1978)
3	$C_6H_{13}O_2N$ (Ile) + $2H_2O$ → $C_5H_{10}O_2$ (2-methylbutyrate) + NH_3 + CO_2 + $2H_2$ + ATP	Stickland		Elsden & Hilton (1978); Mead (1971)
4	$C_5H_{11}O_2N$ (Val) + $2H_2O$ → $C_4H_8O_2$ (2-methylpropionate) + NH_3 + CO_2 + $2H_2$ + ATP	Stickland		Elsden & Hilton (1978); Mead (1971)
5	$C_9H_{11}O_2N$ (Phe) + $2H_2O$ → $C_8H_8O_2$ (phenylacetate) + NH_3 + CO_2 + $2H_2$ + ATP	Stickland	Oxidation	Elsden & Hilton (1976)
6	$C_9H_{11}O_2N$ (Phe) + H_2 → $C_9H_{10}O_2$ (phenylpropionate) + NH_3	Stickland	Reduction	Elsden & Hilton (1976)
7	$C_9H_{11}O_2N$ (Phe) + $2H_2O$ → C6H6 (phenol) + $C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2 + ATP	Non-Stickland		Elsden & Hilton (1976)
8	$C_9H_{11}O_3N$ (Tyr) + $2H_2O$ → $C_8H_8O_3$ (hydroxyphenyl acetate) + NH_3 + CO_2 + $2H_2$ + ATP	Stickland	Oxidation	Elsden & Hilton (1976)
9	$C_9H_{11}O_3N$ (Tyr) + H_2 → $C_9H_{10}O_3$ (hydroxyphenyl propionate) + NH_3	Stickland	Reduction	Elsden & Hilton (1976)
10	$C_9H_{11}O_3N$ (Tyr) + $2H_2O$ → C_6H_6O (cresol) + $C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2 + ATP	Stickland	Oxidation	D'Ari & Barker (1985); Elsden & Hilton (1976)
11	$C_{11}H_{12}O_3N_2$ (Trp) + $2H_2O$ → $C_{10}H_9O_2N$ (indole acetate) + NH_3 + CO_2 + $2H_2$ + ATP	Stickland	Oxidation	Elsden & Hilton (1976)
12	$C_{11}H_{12}O_3N_2$ (Trp) + H_2 → $C_{11}H_{11}O_2N$ (indole propionate) + NH_3	Stickland	Reduction	Elsden & Hilton (1976)
13	$C_{11}H_{12}O_3N_2$ (Trp) + $2H_2O$ → C_8H_7N (indole) + $C_2H_4O_2$ (acetic acid) + NH_3 + CO_2 + H_2 + ATP	Non-Stickland		Elsden & Hilton (1976)
14	$C_2H_5O_2N$ (Gly) + H_2 → $C_2H_4O_2$ (acetate) + NH_3	Stickland		Seto (1980)
15	$C_2H_5O_2N$ (Gly) + 1/2H_2O → 3/4 $C_2H_4O_2$ (acetate) + NH_3 + 1/2CO_2 + 1/4 ATP	Non-Stickland		Lebertz & Andreesen (1988)
16	$C_3H_7O_2N$ (Ala) + $2H_2O$ → $C_2H_4O_2$ (acetate) + NH_3 + CO_2 + $2H_2$ + ATP	Stickland		Bader et al. (1982); Andreesen et al. (1989)
17	$C_3H_6O_2NS$ (Cys) + $2H_2O$ → $C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2S + 1/$2H_2$ + ATP	Stickland		Barker (1961)
