Biological Stoichiometry and Bioenergetics of *Fusarium oxysporum* Ekt01/02 Proliferation Using Different Substrates in Cyanidation Wastewater

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Cyanidation wastewater contains heavy metals, including high concentrations of ammonia and free cyanide (CN^-). Aerobic growth of *Fusarium oxysporum* EKT01/02 in synthetic gold mine wastewater under different substrates was examined using biological stoichiometry and thermodynamic models in batch systems. The molecular weight of the dry biomass obtained was 23.03 g/C-mol, 33.14 g C-mol^{-1}, and 27.06 g/C-mol in glucose with ammonia (GA), *Beta vulgaris* with ammonia (BA), and *B. vulgaris* with cyanide (BCN) cultures, respectively. The microbial growth model showed the highest biomass yield of 0.69 g dry cell/g substrate in BA cultures. The heat of reaction (ΔH^O_{RX}) and Gibbs energy dissipation per mole of biomass formed (ΔG^O_{RX}) were −652.55/−432.11 kJ/C-mol, −132.59/−471.19 kJ/C-mol, and −370.34/−225.35 kJ/C-mol for GA, BA, and BCN cultures, respectively. The total Gibbs energy dissipated increased steadily over time and the metabolic rate of the *F. oxysporum* used was minimally adversely affected by the cyanidation wastewater as shown by the degree of reduction including the respiratory quotient quantified. The *F. oxysporum* proliferation was determined to be enthalpically driven in the cultures studied. This study revealed that the use of *B. vulgaris* agro-waste for the bioremediation of cyanidation wastewater is feasible and could engender sustainability of gold mining wastewater treatment processes.

Keywords: *Beta vulgaris*, biodegradation, bioenergetics, cyanide, *Fusarium oxysporum*

INTRODUCTION

Cyanidation wastewater from gold mining operations contains high concentrations of heavy metals, ammonia, and cyanide. Although the wastewater can be bioremediated, few studies report on the stoichiometric and thermodynamic analysis of such processes.

Thermodynamic analysis can predict the feasibility of a metabolic reaction and suitable conditions under which such a reaction can occur, thus addressing the feasibility of the process being studied.[1–3] Similarly, few studies report on the stoichiometric analysis of microbial proliferation and yield in bioremediation processes, although these factors determine the effectiveness of such bioprocesses because they are dependent on the microbial metabolic functions including cellular respiration of the isolates used. Furthermore, the stoichiometric coefficients define the efficiency of a specific microbial species in a defined process. Introducing bioenergetic analysis in such processes can further elucidate the feasibility of reactions under observation.

Gibbs energy dissipation per C-mol of biomass produced has been used to determine the balance between growth efficiency and metabolic rates using different thermodynamic models of microbial growth to justify the relationship between Gibbs energy dissipation and other parameters as the driving force for microbial growth and biomass yield in the presence of toxicants.[3–5] Available literature on bioenergetics, including stoichiometric analysis of microbial growth, has largely focused on the use of refined carbon sources such as glucose, sucrose, ethanol, and acetate, as substrates and/or electron donors, mostly in batch or fed-batch processes.[6–11] Although this approach can quantify the amount of Gibbs energy required to generate suitable quantities of biomass to support bioremediation reactions by varying the carbon sources used, it does not adequately describe systems in which a green chemistry approach is advocated for.[12] Thus, the challenge is to determine the energy requirements for a system in which a microorganism is grown on an agro-waste in the presence of a metabolic inhibitor such as cyanide, particularly for the bioremediation of cyanidation wastewater.

There are several reports on microbial remediation of industrial wastewater with numerous species of bacteria, fungi, algae, and protozoa for the treatment of cyanidation wastewater.[13–15] Although the process is judged to be robust and environmentally benign, few mineral processing industries have adopted this treatment process due to the nutritional requirements essential for microbial growth on a large scale. The future of microbial remediation of wastewater depends on studies that identify renewable substrates such as agro-waste for microbial growth in bioremediation systems on a large scale. With the large quantity of agro-waste generated annually from processing of agricultural produce, this challenge can be mitigated.[16] For bioremediation processes, agro-waste can be used to provide sufficient macro- and micro-nutrient and/or carbon sources for microbial growth including biocatalytic functions to decontaminate wastewater. The presence of micro- and macro-nutrients such as proteins,

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soluble sugars, and minerals in agro-waste can replace the use of refined carbon sources.[17] Furthermore, applying bioenergetics and biological stoichiometric analyses to systems in which agro-waste is used can further demonstrate the appropriateness of using suitable agro-waste in large scale wastewater treatment plants.

