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Whole-cell biotechnological applications with thermoacidophilic *Crenarchaeota*: Opportunities and challenges

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ABSTRACT

Extremophiles are microorganisms that thrive in harsh environmental conditions where no life exists. These environmental conditions can involve temperature, acidity, salinity, pressure, or radiation that are typically uninhabitable to most microorganisms. The thermoacidophilic Archaea (e.g., *Acidianus*, *Metallosphaera* and *Sulfolobus)* can thrive at high temperatures and low pHs. These organisms are capable of autotrophic, lithotrophic, heterotrophic, chemoheterotrophic, and chemolithoautotrophic lifestyles, and thus can easily be cultivated on many different substrates. Additionally, their innate capacities to oxidise ferrous iron and/or reduced inorganic sulfur compounds have gained recognition for their utility in biomining operations. Members of *Acidianus* and *Metallosphaera* have been applied in bioleaching operations to extract valuable metals from low-grade ores and mineral concentrates. The advances in *Sulfolobus* genetics have presented opportunities for their application as platform organisms in biotechnological and biorefinery processes due to the ability to cultivate these extremophilic organisms under non-sterile conditions. Furthermore, utilising inexpensive and sustainable feedstock such as lignocellulosic biomass is one key advantage of lowering cultivation costs. This review presents current research developments on thermoacidophilic Sulfolobales members, emphasising their whole-cell applications for biomining operations, biotechnology and biorefinery. Several laboratory-scale studies found these organisms promising for large-scale deployment based on their unique characteristics.

1. Introduction

Extremophilic Archaea can thrive at environmental extremes, such as high temperatures, low pH values, high salt concentrations, or combinations thereof [1–3]. Members of thermoacidophilic Sulfolobales have evolved mechanisms to successfully adapt to extraordinarily hot, acidic environments, with optimal pH $\leq$ 4 and temperature $\geq$ 55°C and oxidative stress caused by high levels of metals in mining environments [4,5]. The unusual pathways and enzymes in these organisms offer a unique potential for application in biocatalysis, enzyme cascades and platform organisms (chassis) for metabolic engineering and synthetic biology [2]. The extremozymes/thermozymes produced by extremophiles are functional under extreme conditions due to enhanced enzyme rigidity and stability [2,4]. These enzymes are active in organic solvents and ionic liquids due to enhanced rigidity and stability [2,4]. In addition, Sulfolobales (e.g., *Sulfolobus*, *Metallosphaera*, and *Acidianus*) can convert insoluble metals into a soluble state, showing potential application in biomining operations at laboratory and industrial scales [6–9]. Members of *Acidianus*, such as *Acidianus manzaensis*, have been employed on an industrial scale for in situ remediation of metal contamination of acid mine drainages [8].

On the other hand, the metabolism of *Sulfolobus (e.g., S. acidocaldarius* and *S. solfataricus)*, especially sugar transport and degradation pathways of hexoses and pentoses, are well understood, and many of the involved pathways have been characterised [2,10]. *Sulfolobus* exhibits a broad substrate spectrum that includes D-glucose, D-xylose, L-arabinose, starch, dextrin, saccharose, tryptone, NZ amine, single amino acids and cellulose [2,11]. Interestingly, *S. acidocaldarius* was reported to co-utilise D-glucose and D-xylose with no diauxic effects [12]. *S. acidocaldarius* is the most widely used model organism due to the availability of its genome sequence [13,14] coupled with the development of a powerful genetic toolbox enabling detailed study of gene functions [15,16]. *S. acidocaldarius* is an obligately aerobic organism growing optimally under extreme conditions of low pH values (2.0–3.5)

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and high temperatures (75°C to 80°C). This organism is genetically tractable [3,17], enabling metabolic engineering for potential applications in industrial processes [1,3,18]. Furthermore, the advent of defined medium (e.g. Vienna Defined Medium) has opened the door for batch (shake flask) and continuous (stirred-tank reactor) cultivations of *S. acidocaldarius* [14,19], thus enabling the acquisition of reliable and reproducible process data [14,20] capable of supporting biotechnological applications.

However, genome sequences of members of thermoacidophilic Archaea have opened prospects for transcriptomics, proteomics, metabolomics, systems biology and metagenomics [5,21–27], and provided further insights into life in hot acid [5]. In the past decade, significant progress has been recorded in archaeal genetic studies, which has led to the development of efficient host-vector systems and novel and conventional genetic engineering methods for several archaeal models [28–31]. Nonetheless, the three members of *Sulfolobus* (*Sulfolobus islandicus*, *Sulfolobus acidocaldarius*, and *Sulfolobus solfataricus*) remain the only genetically tractable members of the phylum *Crenarchaeota* [31].