18	$C_5H_{11}O_2NS$ (Met) + $2H_2O$ → $C_3H_6O_2$ (propionate) + CO_2 + NH_3 + CH_4S + H_2 + ATP	Stickland		Wiesendanger & Nisman (1953)
19	$C_3H_7O_3N$ (Ser) + H_2O → $C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2 + ATP	Either		Carter & Sagers (1972)
20	$C_4H_9O_3N$ (Thr) + H_2O → $C_3H_6O_2$ (propionate) + NH_3 + CO_2 + H_2 + ATP	Non-Stickland		Tokushigo & Hayaishi (1972)
21	$C_4H_9O_3N$ (Thr) + H_2 → $C_2H_4O_2$ (acetate) + 1/2$C_4H_8O_2$ (butyrate) + NH_3 + ATP	Stickland		Hardman & Stadtman (1960a)
22	$C_4H_7O_4N$ (Asp) + $2H_2O$ → $C_2H_4O_2$ (acetate) + NH_3 + $2CO_2$ + $2H_2$ + 2 ATP	Either		Meister et al. (1951); Brock & Madigan (1991)
23	$C_5H_9O_4N$ (Glu) + H_2O → $C_2H_4O_2$ (acetate) + 1/2$C_4H_8O_2$ (butyrate) + NH_3 + CO_2 + 2ATP	Stickland		Hardman & Stadtman (1960a)
24	$C_5H_9O_4N$ (Glu) + $2H_2O$ → $C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2 + 2ATP	Non-Stickland		Barker (1961)
25	$C_6H_9O_2N3$ (His) + $4H_2O$ → CH_3ON (formamide) + $C_2H_4O_2$ (acetate) + 1/2$C_4H_8O_2$ (butyrate) + 2NH_3 + CO_2 + 2ATP	Stickland		Barker (1961); Pickett (1943)
26	$C_6H_9O_2N3$ (His) + $5H_2O$ → CH_3ON (formamide) + 2$C_2H_4O_2$ (acetate) + 2NH_3 + CO_2 + H_2 + 2ATP	Non-Stickland		Barker (1961); Pickett (1943)
27	$C_6H_{14}O_2N_4$ (Arg) + $6H_2O$ → $C_2H_4O_2$ (acetate) + $4NH_3$ + $2CO_2$ + $3H_2$ + 2ATP	Stickland	Oxidation*	Andreesen et al. (1989); Barker (1981)
28	$C_6H_{14}O_2N_4$ (Arg) + $3H_2O$ → 1/2$C_2H_4O_2$ (acetate) + 1/2$C_3H_6O_2$ (propionate) + H_2 + 1/2$C_5H_{10}O_2$ (valerate) + $4NH_3$ + CO_2 + ATP	Stickland	Reduction*	Hardman & Stadtman (1960b); Barker (1961); Mead (1971)
29	$C_5H_9O_2N$ (Pro) + H_2O + H_2 → 1/2$C_2H_4O_2$ (acetate) + 1/2$C_3H_6O_2$ (propionate) + 1/2$C_5H_{10}O_2$ (valerate) + NH_3	Stickland		Mead (1971); Elsden & Hilton (1979)
30	$C_6H_{14}O_2N_2$ (Lys) + $2H_2O$ → $C_2H_4O_2$ (acetate) + $C_4H_8O_2$ (butyrate) + $2NH_3$ + ATP	Either		McInerney (1988); Elsden & Hilton (1979), Barker (1981)