Therefore, this study seeks to promote the use of renewable feedstock in the bioremediation of cyanidation wastewater by applying biological stoichiometric and bioenergetic models to determine the functionality including requirements for a *F. oxysporum* species previously determined to be suitable for cyanide degradation.[12] Growth of the species on different substrates, namely glucose with ammonia as a nitrogen source (GA) for primary control experiments, *Beta vulgaris* (red beetroot) with ammonia as a nitrogen source (BA) for secondary control experiments, and *Beta vulgaris* with cyanide as a nitrogen source (BCN), was undertaken for stoichiometric and bioenergetic experiments with free cyanide being the targeted contaminant for bioremediation.

EXPERIMENTAL METHODS

Inoculum Preparation

An isolated *Fusarium oxysporum* EKT01/02 (Accession no: KU985430/KU985431) was cultivated in a synthetic gold mine wastewater containing metal concentrations similar to those reported in a previous study.[18] The wastewater had the following constituents (per litre): 47 mg $CuSO_4 \cdot 5H_2O$, 42 mg $Fe_2(SO_4)_3 \cdot H_2O$, 278 mg $(NH_4)_2SO_4$, 27 mg KH_2PO_4, 3 mg $ZnSO_4 \cdot 7H_2O$, 0.9 mg $PbBr_2$, and 40 mg $Na_2HAsO_4 \cdot 7H_2O$. A loopful of *F. oxysporum* grown on potato dextrose agar (PDA) was inoculated in 80 mL of the synthetic wastewater in a 200 mL multiport flask. The microbial growth was observed in both glucose (Merck) and *Beta vulgaris* cultures (without CN^-) at an average feed rate of 0.05 g L^{-1} h^{-1}. The cultures were incubated at 25 °C in a rotary shaker at 160 rpm (ZHICHENG® model ZHWY-200D, Shanghai, China) and pH 11, previously determined to be the highest pH in cyanidation wastewater. Uninoculated bioreactors were used in control experiments. Samples were taken periodically for biomass concentration measurements in a Jenway 6715 UV/Visible spectrophotometer at wavelength of 300 nm in triplicate using a calibration curve relating optical density (OD) to dry biomass weight.[12] The results showed the limiting substrate concentration for growth of *F. oxysporum* EKT01/02 was 300 mg/L on both glucose and *B. vulgaris*.[19] All reagents were of analytical grade from Merck (Germany).

Agro-Waste Preparation

The *B. vulgaris* agro-waste was obtained from an agro-processing facility in close proximity to Cape Peninsula University of Technology, Cape Town, dried at 80 °C for seven days, and pulverized to less than 100 μm in a grinder (Bosch MKM 7000, Germany).

Experimental Culture Conditions

The cultivation was carried out in a 1 litre stirred tank reactor at ambient temperature i.e. 25 ± 2 °C. A 10 % (v/v) *F. oxysporum* culture (48 h old), was inoculated on synthetic wastewater containing 300 mg glucose as refined carbon source, followed by experiments on 300 mg pulverized *B. vulgaris* agro-waste and subsequently on 300 mg pulverized *B. vulgaris* with 100 mg CN^- /L in the form of KCN added to the synthetic wastewater. An overhead stirrer fitted with a four blade propeller at 250 rpm provided mixing, and aeration was at 0.4 L/min. Biomass was harvested once the carbon source was exhausted and/or when the stationary microbial growth phase was reached. Harvested biomass was centrifuged at 10 000 rpm for 10 min at 4 °C in an Avanti® J-E centrifuge (Beckman Coulter, Inc. USA), washed thrice in sterile distilled water, dried for at least 12 h in a Duran® vacuum desiccator (DURAN Group GmbH, Germany) until the sample weight was constant, and stored at −20 °C for further analyses. All procedures were repeated until a suitable quantity of dry biomass was obtained.

Analytical Procedures

Biomass concentration was determined daily and expressed in grams dried biomass per litre culture medium (g/L). The dry samples from the desiccator were further dried at 100 °C for 24 h in an oven to remove residual moisture, before milling with a mortar and pestle prior to elemental analysis for *C*, *H*, and *N* by a Thermo Flash EA 1112 series analyzer in a Helium carrier gas (Thermo Fisher Scientific Inc. Waltham, USA). The analyzer combusts the sample with oxygen to produce CO_2, H_2O, and N_2 which are separated in a gas chromatograph and analyzed by a thermal conductivity detector. The peaks were integrated and percentages calculated for *C*, *H*, and *N*. All measurements were performed in triplicate.

The heat of combustion of the biomass was determined in an e2k oxygen bomb calorimeter (Digital Data Systems Pty. Ltd., South Africa) in triplicate. A pre-cut firing cotton thread (Part No. CAL2K-4-FC) was looped over the firing wire (Part No. CAL2K-4-FW) and twisted at the ends. A mass of 0.30 g of the dried biomass was weighed in a crucible and inserted into the crucible, ensuring that the firing cotton touches the sample. The electrode assembly was loaded into the vessel body and slightly tightened. The vessel was kept upright, filled with 3000 kPa oxygen, removed from the filling station, and allowed to stabilize for 1 min prior to insertion into the calorimeter. The calorimeter was calibrated with analytical grade Benzoic acid (Part No. CAL2K-BA).