Several review articles have previously addressed aspects of this topic [4,18,32–34], primarily focused on glycolytic enzymes and proteins from thermoacidophilic Archaea with potential industrial application, including a perspective on the potential of *Sulfolobus* in biorefinery. In contrast, the current review offers a distinct and timely contribution by emphasising the recent advances in *Sulfolobus* cultivation strategies and application of thermoacidophilic archaeal whole-cell systems in lignocellulosic biorefinery, which were not comprehensively addressed in previous reviews. Thus, the present review involved the synthesis of various literature offering a fresh perspective, covering the most recent literature up to 2024, and capturing significant advances in lignocellulose degradation and shifts in the field that earlier reviews do not reflect. Together, these elements make this review a timely and original contribution that will be of broad interest to researchers, practitioners, and policymakers in bioenergy.

2. Native lifestyle of thermoacidophilic *Crenarchaeota*

The thermoacidophilic *Crenarchaeota* thermoacidophiles from the crenarchaeal phylum are composed of three major clades, spanning three orders: Acidilobales, Sulfolobales and Thermoproteales (Fig. 1). However, the Sulfolobales are the most studied archaeal lineage (over 30 named species, across seven genera and >20 distinct genomes), while the other two lineages, Acidilobales (containing Caldisphaera and Acidilobus) and Thermoproteales (only the Caldivirga are thermoacidophiles), include only a few named, characterised strains [4]. Sulfolobales were one of the first archaeal lineages discovered by Thomas Brock from his excursions to Yellowstone in the 1960s [35]. In the past decades, several interesting microorganisms have emerged from terrestrial hot springs all over the world, representing the seven named genera from the order: *Acidianus*, Metallosphaera, *Saccharolobus*, *Stygiolobus*, *Sulfodiicoccus*, *Sulfolobus*, *Sulfuracidifex* and *Sulfurisphaera* [36]. These comprised organisms with a wide array of physiological traits, ranging from extreme to moderate acidophily (pH 0.7–4.5), thermophily (65–88°C), obligate and facultative aerobes, obligate anaerobes, metal oxidisers, sulfur reducers/oxidisers, chemoheterotrophs and chemolithoautotrophs [4].

Members of the genus *Acidianus* grow anaerobically, reducing sulfur in its various forms, or aerobically, oxidising sulfur [37]. These include *Acidianus sulfidivorans* - the most acidophilic member (pH optimum ~ 0.7) and *Acidianus infernus* - the most thermophilic member (Temperature optimum ≈ 88°C) [4,37,38]. Several members of Sulfolobales have metal biooxidation capabilities [39,40], including *Acidianus ambivalens*, which has long been used as a model organism to study sulfur biotransformation within the Sulfolobales [4,41–44]. However, *Metallosphaera* (*Metallosphaera sedula* and *Metallosphaera yellowstonensis*) are model systems for metal biooxidation by extremely thermoacidophilic Archaea [45,46]. Besides, *M. sedula* has also been studied for autotrophy catalysed by the 3-hydroxypropionate/4-hydroxybutyrate cycle [47], which has shown promise for metabolic engineering of biosynthetic pathways [4,33,48,49].

The metabolism of the genus *Saccharolobus* depends more on sugar catabolism, unlike other genera of the Sulfolobales, which rely on lithotrophic pathways [4,50]. Members of the genus *Saccharolobus* are predominantly aerobic, thermophilic (optimum temperature $\geq$ 80°C) and acidophilic (pH optimum $\geq$ 3.0) [4,11,50,51]. *Sulfolobus acidocaldarius*, a member of the *Sulfolobus,* has a limited range of carbohydrate utilisation. *S. acidocaldarius* grows optimally at 75°C and pH 3.0, using only amino acids, sucrose, dextrin and starch [11]. This Archaeon was originally thought to oxidise sulfur in the sulfur-rich pools of Yellowstone National Park [35]. Still, the sulfur biooxidation capacity in strains currently available from culture collections is limited. Nevertheless, sulfur oxidation capability in *S. acidocaldarius* DSM 639 can be restored by inserting genes encoding sulfur oxygenase reductase (SOR) and thiosulfate:quinone oxidoreductase (TQO) [52]. *S. acidocaldarius* has a tractable genetic platform to understand the physiological features of the Sulfolobales, such as pili structure controlling motility [53], UV-stress response [15], biofilm formation [54], and cellular division [55]. Currently, *S. acidocaldarius*, *Sulfolobus solfataricus* (renamed *Saccharolobus solfataricus)* and *Sulfolobus islandicus* (renamed *Saccharolobus islandicus*) are the only members of Sulfolobales with tractable genetic systems [33].