*Ornithine is the intermediate that is either reduced or oxidised in the fermentation of arginine.

catabolic reaction step, mediated conceptually by a single bacterial group. The stoichiometric coefficients for this reaction must then be determined.

Step 2. Determine the protein amino acid content and molecular formula

The amino acids that make up casein and their respective composition was determined using literature values (Brunner 1977) as shown in Columns 1 and 2 of Table 3. The molecular formula for casein was then calculated from this information as $CN_{0.23}H_{1.9}O_{0.51}$.

Step 3. Select dominant amino acid fermentation reactions

For some amino acids, such as isoleucine, valine, alanine, cysteine, methionine, serine, aspartate, proline and lysine, there is only one known degradation pathway. In these cases, these reactions were chosen as the dominant fermentation pathway for an anaerobic wastewater treatment system.

In the remaining cases, more than one reaction was known to exist for the fermentation of the amino acid. For these amino acids, the degradation reactions that involved Stickland bacteria were chosen as dominant reactions. This was the case for histidine, glutamate, threonine, and glycine. It was noted that these Stickland reactions also either consumed or had lower hydrogen production while producing similar amounts of ATP than the other oxidation reactions and therefore may be more energetically favourable under anaerobic conditions.

For situations where there was a choice between Stickland reactions, preference was given to those pathways that involved the more common proteolytic bacteria. For example, the reaction for arginine that has been known for a range of preteolytic bacteria was chosen over the one that involved the specialist bacteria *C. sticklandii*. There was little evidence to decide the choice of the dominant reactions for the remaining four amino acids. Batch studies such as Eslden and Hilton (1978) generally indicated that the type of Stickland reaction, ie. oxidation or reduction, depends on microbial species and growth media. Nonetheless, it was therefore assumed that the oxidation reactions that produced ATP would be favoured in an anaerobic wastewater treatment system due to the common presence of hydrogen- utilising methane bacteria (that may reduce the need for electron acceptors), and the continuous nature of the system (that may select for the faster growing organisms). The oxidation reactions for leucine, phenylalanine, tyrosine, and tryptophan were therefore selected as dominant reactions. It was also assumed that tyrosine was degraded to *p*-cresol as this reaction produces less hydrogen with the same ATP yield as the other oxidation reaction.

Step 4. Determine the overall stoichiometry for protein degradation to acids

Based on Steps 1, 2, and 3, the overall stoichiometry for protein degradation to acids was determined as illustrated in Table 3. In the table, each column represents a product of amino acid fermentation. Stoichiometric coefficients for each reaction were placed in each corresponding product column. The coefficients were then multiplied by the amino acid contents, which are based on one carbon mole of protein, and summed to give an overall stoichiometric coefficient for that product.

All aromatic acids produced from amino acid fermentation were lumped together as one compound. An empirical formula ($C_{7.25}H_7O_{1.5}N_{0.25}$) for the lumped aromatic acids was developed using the predicted proportions of aromatic product from the equations shown in Table 3 and the composition of casein. It is necessary to include these aromatic components to maintain a correct carbon balance since aromatic amino acids can account for more than 10 percent of the protein carbon. The overall summation of the individual reactions for amino acid fermentation was then represented by the following reaction step, with each α given in Table 3:

$$\begin{aligned} CN_{0.23}H_{1.9}O_{0.51} + \alpha_{water}H_2O \rightarrow \\ \alpha_{acet}CH_3COOH + \alpha_{prop}CH_3CH_2COOH \\ + \alpha_{buty}CH_3CH_2CH_2COOH \\ + \alpha_{vale}CH_3CH_2CH_2CH_2COOH \\ + \alpha_{arom}C_{7.25}H_{7.2}O_{1.2}N_{0.2} + (0.23 - 0.2\alpha_{arom})NH_3 \\ + \alpha_{CO2}CO_2 + \alpha_{H2}H_2 + 0.219\ ATP \end{aligned}$$

Experimental validation

A laboratory-scale, continuously-fed stirred tank reactor (CSTR) made from a plexiglass column with a constant volume of 2.35 litres was used in this study. The pH was measured continuously using a combination pH electrode and display. The temperature of the reactor was controlled continuously at 35 °C for all experiments. Gas production was measured using a wet-type gas flow meter.

Wastewater was artificially prepared using casein powder as the protein substrate and fed to the reactor

Table 3. Determination of stoichiometric coefficients for protein degradation

Amino acid (AA)	Content[#] (mole AA/ c-mole protein)	Acetic acid (mole/ mole AA)	Propionic acid (mole/ mole AA)	Butyric acid (mole/ mole AA)	Valeric acid (mole/ mole AA)	Aromatic acids (mole C7.25/ mole AA)	Ammonia (mole NH_3/ mole AA)	Carbon dioxide (mole CO_2/ mole AA)	Hydrogen (mole H_2/ mole AA)	ATP (mole/ mole AA)	Equation used (from Table 2)
Arginine	0.0053	0.5	0.5		0.5		4	1	−1	1	28
Histidine	0.0050	1		0.5			2	1		2	25
Lysine	0.0122	1		1			2			1	30
Tyrosine	0.0082	1				0.828	1	1	1	1	10
Tryptophan	0.0016					1.379	1	1	2	1	11
Phenylalanine	0.0078					1.103	1	1	2	1	5
Cysteine	0.0003	1					1	1	0.5	1	17
Methionine	0.0049		1				1	1	1	1	18
Threonine	0.0075	1		0.5			1		−1	1	21
Serine	0.0147	1					1	1	1	1	19
Leucine	0.0167				1*		1	1	2	1	1
Isoleucine	0.0106				1*		1	1	2	1	3
Valine	0.0128			1*			1	1	2	1	4
Glutamate	0.0369	1		0.5			1	1		2	23
Aspartate	0.0124	1					1	2	2	2	22
Glycine	0.0058	1					1		−1		14
Alanine	0.0076	1					1	1	2	1	16
Proline	0.0217	0.5	0.5		0.5		1		−1		29
TOTAL (α)	mole/c-mole protein	0.124	0.018	0.050	0.041	0.018	0.225	0.157	0.134	0.219	