The percentage of ash in dry biomass was determined by drying at 100 °C in an oven to constant weight as previously explained. The dried biomass was ashed in an EMF 260 furnace (Kiln Contracts (Pty) Ltd., Cape Town, South Africa) at 550 °C for 2 h done in triplicate. The fraction of oxygen was computed by difference from the total dry weight as follows:

$$f_O = 1 - (f_C + f_H + f_N + f_{ash}) \quad (1)$$

where $f_O, f_C, f_H, f_N,$ *and* f_{ash} are fractions of –O–, –C–, –H–, –N–, and ash respectively, on a dry biomass basis.

Statistical Analysis

Since all experiments were performed in triplicate, reproducibility was expressed as a standard deviation obtained from the dataset ($n = 3$). Normality of sample distribution was assessed using Shapiro-Wilk's test ($p > 0.05$)[20,21] with inspection of skewness and kurtosis measures and standard errors,[22,23] including visual inspection of box plots, histograms, and normal Q-Q plots. Test of equality of variances in samples (homogeneity of variance) ($p > 0.05$) was done using parametric and non-parametric Levene's test for approximately normally and non-normally distributed sample data respectively.[24,25] The statistical analyses were performed in an IBM Statistical Package for the Social Sciences (SPSS) software v24.0.

THEORY

Stoichiometric Microbial Analysis

Microbial growth models represent a material balance of the system in compliance with the law of conservation of mass. The overall stoichiometry of a biological reaction can be estimated using either the method of half reactions or regularities.[10,26] The general form of such a biological stoichiometric reaction can be described by Equation (2).[3]

$$\frac{1}{Y_{X/S}}S + Y_{A/X}A + Y_{N/X}NS \rightarrow X + Y_{P/X}P + Y_{C/X}CO_2 \quad (2)$$

By sequentially decoupling the overall reaction into catabolic and anabolic reactions, assuming the electron donor is first completely catabolized and a fraction of catabolism products is used to synthesize new biomass,[3] we have Equations (3) and (4):

$$\text{Catabolism: } S + Y_A^{cat}A \rightarrow Y_P^{cat}P + Y_C^{cat}CO_2 \quad \left(\Delta G_{cat}^0\right) \quad (3)$$

$$\text{Anabolism: } Y_p^{ana}P + Y_C^{ana}CO_2 + Y_N^{ana}NS \rightarrow X + Y_A^{ana}A \quad \left(\Delta G_{ana}^0\right) \quad (4)$$

where *S, A, NS, X*, and *P* represent the energy source, electron acceptor, nitrogen source, dry biomass, and reduced electron acceptor – i.e. the byproducts. For an aerobic culture, *S* can be any reducible carbon source such as glucose, fructose, methanol, methane, etc., which acts as an electron donor being primarily oxidized to CO_2 during the catabolic reaction. In this study, *P* was annotated to represent water while *A* was denoted to represent oxygen.

Furthermore, each empirical model was converted to a unit carbon elemental formula and the molecular weights were estimated from data in Table 2. For the microbial growth model using glucose (GA cultures), the molar yield coefficient (MYC) expressed as the ratio of molar growth yield (dry weight of biomass grown per mole of substrate utilised) to the molecular weight of biomass containing a unit carbon, was 3.126 C-mol per mole of glucose. Thus, the growth equation became (Equation (5)):

$$C_6H_{12}O_{6(aq)} + aNH_{3(aq)} + bO_{2(aq)} \rightarrow dCO_{2(aq)} + eH_2O_{(l)} + 3.126CH_{1.167}N_{0.067}O_{0.558} \quad (5)$$

The four unknown stoichiometric coefficients (*a, c, d,* and *e*), were estimated from an elemental balance using the conservation of mass relationship. However, since the molecular weight of the agro-waste, i.e. *B. vulgaris*, is unknown, a different approach was used. *B. vulgaris*, contains about 9.56 % readily oxidizable carbohydrates.[27] Hence, the general formula for a carbohydrate $n(CH_2O)$ was used to represent the agro-waste in models used. The experimental microbial yield based on the quantity of carbon source used $\left(Y_{X/S}\right)$ determined as 0.623 and 0.425 for ammonia as the nitrogen source (BA cultures) and *B. vulgaris* agro-waste with cyanide (CN^-) as the nitrogen source (BCN cultures) experiments respectively, were used. The reciprocal of the experimental biomass yield accounts for the C-mole of $n(CH_2O)$ consumed to produce 1 C-mole of biomass which was thus quantifiable. The stoichiometric coefficients (*p, q, r, s, v, w, x*, and *y*) in Equations (6) and (7) were thus stoichiometrically balanced using an elemental analysis approach.