Members of Sulfolobales can grow heterotrophically, using a variety of substrates as carbon and energy sources. Some organisms are facultative heterotrophs that can grow autotrophically by sulfur, metal or hydrogen oxidation. Furthermore, the heterotrophic lifestyle of crenarchaeal Sulfolobales has been studied extensively using *S. solfataricus* and *S. acidocaldarius* as model organisms for metabolic network reconstruction [4]. *S. solfataricus* exhibits high metabolic versatility and can utilise a wide range of substrates, including monosaccharides (e.g., D-glucose, D-galactose, L-fucose, D-fructose, D/L-arabinose and D-xylose), disaccharides (e.g. cellobiose, maltose, sucrose, trehalose and lactose), oligo- and polysaccharides (e.g., β-glucans, starch and dextrin), amino acids (e.g., glutamate), peptides and proteinaceous substrates (e.g. tryptone), and alcohols including aromatics (e.g., ethanol, phenol) and organic acids (e.g., formic acid) [2,18,56]. Conversely, the metabolism of *S. acidocaldarius* is limited to a narrower spectrum of substrates that include D-glucose, L-arabinose, D-xylose, sucrose, maltotriose, dextrin, starch, wheat bran, several fatty acids, peptides and amino acids [11,57].

Sulfolobales often thrive in harsh environments with limited availability of organic carbon; thus, many species within this order depend on the autotrophic fixation of CO_2 to support growth. Currently, six mechanisms for CO_2 fixation are known throughout the domains of life. Several of these mechanisms build carbon-carbon bonds by fixing CO_2 using oxygen-sensitive carboxylases, or in the case of the Calvin-Bassham-Benson cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) [58]. However, the autotrophic pathway in the Sulfolobales avoids the dependence on RuBisCo by incorporating bicarbonate molecules rather than CO_2 [59]. This pathway (Fig. 2), named the 3-hydropropionate/4-hydroxybutyrate (3-HP/4-HB) cycle, is highly conserved within the Sulfolobales [47]. However, *S. acidocaldarius* is the only member of Sulfolobales that lacks the capacity for CO_2 fixation; hence, *S. acidocaldarius* DSM 639 - a commonly used lab strain is considered a strict heterotroph [52]. Members of the Sulfolobales tend to rely on metals and inorganic compounds that exist in their natural environments to maintain their autotrophic lifestyle [4]. Chemolithoautotrophs can support iron oxidation to drive the electron transport chain despite a positive reduction potential (+0.77 V for Fe^{3+}/Fe^{2+}) [60].

