1. Introduction

Chemical and pharmaceutical industries are manufacturing fine chemicals in use of organic solvents and acids. Acetone is a solvent in the production of cordite, a smokeless ammunition propellant. Ethanol is a gasoline additive to increase octane and improve vehicle emissions. Butanol is used as an alternative liquid transportation fuel and can be catalytically converted into jet fuels. Moreover, butanol has proved to be a better biofuel than acclaimed ethanol owing to its less corrosive advantage to the biofuel production from various feedstock such as carbohydrates, lipids and proteins.

1.1. Carbohydrate-based substrates

Acetone–butanol–ethanol (ABE) fermentation is a known metabolic process of Clostridium sp. for the production of acetone, n-butanol, and ethanol from lignocellulosic biomass degradation [1,6,7]. Starches and simple sugars derived from sugar cane and corn are the most commonly used feedstock for the industrial production of biofuels. Microbial fermentation converts sugars produced from lignocellulosic biomass into biofuels or biorefineries using Clostridium sp. The theoretical maximum calculated to be 0.939 mol butanol/1 mol glucose by ABE fermentation [8] and ~ 1.33 mol butanol/1 mol glucose by mixotrophic fermentation [9]. Clostridium acetobutylicum has been studied as a model organism of biofuel production for several decades [10]. C. acetobutylicum ATCC 824 and C. beijerinckii BA101 were proved to increase n-butanol which are used as fuels, commodity chemicals, fine chemicals, and polymers [4]. Genetically engineered microbial strains should have a metabolic capability of utilizing multiple substrates with variable composition and without catabolite repression, which are crucial for the development of economic processes [5]. A microbial strain that has diverse substrate utilization potential would offer a major competitive advantage to the biofuel production from various feedstock such as carbohydrates, lipids and proteins.
tolerance and for \( n \)-butanol production from carbohydrates [6,7]. Butanol production was previously commercialized from molasses produced from sugar cane industry using \( C. \) acetobutylicum [11]. \( C. \) beijerinckii BA101 mutant produced the highest concentration of \( n \)-butanol (17–21 g/L) from glucose across all microorganisms [12]. However, lignocellulosic biomass has to be pretreated for biofuel production under harsh conditions that requires a large amount of energy consumption [13,14].

1.2. Lipid-based substrates

Compared to cellulosic biomass, food waste holds several significant advantages to produce butanol, since it comprises with significant quantities of sugar, starch, fatty acids, proteins and minerals. The considerable amounts of these functionalized substrates can act as nutrients to proliferate the culture growth during ABE fermentation [15,16]. Even if lipids used for biodiesel generation using transesterification process, current production technology is not being economical to make biofuels through lipid fermentation [17]. However, a co-product derived from high-value fatty acids could potentially make the biofinery process economical [18]. The maximum theoretical yield of ethanol obtained from palmitic acid (1.38 g ethanol/g palmitic acid) is significantly higher than that obtained from glucose (0.51 g ethanol/g glucose) [19,20]. Clomburg and Gonzalez [19] calculated the maximum theoretical yield of 1 mol each of ethanol and hydrogen per mole of glycerol fermented with an ethanol production of 4.6 mmol/L/h.

1.3. Protein-based substrates

Bioconversion process for releasing protein/amino acids from algal biomass or protein-rich waste may be easier than breaking down lignocellulosic to fermentable sugars [21]. The conversion efficiency of total sugar in food waste was up to 88%, but of protein was 40–70% [22]. Therefore, the conversion of proteins into biofuel is usually the rate limiting step during acidogenesis and solventogenesis phases [23]. Amino acids released by proteolysis of a protein-based substrate are catabolized to produce keto acids, which are synthesized into biofuels (ethanol, isobutanol, 2-methyl-1-butanol, and 3-methyl-1-butanol) [24]. Valine, leucine, threonine and isoleucine biosynthetic pathways have been overexpressed in \( E. \) coli host for the production of isobutanol [25], 2-methyl-1-butanol [26], and 3-methyl-1-butanol [27]. Engineered \( E. \) coli achieved a yield of \( \sim 4 \) g/l of alcohols from a yeast extract containing 21.6 g/l of amino acids at 56% of the theoretical yield [28]. Rerouting nitrogen flux in \( E. \) coli was allowed to producing up to 4,035 mg/L of alcohols from \( S. \) cerevisiae, \( E. \) coli, \( B. \) subtilis and microalgae that can be used as protein sources containing \( \sim 22 \) g/l of amino acids [21]. \( E. \) coli carrying Clostridial butanol dehydrogenase (bdh) gene metabolically engineered for the conversion of protein hydrolysate to \( n \)-butanol and \( n \)-pentanol [28]. Molecular and biochemical characteristics of many Clostridium species have been extensively studied for biofuel production. However, industrial and economic importance of them on the protein-based waste are hindered due to a lack of knowledge of their complex nature of metabolic and regulatory networks at a genome-scale. Hence, systems metabolic engineering of industrially important Clostridium would advance in the development of economically viable processes.