$$1.605CH_2O_{(aq)} + pNH_{3(aq)} + qO_{2(aq)} \rightarrow rCO_{2(aq)} + sH_2O_{(l)} + CH_{2.377}N_{0.091}O_{1.093}(cells) \quad (6)$$

$$2.353CH_2O_{(aq)} + vCN^-_{(aq)} + wO_{2(aq)} \rightarrow xCO_{2(aq)} + yH_2O_{(l)} + CH_{1.82}N_{0.027}O_{0.804}(cells) \quad (7)$$

Energy Balances for Biological Systems

The biological stoichiometry of a defined process is incomplete without the exploratory analysis of an energy balance. The standard enthalpy of formation $\left(\Delta H_f^O\right)$ and Gibbs energy $\left(\Delta G_f^O\right)$ values (at pH = 7, 101.325 kPa, and 298 K) available in literature (Table 1) were used for bioenergetic models taking into account the stoichiometric coefficients from the microbial models to determine the heat of reaction $\left(\Delta H_{RX}^O\right)$. Furthermore, to determine experimental values for biomass enthalpy of formation $\left(\Delta H_f^{cell}\right)$, including heat of combustion $\left(\Delta H_c^{cell}\right)$ were obtained as described above, from which a model representing the combustion of a unit mass of biomass can be derived. The biomass enthalpy of formation was calculated for an ion containing carbon mole (ICC/mole) by multiplying the heat of combustion of the dry biomass with the mass of 1 C-mole biomass as shown in Equation (8):

$$\Delta H_f^{cell}\left(\frac{kJ}{mol}\right) = \Delta H_c^{cell}\left(\frac{kJ}{g}\right) \cdot M_X \quad (8)$$

where M_X is the mass of 1 C-mole of the dry biomass. The heat of reaction evolved in the synthesis of 1 C-mole of biomass was calculated using Hess's Law (Equation (9)):

$$\Delta H_{RX}^O = \sum n\left(\Delta H_{products}\right) - \sum n\left(\Delta H_{reactants}\right) \quad (9)$$

where *n* are the appropriate stoichiometric coefficients.

The Gibbs energy is the major driving force of microbial growth.[1,3,5,9,29] The energy exchange that accompanies a biological growth process can be well defined from the initial state to completion under both isothermal and isobaric conditions:

$$\Delta G = \Delta H - T\Delta S \quad (10)$$

where $\Delta G, \Delta H, and\, \Delta S$ are the Gibbs energy, enthalpy, and entropy changes respectively, accompanying microbial growth. Once the bioenergetic properties of the inputs and outputs are known, values of ΔG, ΔH, and ΔS can be estimated for microbial

Table 1. Thermodynamic properties of compounds used at 298.15 K and 101.325 kPa[10]

Substance	Formula	ΔH_f^O (kJ/mol)
Glucose	$C_6H_{12}O_{6(aq)}$	−1263.07
Ammonia	$NH_{3(aq)}$	−80.29
Oxygen	$O_{2(aq)}$	−12.09
Water	$H_2O_{(l)}$	−285.83
[a]Cyanide ion	$CN^-_{(aq)}$	140.3

[a]The data was adapted from Finch et al.[28]

Table 2. Elemental analysis of dry biomass as a mass fraction (g/100 g dry biomass) measured in triplicate. The standard deviation is indicated in parentheses (n = 3)

Substrate	f_{ash}	f_C	f_H	f_N
GA	12.98 (±0.07)	45.24 (±0.05)	4.40 (±0.09)	3.53 (±0.06)
BA	10.73 (±0.04)	32.33 (±0.03)	6.41 (±0.02)	3.42 (±0.03)
BCN	10.19 (±0.04)	39.82 (±0.05)	6.04 (±0.05)	1.24 (±0.04)

growth models. The quantity of Gibbs energy needed to synthesize 1 C-mole of microbial biomass has been previously modelled by Heijnen and van Dijken[30,31] using an empirical correlation. Their findings indicated Gibbs energy of a reaction (ΔG^O_{RX}) for synthesizing 1 C-mole of biomass depends mostly on the degree of reduction (γ_s) of the carbon donor and the number of carbon atoms as expressed in the model (Equation (11)).[30,31]

$$-\Delta G^O_{RX} = 200 + 18(6 - C)^{1.8} + \exp\left[(3.8 - \gamma_s)^{0.32} \cdot (3.6 + 0.4C)\right] \quad (11)$$