AA – amino acid; α – stoichiometric coefficients for protein equation in Equation 1; TOTAL equals the sum of (AA content × Organic acid stoichiometric coefficient), [#] content based on 55% α, 33% β, and 12% κ casein from Brunner (1977); *represents isomer compounds.

by a peristaltic feed pump from a 20 litre glass feed bottle, placed inside a drum kept cool by temperature controlled recirculation system. Components of the artificially prepared wastewater included casein, phosphorous in the form of potassium dihydrogen orthophosphate (KH_2PO_4) and trace metals. Phosphorous was added to meet the ratio of C : P of 100 : 2 on a weight basis. No nitrogen source other than protein was added to the system.

Performance measures for the reactor included gas generation rates and concentrations of the gas, and pH, ammonia concentrations and organic acid concentrations of the liquid. Organic acids were analysed using a Perkin-Elmer Autosystem Gas Chromatograph (GC) with autosampler and Flame Ionisation Detector (FID). The column used was a J&W DB-FFAP (Nitroterphthalic Acid Modified Polyethyleneglycol) Megabore (Part Number 125-3212). The analyses of the unknown acid peaks was performed using the GC described above in conjunction with a QMASS-910 Benchtop Quadrupole Mass Spectrometer. Individual organic acid carbon concentrations were calculated from GC analyses of filtered samples. Ammonia concentrations were calculated from diluted samples using an ammonia probe (Orion Ammonia Electrode, Model 95-12).

The results from two dynamic experimental runs were used in this paper. The first run involved a 100% increase in organic feed concentration for 36 hours from steady state at nominal operating conditions. The second run involved a 100% increase in feed flowrate for 24 hours from steady state at nominal operating conditions. In each case after the imposition of the organic overload or hydraulic overload the feed conditions were brought to nominal operating conditions. The duration of each run was approximately five days. The nominal operating conditions were a protein feed concentration of 6 g/l and flowrate of 6 l/day. Twenty sampling events for organic acids and ammonia were taken during each run.

From the organic acid and ammonia concentrations, a molar ratio of each acid to ammonia was calculated at each sampling event of the dynamic experiment. Stoichiometric coefficients were determined by multiplying each ratio by 0.225 mole of ammonia (i.e., stoichiometric yield of ammonia per mole of amino acid fermented from Table 3), and then averaged over all sampling events. It was assumed that very little methanation was occurring in the reactor because of minimal gas production rates. Further, anabolic reactions were assumed not to require or produce any significant quantities of ammonia. Therefore, the acid and ammonia concentrations were assumed to be the total of what was produced from amino acid fermentation.

The proposed stoichiometric values for casein degradation to organic acids are compared in Table 4 to experimental values for the two runs. The values shown for measured stoichiometry are mean and error values (1.95 standard deviation). The comparison is made on the basis of ammonia. The term aromatics include the summation of a number of compounds found in the system. These were shown by the GCMS to resemble *p*-cresol, phenylacetate and indoleacetate. No significant amounts of any other compounds including caproic acid were measured during either of the experimental runs.

Discussion

The comparison between experimental and theoretical stoichiometry shows that each coefficient is within the range of the experimental variation, with the exception of propionic acid, where a lower ratio was found for the experiments. From examining Table 3, the degradation of δ-aminovalerate, which is both an intermediate of arginine and proline degradation, makes up approximately 70 percent of the theoretical propionic acid production (0.013 of the 0.018 in Table 3). From this it could be inferred that an alternative degradation pathway for δ-aminovalerate may have occurred. However, the alternative product to propionic acid is not obvious from the results shown in Table 4. Nonetheless, this discrepancy accounts for only a relatively small fraction of the total acids within the system.

The GCMS results for the aromatic compounds validated the choice of reactions for the three aromatic amino acids in Step 3 of the methodology. Similarly for leucine, the fact that caproic acid was not detected in significant quantities validates the reaction choice for this amino acid.