2. \( C. \) sticklandii genome

\( C. \) sticklandii is a Gram-positive, anaerobic, motile and non-pathogenic bacterium isolated from mud of the San Francisco Bay Area [29]. It utilizes amino acids as an important carbon and energy sources. It also catabolizes purines, glucose, maltose and galactose through a fermentation process, but these compounds are only minor substrates for its energy and growth [30,31]. It contains a single circular chromosome of 2,715,461 bp. It encompasses 2573 coding sequences, 77 RNA genes and 38 pseudogenes. Its coding sequences contain high protein coding density of 89.2% and 2.1% repeated regions [32]. \( C. \) sticklandii genome consists of 199 pathways, 1103 metabolic reactions, 829 metabolites and 739 enzymes. It shares the highest number of genes that are homologous to the pathogenic \( D. \) difficile and hype-ammonia producing Clostridium species. In this genome, 33% coding sequences are annotated as conserved hypothetical proteins.

2.1. Comparative genomic analysis

The genomes of Clostridium genus are highly diverse in morphology, the \( G + C \) content of the DNA (24–55 mol%), and metabolic activity [33]. \( C. \) acetobutylicum, \( C. \) beijerinckii and \( C. \) ljungdahlii have 250 pathways, 1300 metabolic reactions and 1000 metabolites, which exhibited their complex nature of metabolic and regulatory networks (Fig. 2). \( C. \) sticklandii, \( C. \) bifermentans and \( C. \) aminophilum consist of a typical transporter system that is able to transport most of the amino acids and amine-derived compounds across membranes. \( C. \) beijerinckii has a well-established transcriptome structure, but its transcript boundaries are not well understood. It is still not clear in other Clostridial genomes [34]. Genes involved in carbohydrate, lipid and nucleotide metabolism and their annotated features are extensively available for \( C. \) acetobutylicum, \( C. \) beijerinckii, \( C. \) cellulolyticum and \( C. \) ljungdahlii to date (Fig. 3). Energetic metabolism is vastly studied to know ion-gradient driven chemiosmotic ATP generation in all Clostridial genomes excluding \( C. \) bifermentans and \( C. \) aminophilum. Genes

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Fig. 1. International Energy Statistics for global biofuel production and consumption.

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Biotechnology Reports 16 (2017) 32–43
involved in amino acid metabolism of many Clostridial genomes including \textit{C. sticklandii} are greatly annotated with detailed functional information, suggesting their physiological importance in the growth and catabolism in different environmental niche.

3. General metabolism

The simultaneous presence of genes encoding for the complete Wood-Ljungdahl pathway and the glycine synthase is a particular metabolic feature of \textit{C. sticklandii} [32]. Genes coding for sulfate assimilate pathway are absent in this genome. Biotin and pantothenate are the absolute requirement for its growth, as those vitamins biosynthesis pathways absent in this genome [35]. The genes of the non-oxidative pentose phosphate pathway are present. The Rnf complex, Na$^+$-dependent FOF1-ATPase and an additional V-ATPase perform ion-gradient driven chemiosmotic ATP generation via electron-transport phosphorylation [32,36]. Superoxide reductase, Mn-superoxide dismutase, PerR homolog, glutathione peroxidases, seleno-peroxiredoxin and thioredoxin-dependent peroxidase are defense systems that enhance its survival under microaerophilic conditions [37].

3.1. Amino acid catabolism

3.1.1. Protein degradation and transport

Proteins are initially hydrolyzed by exogenous proteases to small peptides and amino acids. \textit{C. sticklandii} can be able to ferment amino acids to ammonia, CO$_2$, hydrogen, acetate and short chain fatty acids by the catalytic action of different metabolic enzymes as presented in Table 1. It has ABC transporters for oligopeptides, methionine, and branched-chain amino acids, serine/threonine symporter and also arginine/ornithine antiporter, which can mediate amino acid transport across the membrane [32]. It has a typical system for amino acid catabolism (Fig. 4).