This model was used to determine the Gibbs energy needed to synthesize 1 C-mole of biomass and the degree of freedom can be estimated using Equation (12):

$$\gamma_s = (4nC + nH - 2nO - 3nN)/nC \quad (12)$$

Quantifying Microbial Growth and Bioenergetic Kinetic Parameters

The Gibbs energy dissipation for biomass growth and maintenance $(1/Y_{GX})$ was estimated:[11]

$$\frac{1}{Y_{GX}} = \frac{1}{Y^{max}_{GX}} + \frac{m_G}{\mu} \quad (13)$$

where $1/Y^{max}_{GX}$ was the Gibbs energy requirement for synthesizing a unit C-mole of biomass as defined in Equation (12), with m_G being the maintenance Gibbs energy, approximated to 4.5 kJ C-mol^{-1} h^{-1} at 298 K. The specific microbial growth rate (μ) was estimated using Equation (14):

$$\mu = \frac{1}{t_n - t_{n-1}} \ln\left(\frac{X_n}{X_{n-1}}\right) \quad (14)$$

where X_n and X_{n-1}were biomass concentrations (g dry biomass weight/L) at times t_n *and* t_{n-1} (h), respectively.

RESULTS AND DISCUSSION

Elemental Analysis

Elemental analysis of the biomass (Table 2) showed the percentages of ash, *C, H, N,* and *O* are similar for all cultures studied. The mass fraction of sulphur, potassium, phosphorus and other ions was not considered. Previous studies have shown that these constituents only contribute minorly to the empirical formula as their inclusion only affects the composition associated with the oxygen fraction of the biomass.[26,32] The hydrogen and carbon fractions of the *F. oxysporum* biomass grown on BA and BCN was determined to be relatively constant (6.22 % and 36.07 %, respectively) by dry weight. In contrast, the nitrogen content differed for cultures grown on BA and grown on BCN, which was attributed to the different nitrogen source (ammonia and cyanide). The nitrogen content of the dry biomass for GA and BA cultures was statistically similar (average = 3.47 %). The ash content for cultures grown on *B. vulgaris* (average = 10.46 %) differed from those that were grown on glucose (12.98 %). Although the average values of ash and hydrogen in BA and BCN differ, the t-test showed that the results were statistically indifferent.

The mass of 1 C-mole of biomass and the elemental formula were quantified to be within the range of previous research (Table 3). The higher C-molar mass (33.14 g C-mole) observed when cultures were grown on agro-waste can be attributed to the excess macro- and micro-nutrients available within *B. vulgaris* which were not present in the refined carbon source used and/or the rigidification of the fungal cell membranes including accumulations of extracellular polymeric substances, as the biomass strived to protect itself from cyanide toxicity. The degree of reduction indicates there are more available electrons during cyanide biodegradation which may be linked to the constituents available in *B. vulgaris*.[33] The degree of reduction on agro-waste (BA) was also similar to cultures in which glucose was used, an indication that use of agro-waste as a carbon source has minimal impact on the performance of the cultures. In comparison with similar filamentous fungi reported by Duboc et al.,[26] the degree of reduction and dry biomass weight of GA agrees with their report.

Microbial Growth Model

The microbial growth models used to represent aerobic growth of *F. oxysporum* on GA, BA, and BCN are shown in Table 4, organized into catabolic, anabolic, and overall metabolic stoichiometric reactions. The catabolic equations represent the oxidation of the carbon source (glucose or *B. vulgaris* waste). The nitrogen source (ammonia or cyanide) reacts with the catabolic products to

Table 3. Elemental formula of filamentous fungi and mass of 1 C-mole for dry biomass (M_X) and the degree of reduction (γ). The standard deviation is indicated in parentheses (n = 3)

Fungi	Carbon source	Elemental formula	(M_X)	γ
[a]*F. oxysporum*	Refined (GA)	$CH_{1.167}N_{0.067}O_{0.558}$	23.03 (±0.12)	3.850 (±0.05)
[a]*F. oxysporum*	Agro-waste (BA)	$CH_{2.377}N_{0.091}O_{1.093}$	33.14 (±0.31)	3.918 (±0.04)
[a]*F. oxysporum*	Agro-waste (BCN)	$CH_{1.82}N_{0.027}O_{0.804}$	27.06 (±0.28)	4.131 (±0.06)
[b]*N. crassa*	Refined	$CH_{1.80}N_{0.13}O_{0.45}$	24.91	4.52
[b]*P. chrysogenum*	Refined	$CH_{1.87}N_{0.08}O_{0.22}$	23.47	5.18
[b]*M. rouxii*	Refined	$CH_{1.79}N_{0.07}O_{0.43}$	23.83	4.74
[b]*A. niger*	Refined	$CH_{1.60}N_{0.10}O_{0.55}$	25.98	4.22