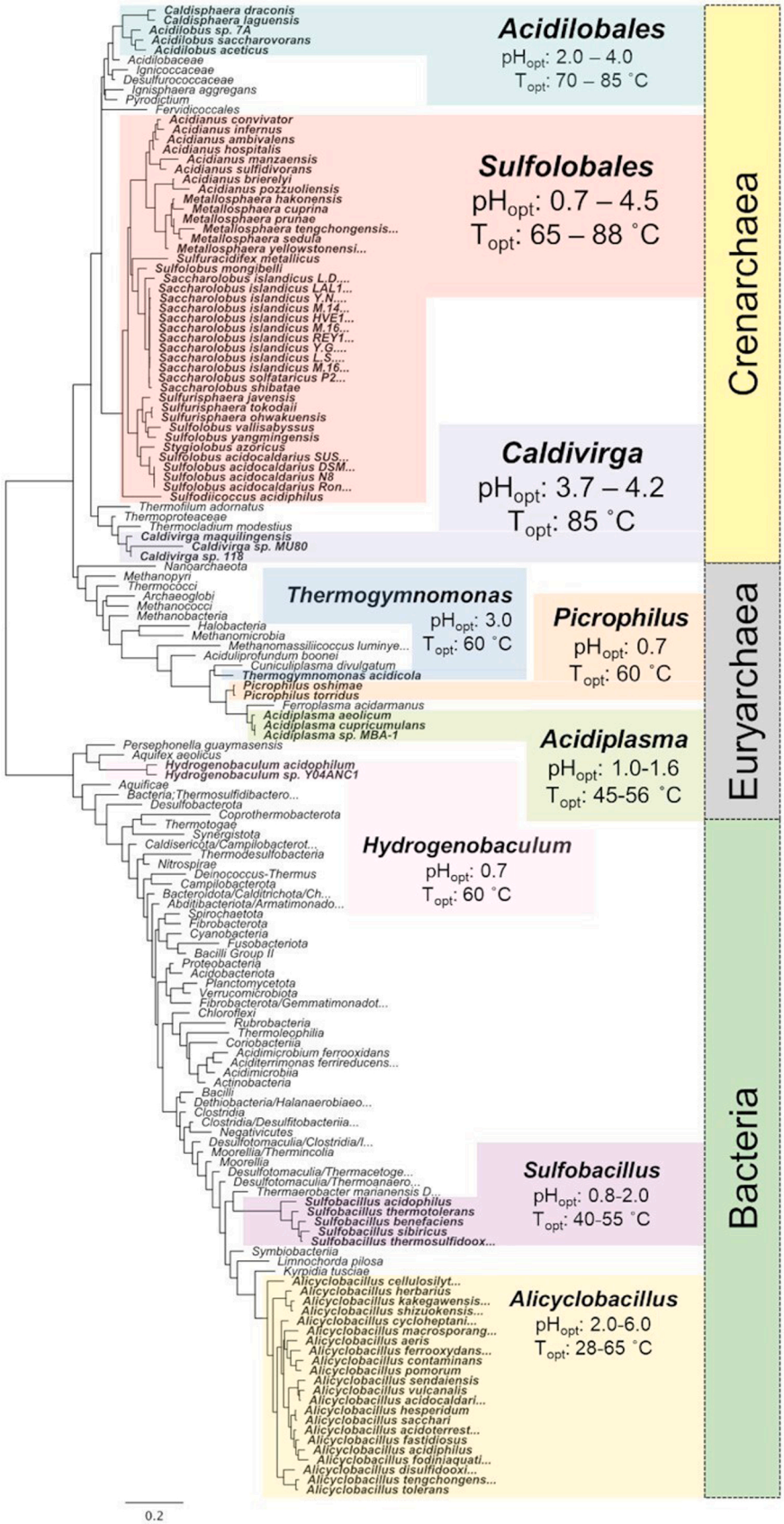

Fig. 1. 16S phylogeny tree of thermoacidophilic organisms.