3.1.2. Stickland reactions

Stickland reaction is a particular kind of fermentation of amino acids in the hype-ammonia producing Clostridium species. It is characterized by simultaneous oxidation of one amino acid and reduction of another amino acid [38,39]. Approximately 0.5 mol ATP is generated per mole amino acid transformed in Stickland reaction [40]. The δ-aminovalerate is an intermediate of arginine and proline degradation, which makes up approximately 70 percent of the theoretical propionic acid production. The theoretical stoichiometric coefficient for H$_2$ is 0.134, which produces about 20% H$_2$ from amino acid fermentation consumed by amino acids reduction [41]. \textit{C. bifermentans}, \textit{C. sordelli}
Fig. 3. Central metabolic information of *Clostridium sticklandii* DSM 519 and related *Clostridium* species, collected from the MetaCyc database. In our study, various subsystems involved in the metabolism of carbohydrate (a), amino acid (b), lipid (c), nucleotide (d) and energetic process (e) were compared to capture the metabolic abundance in environmental niche across the closely related Clostridial genomes (CST: *C. sticklandii*; CBI: *C. bifermentans*; CAM: *C. aminophilum*; CAC: *C. acetobutylicum*; CBE: *C. beijerinckii*; CCE: *C. cellulolyticum*; CLJ: *C. ljungdahlii*; CTH: *C. thermocellum*).
and C. sticklandii are belonged to Group IA strains. All strains utilize serine and threonine. The most strains produce α-amino butyrate and γ-amino butyrate [42]. Amino acid fermentation pattern is remarkably similar in C. sticklandii, C. aminophilum, C. aminovalericum, C. acidurici and C. barkeri, but their growth patterns are varied in some extent [43]. These bacteria were produced up to 20-fold more ammonia than other amino acids [45,47]. Methylene-tetrahydrofolate, CO2 and ammonia oxidizes ornithine, arginine, threonine, serine and threonine. The most strains produce 5-aminovalerate, which is subsequently excreted [54,56]. Proline reductase, proline racemase and proline racemase are key enzymes involved in the Stickland reaction by 2,4-diaminopentanoate dehydrogenase (EC 1.4.1.12).

### 3.1.3. Aliphatic amino acids

Glycine reductase, glycine acetyltransferase, phosphotransacetylase and acetate kinase are key enzymes involved in the γ-glutamate catabolism in C. sticklandii [46,47]. Methylenetetrahydrofolate, CO2 and ammonia are produced from glycine by direct oxidation of NAD⁺ via either glycine cleavage system or reduced to the acetyl phosphate by glycine dehydrogenase [48]. α-Alanine is deaminated to acetate and ammonia with reduction of NADH as NAD⁺ by L-alanine dehydrogenase (EC 1.21.4.2) reduces γ-glycine alone to acetate with the release of ammonia via intermediate acetyl phosphate. Reduced thiorodoxin and ATP molecules are produced as end products by glycine reductase and acetate kinase, respectively. Acetate is formed from pyruvate via acetyl-CoA and acetate phosphate by a combined action of phosphate acetyltransferase and acetate kinase. Pyruvate-formate lyase converts pyruvate into acetyl-CoA. Pyruvate-formate lyase transfers an acetyl group with the use of electron donor NAD⁺, which reduced to NADH2 in the conversion of pyruvate into acetyl-CoA.

C. sticklandii has two adaptable catabolic systems such as ornithine degradation I (proline biosynthesis) and ornithine degradation II (Stickland reaction) for the degradation of l-ornithine. It contains all important genes coding for ornithine carbamoyltransferase, carbamate kinase, ornithine cyclodeaminase, ornithine aminotransferase, acetyl-CoA carbonyltransferase, and pyrroline-5-carboxylate reductase for ornithine catabolism. Ornithine racemase, adenosylcobalamin-dependent Δ-ornithine aminomutase and Δ-ornithine 4,5-aminomutase are identified and characterized for their enzymatic functions [49-53]. l-Proline is directly produced in the exponential phase from the breakdown of l-ornithine by a catalytic action of ornithine cyclodeaminase. Ornithine can be reduced to 5-aminovalerate through the formation of D-proline as an intermediate [45,54,55]. This organism has a typical operon that performs the reductive ring cleavage of l-proline into 5-aminovalerate, which is subsequently excreted [54,56]. Proline reductase, proline racemase and proline racemase are key enzymes involved in proline catabolism [57-60]. l-Alanine-γ-glycine pair could not be catabolized in the Stickland