[a]This study, [b]Adapted from Duboc et al.[26]

Table 4. Microbial growth equations for aerobic growth of *F. oxysporum* on GA, BA, and BCN based on suggested model Equations (3) and (4)[3]

Growth on GA
Catabolism:
$C_6H_{12}O_{6(aq)} + 6O_{2(aq)} \rightarrow 6H_2O_{(l)} + 6CO_{2(aq)}$
Anabolism:
$1.511H_2O_{(l)} + 3.126CO_{2(aq)} + 0.209NH_{3(aq)} \rightarrow 3.126CH_{1.167}N_{0.067}O_{0.558}(cells) + 3.009O_{2(aq)}$
Metabolism:
$C_6H_{12}O_{6(aq)} + 0.209NH_{3(aq)} + 2.991O_{2(aq)} \rightarrow 2.874CO_{2(aq)} + 4.489H_2O_{(l)} + 3.126CH_{1.167}N_{0.067}O_{0.558}(cells)$
Growth on BA
Catabolism:
$CH_2O_{(aq)} + O_{2(aq)} \rightarrow H_2O_{(l)} + CO_{2(aq)}$
Anabolism:
$0.655H_2O_{(l)} + 0.623CO_{2(aq)} + 0.057NH_{3(aq)} \rightarrow 0.623CH_{2.377}N_{0.091}O_{1.093}(cells) + 0.61O_{2(aq)}$
Metabolism:
$CH_2O_{(aq)} + 0.057NH_{3(aq)} + 0.390O_{2(aq)} \rightarrow 0.377CO_{2(aq)} + 0.345H_2O_{(l)} + 0.623CH_{2.377}N_{0.091}O_{1.093}(cells)$
Growth on BCN
Catabolism:
$CH_2O_{(aq)} + O_{2(aq)} \rightarrow H_2O_{(l)} + CO_{2(aq)}$
Anabolism:
$0.387H_2O_{(l)} + 0.414CO_{2(aq)} + 0.011CN^-_{(aq)} \rightarrow 0.425CH_{1.82}N_{0.027}O_{0.804}(cells) + 0.436O_{2(aq)}$
Metabolism:
$CH_2O_{(aq)} + 0.011CN^-_{(aq)} + 0.564O_{2(aq)} \rightarrow 0.586CO_{2(aq)} + 0.613H_2O_{(l)} + 0.425CH_{1.82}N_{0.027}O_{0.804}(cells)$

produce biomass as shown in the anabolic equations. In reality, catabolic and anabolic processes are interdependent during growth, although they are theoretically constructed independently to elucidate the metabolism process. The overall metabolic description of a process is what is required to describe the actual biomass generated for bioremediation studies.

During catabolism, oxidation of the carbon source provides the ATP required to catalyze the anabolic mechanisms. In turn, anabolism conserves the chemical form of the non-thermal energy contained within the carbon source. Therefore, metabolism can be said to be an energy conservation process, i.e. all the non-thermal energy remaining within the carbon source for microbial growth processes.[29] Meanwhile, for complete aerobic oxidation of the substrate, a non-conservative process is followed with minimal non-thermal energy being required for the conservation of energy within biomass. The growth efficiency can be estimated as a quantifiable ratio between available electrons (AE) in conserved biomass to those that are available in the non-conservative reactions. The AE can be classified as a degree of reduction for a unit carbon atom.

For growth on BCN, cyanide can be converted to cyanate by cyanide monoxygenase, followed by conversion of cyanate to ammonia and carbon dioxide with cyanate as catalyst. Alternatively, cyanide can be oxidized directly using cyanide dioxygenase to produce ammonia and carbon dioxide as shown in Equation (15).[34,35]

$$HCN_{(aq)} + O_{2(aq)} + 2H^+_{(aq)} + NAD(P)H \xrightarrow{cyanide\ dioxygenase} CO_{2(aq)} + NH_{3(aq)} + NAD(P)^+ \quad (15)$$

The ammonia byproduct can be consumed with other byproducts to generate biomass including the carbon dioxide from cyanide biocatalytic decomposition which accounts for the higher molar production of carbon dioxide observed in BCN cultures compared with those grown in BA. This contributes to the higher AE observed in the BCN cultures.