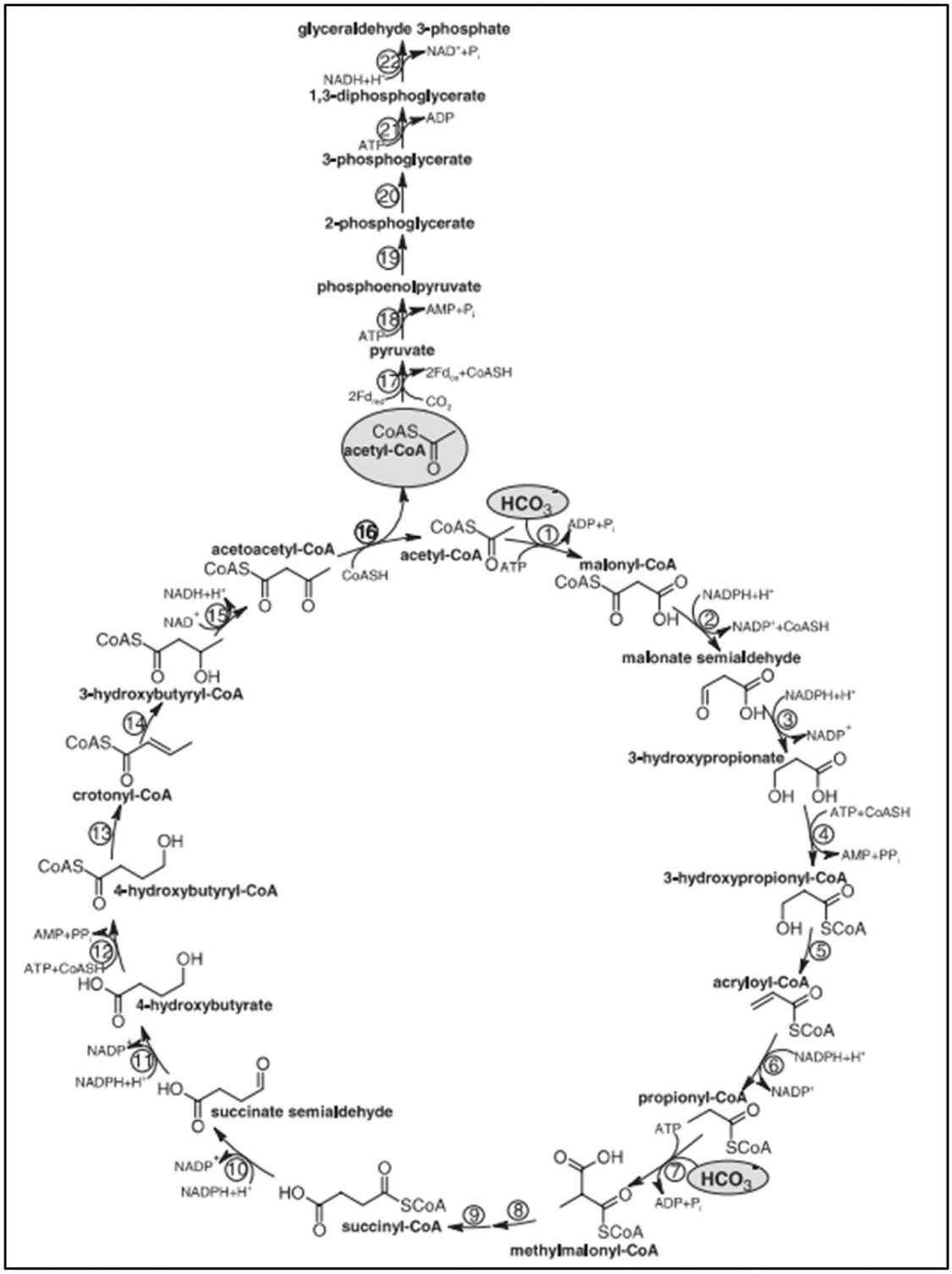

Fig. 2. Proposed autotrophic 3-hydroxypropionate/4-hydroxybutyrate cycle in *M. sedula*. Reactions of the cycle are indicated. Enzymes: 1, acetyl-CoA carboxylase; 2, malonyl-CoA reductase (NADPH); 3, malonate semialdehyde reductase (NADPH); 4, 3-hydroxypropionyl-CoA synthetase (AMP-forming); 5, 3- hydroxypropionyl-CoA dehydratase; 6, acryloyl-CoA reductase (NADPH); 7, propionyl-CoA carboxylase; 8, methylmalonyl-CoA epimerase; 9, methylmalonyl-CoA mutase; 10, succinyl-CoA reductase (NADPH); 11, succinate semialdehyde reductase (NADPH); 12, 4-hydroxybutyryl-CoA synthetase (AMP-forming); 13, 4- hydroxybutyryl-CoA dehydratase; 14, crotonyl-CoA hydratase; 15, 3-hydroxybutyryl-CoA dehydrogenase (NAD+); 16, acetoacetyl-CoA b-ketothiolase. The proposed pathway of glyceraldehyde 3-phosphate synthesis from acetyl-CoA and CO2 is also indicated. Enzymes: 17, pyruvate synthase; 18, pyruvate, water dikinase [phosphoenolpyruvate (PEP) synthase]; 19, enolase; 20, phosphoglycerate mutase; 21, 3- phosphoglycerate kinase; 22, glyceraldehyde 3-phosphate dehydrogenase. The activities of pyruvate synthase and PEP synthase at 75°C were 10–25 nmol min^{-1} mg^{-1} protein (6) and 25 nmol min^{-1} mg^{-1} protein, respectively. Adapted from Berg et al. [47] with a permission.

3. Biotechnological potential of thermoacidophilic Archaea

3.1. Enzyme production

The members of the genus *Sulfolobus* (e.g., *S. acidocaldarius*, *S. islandicus* and *S. solfataricus*) are all potential host 'chassis' on which to build biosynthetic designs of increasing complexity, albeit the genetic stability of *S. acidocaldarius* might be an advantage [4]. In addition, the extreme thermoacidophilic nature of *Sulfolobus* offers many clear advantages for industrial biotechnology applications due to reduced risk of contamination, higher substrate solubility, thermostable enzymes, reduced energy, and process cost [1,2,61,62], as well as flexibility in the utilisation of many different carbon sources. The ease of cultivation of these organisms under aerobic conditions on many different substrates is also an important benchmark for metabolic engineering and biotechnological applications [2].