### Table 1

| Amino acids | Gene   | EC        | Enzyme                                      |
|-------------|--------|-----------|---------------------------------------------|
| Arginine    | speA   | 4.1.1.19  | Arginine decarboxylase                      |
| Arginine    | speB   | 3.5.3.11  | Aspartate transaminase                      |
| Asparaginase| amsA   | 3.5.1.1   | Aspartate transaminase                      |
| Aspartate   | phdD   | 2.6.1.1   | α-Serine ammonia-lyase                      |
| α-Serine    | sdaA   | 4.3.1.18  | γ-Serine ammonia-lyase                      |
| γ-Serine    | sdaAA  | 4.3.1.17  | L-Serine ammonia-lyase                      |
| Glutamate   | gdhA   | 1.4.1.2   | Glutamate dehydrogenase                     |
| Glutamate   | gcaA   | 2.8.3.12  | Glutamate CoA-transferase                   |
| Glutamate   | gcaC   | 4.1.1.70  | Glutaconate CoA-transferase                 |
| Glutamate   | scad   | 1.3.8.1   | Short-chain acyl-CoA dehydrogenase          |
| Glutamate   | acoA   | 2.8.3.8   | Acetate CoA-transferase                     |
| Glutamate   | pdcS|amB   | 3.5.1.2   | Glutaminase                                 |
| Glycine     | gcvP1|gcvPB|acoL | 1.4.4.2 | Glycine dehydrogenase (aminomethyl-transferring) |
| Glycine     | gcvF1|gcvFL|acoL | 2.1.10 | Aminomethyltransferase                       |
| Glycine     | acoL   | 1.8.1.4   | Dihydroylipol dehydrogenase                 |
| Histidine   | hudI   | 4.3.1.3   | Histidine ammonia-lyase                     |
| Histidine   | hudU   | 4.2.1.49  | Urocanate hydratase                         |
| Histidine   | hudT   | 3.5.2.7   | Imidazolonepropionase                       |
| Histidine   | hudG   | 3.5.3.8   | Formimidoylglutaminase                      |
| Isocitrate  | dihE   | 2.6.1.42  | Branched-chain-amino-acid transaminase      |
| Isocitrate  | dihF   | 1.3.8.5   | 2-Methyl-branched-chain-enol-CoA reductase  |
| Isocitrate  | dihG   | 4.2.1.17  | Enol-CoA hydratase                          |
| Isocitrate  | dihI   | 1.1.1.178 | 3-Hydroxy-2-methylbutyryl-CoA dehydrogenase |
| Isocitrate  | atoA   | 2.3.1.9   | Acetyl-CoA-C-acetyltransferase              |
| Lysine      | gpeA   | 4.1.1.18  | Lysine dehydrogenase                        |
| Methionine  | megl   | 4.4.1.11  | Methionine γ-lyase                          |
| Phenylalanine| pah   | 1.14.16.1 | Phenylalanine 4-monooxygenase                |
| Phenylalanine| pchD1 | 4.2.1.96  | 4a-Hydroxysterohydrobiphenolinate dehydratase|
| Threonine   | tdh    | 1.1.1.103 | L-Threonine 3-dehydrogenase                 |
| Threonine   | tdh    | 2.3.1.29  | L-Threonine 3-dehydrogenase                 |
| Threonine   | tdh    | 1.1.1.103 | L-Threonine 3-dehydrogenase                 |
| Threonine   | atoC   | 1.4.3.21  | Primary-amine oxidase                       |
| Threonine   | lthl   | 4.2.1.4(1.4.1.48) | l-Threonine aldolase[low-specificity l-threonine aldolase](1.5.1.12) |
| Threonine   | lthl   | 1.2.1.10  | Acetatedehyde dehydrogenase (acyclating)    |
| Threonine   | arcB   | 2.1.3.3   | Ornithine carbamoyltransferase              |
| Threonine   | arcC   | 2.7.2.2   | Carbamate kinase                            |
| Ornithine   | arcB   | 4.3.1.12  | Ornithine cyclodeaminase                    |
| Ornithine   | orr    | 5.1.1.12  | Ornithine racemase                          |
| Ornithine   | orr    | 5.3.2.7   | Ornithine aminotransferase                  |
| Ornithine   | orr    | 5.4.3.5   | Ornithine 4,5-aminomutase                   |
| Ornithine   | ord    | 1.4.1.12  | 2,4-Diaminopentanoate dehydrogenase         |
| Ornithine   | proC   | 1.5.1.2   | Pyrroline-5-carboxylate reductase           |
| Ornithine   | proE   | 5.1.1.4   | Proline racemase                            |