Bioenergetic Parameters

In addition, changes in thermodynamic properties can be calculated, although not precisely, by using the microbial growth models and known thermodynamic properties of reactants and products except for biomass for which a true standard state is unknown. The ΔH_c^{cell} determinations as described earlier in a bomb calorimeter were -12.23 ± 0.02, -13.15 ± 0.03, -15.54 ± 0.06 kJ/g for biomass obtained from GA, BA, and BCN cultures, respectively. All measurements were performed in triplicate. The experimental enthalpy of combustion for *B. vulgaris* waste was -431.1 ± 0.3 kJ C-mol^{-1} (n = 6). Generally, Thornton's rule[36] can be used for estimating heat of combustion of organic substances, as for many organic substances, their heat of combustion is directly proportional to the number of atoms of oxygen consumed during combustion, as described by Equation (16):

$$\Delta H_c^o = -108.99 \frac{KJ}{eq} X(eq.\ transferred\ to\ oxygen\ during\ bomb\ calorimetric\ combustion) \quad (16)$$

Using Thornton's rule, enthalpy of combustion of *B. vulgaris* waste was estimated as -435.96 kJ C-mol^{-1} which correlated to the experimental value (-431.1 kJ C-mol^{-1}) obtained while the equivalent electron transferred to oxygen using calorimetric value is 3.95. The experimental values from bomb calorimetric combustion with the available thermodynamic properties listed in Table 1 were used to determine the changes in bioenergetic

Table 5. Thermodynamic parameters of *F. oxysporum* growth in different substrates at 298.15 K and 101.325 kPa. The standard deviation is indicated in parentheses (n = 3)

Substrate	ΔH_f^{cell} (kJ C-mol^{-1})	ΔH_{RX}^{O} (kJ C-mol^{-1})	ΔG_{RX}^{O} (kJ C-mol^{-1})	ΔS_{RX}^{O} (kJ K^{-1} C-mol^{-1})
GA	−281.69 (±0.47)	−652.55 (±0.21)	−432.11 (±0.05)	−0.74 (±0.02)
BA	−435.78 (±1.04)	−132.59 (±0.14)	−471.19 (±0.03)	1.14 (±0.03)
BCN	−420.54 (±1.76)	−370.34 (±0.18)	−225.35 (±0.05)	−0.48 (±0.01)

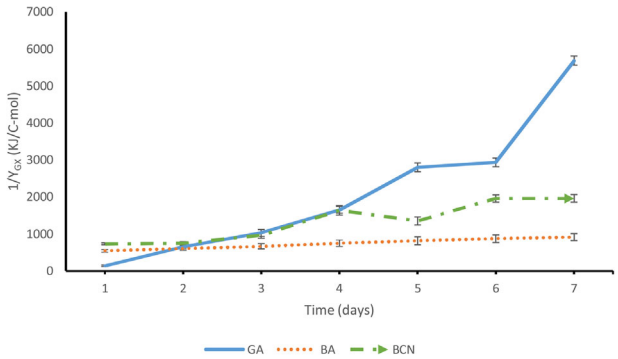

Figure 1. Time behaviour of average total Gibbs energy of biomass $(1/Y_{GX})$ during *F. oxysporum* growth in glucose (GA), in *Beta vulgaris* with ammonia (BA), and in *Beta vulgaris* with cyanide (BCN). The error bars indicate standard deviation (n = 3).

parameters accompanying the aerobic growth of the *F. oxysporum* isolate used as shown in Table 5.

The more exothermic ΔH_f^{cell} calculated using Equation (8) indicated higher values for growth on *B. vulgaris*, than on glucose as previously observed. The accuracy of these values is a function of the validity of the molecular formula of the carbohydrate used to represent the *B. vulgaris* agro-waste which has a direct influence on the accuracy of the bioenergetic parameter determinations. ΔH_{RX}^{o} and ΔG_{RX}^{o} shown in Table 5 is an indication of spontaneous metabolic processes in both refined and agro-waste carbon sources. Furthermore, from bioenergetic analysis, the growth on BA was hypothetically spontaneous at varying temperatures due to negative enthalpy and positive entropy changes for such a system. This may be directly linked to other added nutritional value components such as proteins, vitamins, and other minerals besides the available carbohydrates which are available in the agro-waste used thus can dissociate at different rates depending on the culture temperature. The estimated change in entropy values in all cases was determined to be weak, therefore, the growth processes were observed to be enthalpically driven which is similar to most previous reports.[2,26,29]

The results in Figure 1 show a gradual increase in the total Gibbs energy dissipated over time. Previous reports indicated that the Gibbs energy dissipation for biomass growth including maintenance $(1/Y_{GX})$ increases gradually in batch cultures,[6,37] achieving increasing metabolic rates although resulting in low biomass yield. The microbial growth model showed the highest biomass yield based on substrate and oxygen in BA cultures (Table 6). The results in Table 6 and Figure 1 concur with observations in previous studies that showed an increase in energy requirements is largely due to constraints in synthesizing biomass from a carbon and/or an energy source which causes reduction in specific growth rate as the process approaches the stationary phase.[1,6]

Table 6. Kinetic parameters of *F. oxysporum* on glucose with ammonia (GA), *Beta vulgaris* with ammonia (BA), and *Beta vulgaris* with cyanide (BCN). The standard deviation is indicated in parentheses (n = 3)