The *N*-glycans in Sulfolobales (e.g., *S. acidocaldarius*, *S. solfataricus*, and *S. shibatae)* are composed of a variety of different sugar residues (e.g., glucose, mannose, rhamnose, glucuronic acid, iduronic acid, *N*-acetylgalactosamine, *N*-acetylglucosamine, galactofuranose, sulfoquinovose and galactouronic acid), allowing post-translational modification of proteins in these organisms [63–65]. Post-translational protein modifications improve protein structure, stability, and functions, allowing the application of archaeal enzymes to a broad spectrum of biotechnological and industrial processes [64].

The cytosolic enzymes of *Sulfolobus* are adapted to high temperature and neutral pH, whereas their extracellular enzymes, such as amylases, cellulases and lipases, are also adapted to low pH; hence, these properties are consistent with process designs used in lignocellulosic biomass pre-treatments that involve high temperatures and low pH [4]. Therefore, thermoacidophiles and their enzymes offer certain benefits for industrial bioprocesses because at high temperature, reaction rates and substrate (e.g., starch and lignocellulosic carbohydrates) accessibility increase, thereby enhancing biomass conversion [1,33,66].

Sulfolobus enzymes have been used for biocatalysis, multi-enzyme cascades, and metabolic engineering/synthetic biology approaches [2]. The dihydroxy-acid dehydratase (DHAD) of *S. solfataricus* with broad substrate specificity was successfully used for the conversion of carbohydrates into chemical building blocks (e.g., 2-keto-3-desoxygluconate (KDG), 2-ketovalerate and pyruvate) [67]. As depicted in Fig. 3, DHAD, together with glucose dehydrogenase from *S. solfataricus* and 2-keto-3-desoxy(6-phospho)gluconate aldolase from *S. acidocaldarius*, allowed the establishment of an artificial enzyme cascade for the cell-free production of ethanol and isobutanol from glucose [2,68]. Furthermore, in vitro enzymatic synthesis of myo-inositol and α-glucose 1-phosphate from starch has been performed using the isoamylase from *S. tokodaii* [68,69], and this enzyme has also been used to establish a starch biobattery [70]. The phosphotriesterase-like lactonases from *S. acidocaldarius* and *S. solfataricus* are active in the decontamination of organophosphate pesticides and warfare agents, hence are considered sustainable tools for bioremediation and as bioscavengers [71]. More recently, activity-based protein profiling (ABPP) has been established as a novel methodology for in vivo enzyme identification and screening of members of the serine hydrolase family in extremophilic Archaea with a special focus on esterases in *S. acidocaldarius* [2,72,73]. In addition to proteins, *Sulfolobus* lipids have gained increasing interest due to their stability, e.g., against bile salts and liposomes, which are proposed as suitable vehicles for drug delivery [74]. Table 1 shows the various archaeal extremozymes that have attracted interest in biotechnological applications.

3.2. Biomining applications of thermoacidophilic Archaea

Bioleaching is a well-established industrial and laboratory-scale operation that involves mobilising base, precious, and strategic metals from mineral ores by acidophilic microbes and significantly contributes to mining operations [75–83]. Extreme thermoacidophiles belonging to *Acidianus*, *Metallosphaera* and *Sulfolobus* with innate capabilities to oxidise ferrous iron and/or reduced inorganic sulfur compounds (RISCs) have gained recognition for their utility in bioleaching [7,84,85]. These