C. sticklandii DSM 519. Information of genes and enzymes listed in this table were collected for individual amino acid catabolism from the MetaCyc database.
reaction as it does not have alanine dehydrogenase [61]. L-Valine, L-leucine, and L-isoleucine are probably transaminated to 2-ketoacids. These amino acids are subsequently assimilated in the primary metabolism by branched-chain-amino-acid transaminase, 2-methyl-branched-chain-enoyl-CoA reductase, enoyl-CoA hydratase, and 3-hydroxy-2-methylbutyryl-CoA dehydrogenase [62].

3.1.4. Aromatic amino acids
Aromatic amino acids are catabolized by oxidative system of \textit{C. sticklandii}, but its catabolic pathways is still unknown [63]. However, genes coding for phenylalanine 4-monooxygenase and 4a-hydroxytetrahydrobiopterin dehydratase were recently identified from this genome, which could possibly metabolize L-phenylalanine. This genome contains all the genes (\textit{hutH}, \textit{hutU}, \textit{hutI} and \textit{hutG}) involved in the L-histidine metabolism.

3.1.5. Acidic amino acids
L-Histidine is assimilated into glutamate that is not exogenously used by \textit{C. sticklandii} [42,64]. Glutamate is metabolized to acetyl-CoA with consecutive catalytic action of glutaminase, glutamate dehydrogenase, glutaconate CoA-transferase and glutaconyl-CoA decarboxylase via glutamate degradation V (via hydroxyglutarate) or glutamine degradation I [32].

3.1.6. Neutral amino acids
Branched chain amino acids are catabolized by the oxidative system of this organism [62]. The NAD-dependent \textit{l}-threonine-3-dehydrogenase and \textit{l}-threonine dehydrogenase are identified and characterized from this organism. It indicates the potential of using \textit{l}-threonine as carbon and nitrogen sources for its growth [65,66]. These enzymes oxidize threonine to 2-amino-3-ketobutyrate that is split into glycine and acetyl-CoA [65]. Threonine aldolase transforms threonine to glycine and acetaldehyde. Threonine dehydratase catalyzes threonine to ammonia and 2-ketobutyrate, which are oxidized to propionate and CO$_2$ or reductively aminated to 2-aminovalerate [64]. \textit{l}-Serine catabolism is rapidly mediated in the exponential growth phase by the presence of iron-dependent serine dehydratase and serine racemase [60,67]. This organism is able to catabolize both \textit{d}-serine and \textit{l}-serine due to its genome contains \textit{d}-serine ammonia-lyase and \textit{l}-serine ammonia-lyase. Pyruvate is an intermediate product resulted from dehydrogenation of serine. Asparaginase is a key enzyme involved in the degradation of L-asparagine to ammonia and CO$_2$ via asparagine degradation I pathway.

3.1.7. Basic amino acids
\textit{l}-Arginine is quickly metabolized into ornithine via citrulline and conserves energy without any redox reaction. This organism contains all the genes (\textit{speA} and \textit{speB}) involved in the arginine deiminase pathway as well as the ornithine oxidative-reductive pathway [54,55]. Cobamide coenzyme-dependent beta-lysine mutase, lysine-5,6-amino-mutase and lysine decarboxylase are isolated and studied for understanding its \textit{l}-lysine catabolism [68,69]. Lysine is catabolized into acetate, butyrate, and ammonia by exergonic reduction of crotonyl-CoA to butyryl-CoA [70–73]. After depletion of serine, arginine and cysteine, crotonyl-CoA is formed from the condensation of two acetyl-CoAs via butyrate fermentation during its exponential growth phase [32].

3.1.8. Sulfur-containing amino acids
A putative \textit{l}-cysteine sulfide lyase found to degrade \textit{l}-cysteine into sulfur-containing amino acids via \textit{l}-cysteine sulfi de lyase found to degrade \textit{l}-cysteine into a putative \textit{l}-cysteine sulfide lyase found to degrade \textit{l}-cysteine into...
pyruvate, ammonia and hydrogen sulfide. Methionine sulfoxide reductase is involved in the repair of oxidative damaged methionine, which may enhance its growth under microaerophilic condition. Gene \(( \text{megL} )\) coding for methionine \(\gamma\)-lyase possibly degrades \(\gamma\)-methionine through the methionine degradation II pathway. A selenium-containing tRNA\(^{\text{Glu}}\) was isolated and characterized from this organism for a better understanding of the functional role of selenium in specific tRNA species that modulate the protein synthesis efficiency of certain mRNAs \([74,75]\). The presence of D-selenocystine alpha, beta-lyase supports selenocysteine metabolism \([76]\).