Substrate	$Y_{X/S}$ (g dry cell/g substrate)	Y_{X/O_2} (g dry cell/g O_2)	μ_{max} (h^{-1})	[a]R.Q
GA	0.39 (±0.01)	0.75 (±0.11)	0.0076	0.96
BA	0.69 (±0.03)	1.65 (±0.31)	0.0642	0.97
BCN	0.38 (±0.02)	0.64 (±0.05)	0.0089	1.04

[a]R.Q = respiratory quotient

By comparison, the growth on BA showed the lowest energy requirements for microbial growth with the highest dry biomass yield and maximum specific growth rate as shown in Table 6, while the Gibbs energy dissipated on GA was quantifiably large resulting in a lower maximum specific growth rate and dry biomass yield. The increase in energy requirements occurred at a specific growth rate of 0.0008 h^{-1} after 6 days on GA, meanwhile, prior to that, i.e. after 4 days, there was a decrease in energy requirements on cultures grown in BCN due to an increase in the specific growth rate from 0.0032 to 0.004 h^{-1} prior to cultures reaching the stationary phase. The relatively high biomass yield in BCN compared to GA may be due to the combined effect of an elongated catabolic pathway, presence of stored mucilage in the cells, and the requirement to assimilate micro- and macro-nutrients available in *B. vulgaris*.[4,17] The biomass yield based on oxygen consumption varied but the respiratory quotient (R.Q) was similar for the isolate in all cultures studied, an indication that the metabolic performance of the isolate was largely identical irrespective of the substrate used.

CONCLUSIONS

Clearly, the biological stoichiometry of microbial growth on agro-waste is as feasible as growth on glucose. The bioenergetic parameters even in the presence of an inhibitor, i.e. CN^-, support this claim. The agro-waste used proved to be as efficient as glucose with biomass yield and energy requirements. The respiratory quotient showed the metabolism of the *F. oxysporum* EKT01/02 was not affected by the different carbon sources used. This would encourage the use of agro-waste in wastewater treatment processes. This is the first report on the stoichiometry including bioenergetics of microbial proliferation on agro-waste in the presence of cyanide. However, there is a need to validate Heijnens's model used to estimate the Gibbs free energy of the growth process equation using Battley's approach of determining the heat capacity of dry cells in a low temperature calorimeter.

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NOMENCLATURE

$Y_{i/j}$	yield of i versus j (C-mol/C-mol)
$Y^{cat}_{i=A,P,C}$	stoichiometric coefficients of constituent in catabolic reaction
$Y^{ana}_{i=A,P,C,N}$	stoichiometric coefficients of constituent in anabolic reaction
$f_{i=O,C,H,N,ash}$	percent fraction of constituent in dry biomass
A	electron acceptor
S	energy source, electron donor
NS	nitrogen source
X	dry biomass
P	reduced electron acceptor
μ	specific growth rate of biomass
M_X	molecular weight (g/C-mol)
γ_i	degree of reduction of ith compound
ΔH^{cell}_f	enthalpy of formation of biomass (KJ/C-mol)
ΔH^{o}_{RX}	heat of reaction of formation of a unit C-mole of dry biomass (KJ/C-mol)
ΔH	change in enthalpy (KJ/C-mol)
ΔH^{cell}_c	heat of combustion of dry biomass (KJ/C-mol)
ΔH^{o}_c	standard heat of combustion (KJ/C-mol)
ΔH^{o}_f	standard enthalpy of formation
C_p	heat capacity (KJ/g/K)
T	temperature (K)
ΔS^{o}_{cell}	absolute entropy of biomass (KJ/C-mol/K)
ΔS	change in entropy (KJ/C-mol/K)
ΔS^{o}_{RX}	standard entropy of overall growth reaction per unit C-mole of dry biomass (KJ/C-mol/K)
$1/Y_{GX}$	total Gibbs energy dissipated for biomass growth and maintenance (KJ/C-mol)
$1/Y^{max}_{GX}$	Gibbs energy for synthesising a unit C-mole of biomass (KJ/C-mol)
m_G	maintenance Gibbs energy (KJ/C-mol/h)
ΔG^{o}_f	standard Gibbs energy of formation (KJ/C-mol)
ΔG^{o}_{cat}	standard Gibbs energy of catabolic reaction (KJ/C-mol)
ΔG^{o}_{ana}	standard Gibbs energy of anabolic reaction (KJ/C-mol)
ΔG^{o}_{RX}	standard Gibbs energy of overall growth reaction per unit C-mole of dry biomass (KJ/C-mol)
ΔG	free energy change (KJ/C-mol)
t	time (h)
$R.\ Q$	respiratory quotient

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