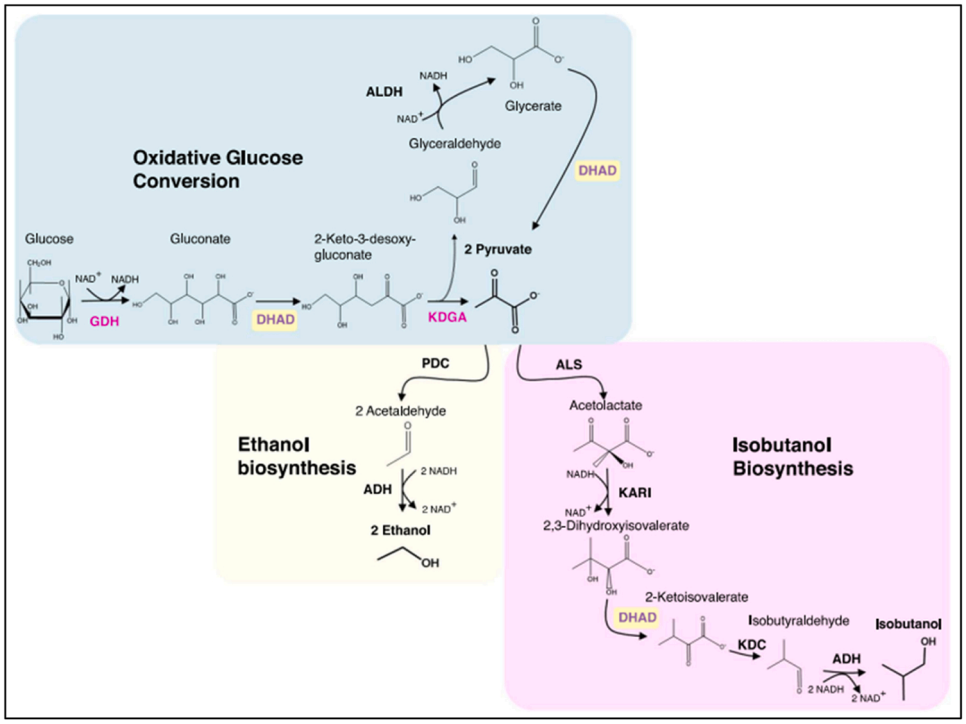

Fig. 3. Artificial enzyme cascade for bio-alcohol production. In particular, the *S. solfataricus* dihydroxyacid dehydratase (DHAD, highlighted in purple with yellow boxes) with broad substrate specificity catalyzes three different steps in the synthetic cascades for ethanol and isobutanol production. Adapted from Schocke et al. [2] with a permission.

Table 1
Enzymes from the genus *Sulfolobus*.

Microorganism	Enzyme	Reference
S. solfataricus	Dihydroxy-acid dehydratase	[67]
S. solfataricus	Glucose dehydrogenase	[2]
S. acidocaldarius	2-keto−3-desoxy(6-phopho)gluconate aldolase	[2,68]
S. tokodaii	Isoamylase	[2,69]
S. solfataricus, S. acidocaldarius	Phosphotriesterase-like lactonases	[71]
S. shibatae	Esterase	[73]
S. acidocaldarius, S. solfataricus	Serine hydrolases	[2,72,73]
S. solfataricus	Xylanase and β-xylosidase	[65]
S. solfataricus	β-glucosidase	[65]

extreme thermoacidophiles have demonstrated versatility in utilising different energy substrate sources for growth [7,86]. Extreme thermoacidophiles can grow mixotrophically by simultaneously utilising yeast extracts and sulfide minerals, Fe^{2+} or RISCs. Therefore, the metabolic versatility of extreme thermoacidophiles means that various energy sources can be adopted for their propagation and then used to inoculate low-grade chalcopyrite ore heaps [87].

Microbial metal mobilisation from sulfides and oxides occurs indirectly [81]. In this process, Fe^{3+} abiotically oxidises the solid material, generating Fe^{2+}, which is subsequently re-oxidised by microbes back to Fe^{3+}, which resembles magnetosome biomineralisation in magnetotactic bacteria [88]. However, most members of well-characterised Sulfolobales cannot oxidise ferrous iron [Fe^{2+}] to ferric iron [Fe^{3+}], which is vital for many types of bioleaching, despite many species subsisting in inorganic chemical-laden environments [81]. The thiosulfate and polysulfide mechanisms are the two different mechanisms proposed for bioleaching various sulfide minerals based on the differences in intermediary sulfur compounds formed [4,76]. Fig. 4A shows sulfur reduction at the surface of the cell membrane by the membrane-bound sulfur reductase. Sulfur reduction occurs through electron transfer from reduced quinols (e.g., *Sulfolobus* quinol). This complex linking hydrogen oxidation with quinone cycling regenerates the oxidised quinone pool and powers the proton export. As shown in Fig. 4B, sulfur oxidation occurs in the cytoplasm by cycling through several reduced inorganic compounds (RISCs). The oxygenase reductase disproportionates zero-valent sulfur into H_2S and sulfite (SO_3^{2-}) without the assistance of any cofactors. The two products (H_2S and SO_3^{2-}) react abiotically to generate thiosulfate ($S_2O_3^{2-}$) as a by-product.