4. Biofuels from amino acid catabolism

In the \(C.\) sticklandii, pyruvate is a major metabolic intermediate produced from amino acid catabolism, which further ferments into organic solvents (ethanol and \(n\)-butanol) and organic acids (acetate and \(n\)-butyrate). The proposed pathway for the solventogenesis and acidogenesis from amino acid catabolism is represented in Fig. 5. 2-Ketoacyl-CoA dehydrogenase converts acetyl-CoA to acetoacetyl-CoA, which is a key metabolic switch to synthesis either \(n\)-butanol or \(n\)-butyric acid. Stickland reactions are a great concern of interest for amino acid catabolism by \(C.\) sticklandii. It has a separate pathway for lysine catabolism that directs \(n\)-butanol and \(n\)-butyric acid production. Hydrogen production is coupled with formate metabolism which may be directed by accumulation of pyruvate in the cytoplasm.

**4.1. Butanol biosynthesis**

\(C.\) sticklandii has many branch points to execute solventogenesis and acidogenesis. Acetyl-CoA, acetoacetoyl-CoA, and butanoyl-CoA are key intermediates produced during biofuel synthesis \([78,79]\). Acetyl-CoA is converted to acetate in a single step, catalyzed by the enzyme acetate-CoA ligase. Acetyl-CoA acetyltransferase converts acetyl-CoA to acetoacetoyl-CoA that forms acetoacetate. Acetone is produced from decarboxylation acetooacetate by acetoacetate decarboxylase. Acetone is reduced to 2-propanol by a reaction catalyzed by NADP\(^+\)-dependent oxidoreductase. Butanoyl-CoA is formed from acetooactoyl-CoA via intermediates 3-hydroxybutanoyl-CoA and crotonoyl-CoA by the consecutive action of 3-hydroxybutanoyl-CoA dehydrogenase, (S)-3-hydroxybutanoyl-CoA hydro-lyase and butyroyl-CoA dehydrogenase. Finally, \(n\)-butanoyl-CoA is produced from butanoyl-CoA through butanoyl-CoA and butanoyl phosphate by two modifying enzymes, phosphate butyroyl-transferase and butyrate kinase. Butanoyl-CoA is repeatedly transformed to \(n\)-butanol in two step mechanisms by butanal: NAD\(^+\)-dependent oxidoreductase or butanal dehydrogenase with NADH as an acceptor and oxidoreductase with the reduction of NADH.

**4.2. Hydrogen production**

\(C.\) sticklandii genome compose genes coding for periplasmic [Fe-Fe] formate hydrogen lyase (FHL) catalyzing the reaction: H\(_2\) + CO\(_2\) \(\rightarrow\) CH\(_3\)CO\(_2\)H + H\(_2\)O.

**Fig. 5.** The proposed pathway for protein catabolism-directed biofuel production in \(Clostridium\) sticklandii DSM 519. We proposed this pathway based on the metabolic reactions collected from the MetaCyc database for amino acid catabolism associated acetate, \(n\)-butanol, \(n\)-butyric acid, ethanol and hydrogen production. Enzyme commission was assigned according to the Kyoto Encyclopedia of Genes and Genomes database entries. Pyruvate was considered as a main metabolic precursor for the synthesis of organic solvents and organic acids in the proposed pathway. Ethanol is simply produced from acetaldehyde by alcohol dehydrogenase. Butanoyl-coA is a key metabolite switch to synthesis either \(n\)-butanol or \(n\)-butyric acid. Stickland reactions are a great concern of interest for amino acid catabolism by \(C.\) sticklandii. It has a separate pathway for lysine catabolism that directs \(n\)-butanol and \(n\)-butyric acid production. Hydrogen production is coupled with formate metabolism which may be directed by accumulation of pyruvate in the cytoplasm.
hydrogenase (hymC), two cytoplasmic iron-only hydrogenases (hydA and hymABC) and [Fe-Fe]-hydrogenase maturation proteins, hydF, hydG and hydE. HydA is clustered together with genes coding for a formate dehydrogenase (fdhAB). The presence of periplasmic [Fe-Fe] hydrogenase catalyzes the reduction of protons to the hydrogen along with translocates protons. The proton concentration is also lowered by the oxidation of formate to CO₂ from which released electrons are transferred directly to the hydrogenase via ferredoxin to reduce H⁺ ions. Hydrogen is generated from reducing equivalents during amino acid catabolism with the proton transport and energy conservation ferredoxin oxidoreductase. It is an alternative energy-conserving system to increase its growth efficiency on formate [44].