The two experimental runs were found to have similar values for stoichiometric coefficients for each organic acid. Individual isomers for both butyric acid and valeric acid were listed separately to provide greater scope for comparisons. The butyric acid and valeric acid coefficients and propionic acid coefficients compared well between the two experimental runs. The acetic acid and aromatics coefficients between the two runs showed the greatest difference,

Table 4. Comparison of theoretical and measured stoichiometric coefficients for a laboratory scale anaerobic treatment system treating casein-based wastewater

Amino acid fermentation product	Measured stoichiometric coefficient Run 1	Measured stoichiometric coefficient Run 2	Theoretical stoichiometric coefficient
Acetic acid	0.142 (±0.011, 7.7%)	0.114 (±0.019, 17%)	0.124
Propionic acid	0.006 (±0.001, 17%)	0.007 (±0.002, 29%)	0.018
Butyric acid	0.048 (±0.006, 13%)	0.045 (±0.007, 16%)	0.050
iso-	0.015 (±0.002, 13%)	0.015 (±0.002, 13%)	0.013
normal-	0.033 (±0.004, 12%)	0.029 (±0.005, 17%)	0.037
Valeric acid	0.045 (±0.005, 1%)	0.046 (±0.008, 17%)	0.041
iso-	0.030 (±0.003, 10%)	0.031 (±0.004, 13%)	0.027
normal-	0.014 (±0.002, 14%)	0.016 (±0.005, 31%)	0.014
Aromatics	0.016 (±0.00, 31%)	0.024 (±0.007, 29%)	0.018

however was still within the errors of the experimental runs.

The change in the stoichiometric coefficients for each organic acid over the course of each experimental run indicated the effect of hydraulic and organic loading on amino acid fermentation pathways. For the first experimental run, the stoichiometric coefficients were found to vary ±13% on average, while the stoichiometric coefficients of the second experimental run varied ±20% on average. The difference in these values is a result of a small absolute change in the coefficients for propionic acid and *n*-valeric acid, and is therefore not of major significance.

The changes in loadings that were imposed on the laboratory-scale system (i.e., 100% organic feed change and 100% hydraulic feed change) were extreme. Considering this point, the results of ±13% and ±20% for stoichiometric coefficients support the assumption that amino acid pathways remain constant and that protein degradation can be described by a single stoichiometric reaction for such a system.

A further observation must be made in relation to the assumption that Stickland reactions were dominant over non-Stickland reactions. Stickland reactions are coupled reactions between a reducing and an oxidising amino acid, and therefore the net production of hydrogen from completely coupled reactions would be zero. However, it can be seen that the theoretical stoichiometric coefficient for hydrogen in Table 3 is 0.134. This coefficient is a result of 0.174 mole H_2 produced by oxidation versus 0.040 mole H_2 consumed by reduction, per carbon mole protein consumed. In other words, only about 20% of the hydrogen produced from amino acid fermentation was consumed by the reduction of amino acids. Therefore, about 60% of fermentation reactions were not coupled to other amino acid reactions. Nonetheless, the chosen reaction pathways provided a good prediction of fermentation products. Therefore, these reactions must be coupled to hydrogen-consuming methane bacteria to be energetically favoured, and would not be termed Stickland reactions. This is contrary to batch studies reported in the literature that had only observed these reactions with Stickland bacteria.

Conclusions

- The theoretically derived stoichiometric coefficients generally compared well to experimentally determined coefficients. The use of a single reaction step for the overall catabolic reaction of protein degradation to organic acids was therefore validated.
- Under changing feed conditions, i.e., a doubling of protein concentration in feed or feed flow rate, fermentation of amino acids was found to occur predominantly by only one pathway.
- The choice of Stickland reactions over uncoupled degradation provided good comparisons. However, based on an electron balance, only about 40% of the amino acids could have proceeded coupled to other amino acid reactions. The remaining amino acid reactions must have relied on

hydrogen-consuming methane bacteria as electron acceptors.

- The discrepancy in the prediction of stoichiometry for propionic acid appeared to result from an alternative pathway for arginine and proline degradation, possibly fermentation of δ-aminovalerate without the production of propionic acid.

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