Molecular hydrogen is produced by the reduction of protons released from the reversible interconversion of protons, electrons and H₂ through the action of hydrogenase. Endogenously produced formate is oxidized into CO₂ and H⁺ to evolve hydrogen by formate hydrogenlyase complex formed from NAD-dependent formate dehydrogenase H (fdhH) and hydrogenase 3 [80,81]. FdhH catalyzes the oxidation of formate generating CO₂ and transferring the electrons to hydrogenase 3. It transfers electrons to the catalytic subunit hydE of hydrogenase complex via hydF and hydG subunits [82]. HydH is required for the conversion of a precursor form of hydE into the mature form. HydA is a repressor protein that represses the hyd operon [83] and increases fdhH and hydrogenase activity [81]. It is likely to control formate oxidation and the hydrogen production rate by manipulating fdhH and hydA [84]. Gene expression analysis suggested that the concentration of formate in the cell determines the biosynthesis of formate hydrogenlyase complex [85]. Endogenous selenium and molybdenum are required for the catalytic activity of formate hydrogenase while nickel is necessary at the active site of hydrogenase 3 [86].

4.3. End-product feedback inhibition

Feedback repression is effected by amino acid end-products acting as co-repressors interfering with transcription initiation in C. sticklandii [87]. Nitrogen metabolite repression, nitrogen catabolite repression and ammonia repression are known nitrogen source regulation in proteolytic Clostridium sp., which typically control proteases, amidases, ureases, nitrogenase, hydrogenase and those that degrade amino acids [88]. It also controls the key ammonium assimilation such as NADP-glutamate dehydrogenase, glutamine synthetase, glutamate synthase and alanine dehydrogenase. The pool sizes of one or more substrates and/or products of these enzymes may suppress the enzymatic activities [89]. TnrA becomes bound to the feedback inhibited form of glutamine synthetase, when nitrogen is exceeded in the culture medium [90]. Biosynthetic intermediates of aspartate kinase can be feedback inhibited by its end product either by lysine or threonine and activated by metabolite from a competing branch as similar to C. acetobutylicum [91]. Engineering nitrogen flux favors the breakdown of amino acids rather than their synthesis, resulting in 2-keto-methylvalerate, 2-keto-isocaproate and 2-keto-isovalerate [28]. This approach is a great concern to overcome their feedback repression and to improve the biofuel production efficiency in E. coli [21].

5. Metabolic simulation for biofuel production

Mathematical models describing the overall physiology of the process, design features, mode of operation along with comparison and validation with experimental results of ABE fermentation were reviewed by Mayank et al. [92]. Ethanol production is optimized in Synechocystis mutant by using the flux balance analysis, method of minimization of metabolic adjustment and regulatory on/off minimization [93]. It is also improved in C. thermocellum by making more electrons available for acetyl coenzyme A reduction. In this approach, hydrogenase active site assembly is eliminated for hydrogen production that redirected carbon flux towards ethanol production [94]. A genetic change in C. phytofermentans demonstrated to improve its ethanol tolerance [95]. E. coli harboring engineered C. acetobutylicum butanol biosynthetic pathway efficiently converted n-butyric acid to n-butanol [96]. Therefore, mathematical models and metabolic simulation tools are under intense development to metabolically engineer Clostridial strains for the biofuel production [1,97–100].

5.1. Genome-scale models for biofuel production

Systems biology is the quantitative analysis of biological systems using predictive mathematical models. It is focused on interpreting biological function and behavior at a cellular level by using very large-
scale datasets [101]. The in silico metabolic reconstruction is a common approach permitting simulations of engineered genotypes to elicit desired phenotypes, as outlined in Fig. 6. Constraint-based metabolic simulation in rational metabolic engineering requires a metabolic network reconstruction and a corresponding in silico metabolic model for the microorganism of interest [102]. A genome-scale metabolic model is fundamentally defined by a list of mass-balanced and charge-balanced reactions, which can simulate regulatory changes, genetic engineering effects, and dynamic cell behavior. Genome-scale metabolic networks have become more refined and complex, allowing for the expanded scopes in systematic metabolic engineering for microbial biofuel production [103–105]. However, integrated models of metabolism will become particularly important for understanding and manipulating biological behavior on a genome scale in the future. To meet industrial biotechnology process development needs, microbial cell factories are now being constructed faster and more efficiently through the use of systems biology resources [102,106].

Genome-scale models have been developed as a computational initiative for unraveling the systems-level metabolic competence of an organism and guiding metabolic engineering strategies for strain development [107]. Using these models, cofactors and energetic requirements can be metabolically balanced for the microbial growth. These models are used to predict the availability of biosynthetic precursors in response to environmental and genetic perturbations [103,104]. Genome-scale models for a range of different Clostridium sp. with varying metabolic capabilities have been published over the last ten years (Table 2). Several successes have been demonstrated for model-driven systems engineering of C. acetobutylicum [10,112–115], C. thermocellum [116], C. cellulolyticum [117], C. beijerinckii [105] and C. ljungdahlii [118] for solventogenesis and acidogenesis. Constraint-based genome-scale metabolic model has been developed in a step-wise optimization procedure by which the solvent production was predicted in the continuous culture of immobilized C. acetobutylicum cells [119]. A genome-scale metabolic model integrated with stress-related specific transcriptomics is used for the prediction of its focal points of gene regulation [120]. The interdependence of gene regulation, metabolism, and environmental cues can be elucidated for ABE fermentation on the basis of its systems metabolic picture [121]. Using its quantitative system-scale model, the biofuel producing steady-state can be determined for a better understanding of the functional regulation of primary metabolism [122].

### 5.2. Model-driven systems metabolic engineering

In this approach, genome-scale metabolic modeling and x-ome technologies are deployed an increasing extent looking into the metabolic landscape to be engineered in order to establish an economically viable process it is necessary to improve the performance of the microorganism [102,106]. Metabolic engineering of C. sticklandii is challenging due to the lack of an efficient gene inactivation system and the existence of complex metabolic networks. Extensive studies on protein catabolism-directed solventogenesis and acidogenesis are needed for guiding metabolic engineering of C. sticklandii to improve the yield of biofuel. Metabolic engineering targets for biofuel production have been evaluated from several Clostridial strains using conventional molecular approaches [1,95,100,123]. Systems-level characterization of C. sticklandii comprehensively examines metabolic networks to be engineered and provides a scaffold for more effective bioprocess development. Molecular complexity and the functional behavior of engineered C. sticklandii that plays a role in the response to alternative substrates have been efficiently explored with advances in an integrated knowledge base of its metabolic interaction networks. Current systems biological understanding of C. sticklandii would make it possible to expansively guide model-driven systems metabolic engineering within context of entire host metabolism, to diagnose stresses due to product synthesis, and provides the rationale to cost-effectively engineering strategies. Even if chemical processing systems are more convenient to produce fuel and refineries, these processes are very toxic to the environment and human health [15]. Concerning the green biotechnology, its systems-level modeling framework would explore the metabolic capabilities to decipher a complex genotype-phenotype relationship governing its biofuel synthesis from protein-based waste.

### 6. Conclusion

C. sticklandii has a great concern of utilizing proteins for producing renewable alternatives to the petroleum-based chemicals and fuels using a metabolic model at the genome-scale. Target genes for guiding metabolic engineering and novel pathways to be engineered have to be elucidated to the scientific community for competent biofuel production at industrial scale. Genome-scale model of C. sticklandii would provide a skeleton for predicting its metabolic behavior and emphasizing metabolic connections to biofuel synthesis. It will afford a guide for parallel reconstruction of genome-scale metabolic networks of related species. Of particular importance is the development of a regulatory circuit that allows a recombinant pathway to become part of a heterologous metabolic network for biofuel production [124] and tolerance [125]. Evolving genetic regulatory circuits would perform a self-regulating gene expression and enzyme activity in response to the precursor and or substrate supply in the host cell [126,127]. Genome-scale model of C. sticklandii will also provide a clue to design a synthetic pathway that needs to be integrated into the metabolic network of heterologous host in order to achieve optimal production levels. Optimized bioprocess parameters would be implemented for biofuel production from waste recycling of protein industry-based biomass in the biotechnology industry with eco-friendly manner. The present review concluded that model-driven systems metabolic engineering approach would advance the broad applicability of using C. sticklandii for biofuel production in the industrial sector.

### Compliance with ethical standards

The article does not contain any studies with human participants or animals performed by any of the authors.
Conflict of interest

The author declares that this article’s content has no conflicts of interest.

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