Pyrite, the most abundant and widely distributed metal sulfide, is dissolved through the thiosulfate mechanism, while chalcopyrite, a recalcitrant primary sulfide, is dissolved through the polysulfide mechanism [7,76]. A variety of sulfide minerals commonly coexist with low-grade chalcopyrite ore in bioleaching heaps; hence, acidophiles residing inside the heap should be flexible to adjust their metabolic pathways to obtain energy through the biooxidation of different energy sources [76]. The exothermic nature of biooxidation of sulfide ores means that the temperature inside the low-grade chalcopyrite ore heap would increase gradually during bioleaching processes [89,90]. The temperature inside large ore heaps can finally reach 60–80°C, as indicated in the copper biomining industry [7,91], hence such high temperature inhibits mesophiles and moderate thermoacidophiles. However, in whole-cell assays, the complete elimination of Fe^{3+} as a mediator is usually impossible due to the presence of trace amounts of iron, hence the difficulty in differentiating between direct biological oxidation and Fe^{3+}-mediated abiotic oxidation of the metal [82,92]. The various metals mobilised by the members of thermoacidophilic Archaea are shown in Table 2.

3.2.1. *Sulfolobus*

Members of Sulfolobales such as *Sulfolobus metallicus* [40] and *Sulfolobus acidocaldarius* [35] mobilise copper, zinc, and uranium [9,93]. Additionally, these species contribute to coal desulfurisation, offering potential strategies for reducing sulfur content in fossil fuels [94–96]. Another key biogeochemical role of *Sulfolobus* is its ability to oxidise arsenite (As^{3+}) to arsenate (As^{5+}), which is relevant for arsenic

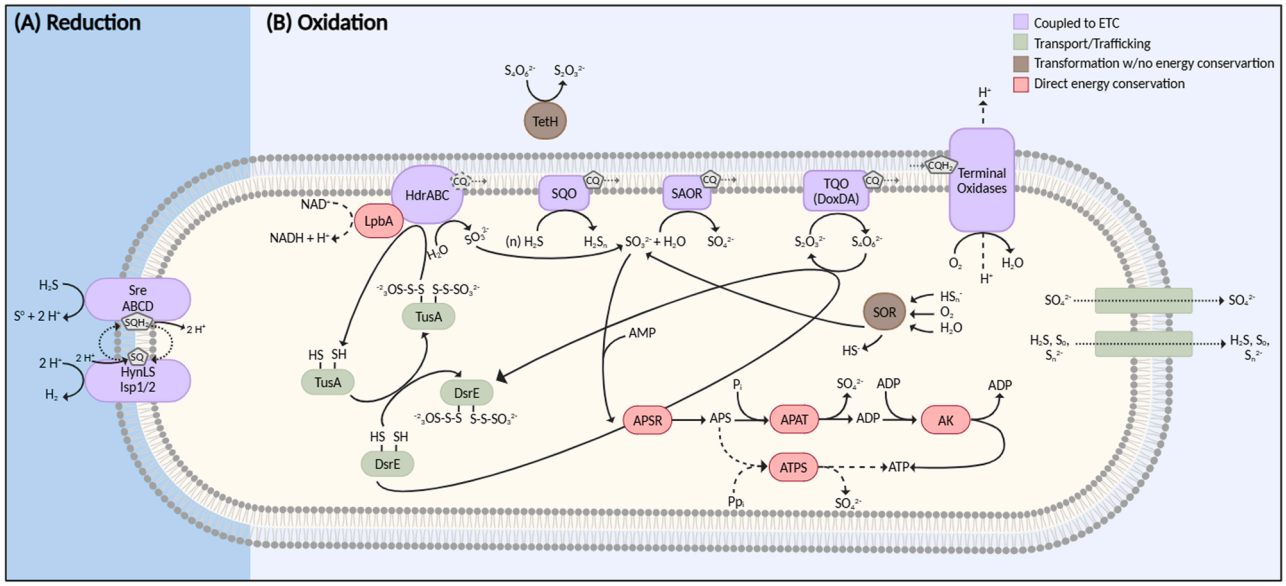

Fig. 4. Current knowledge of the mechanism of sulfur oxidation and reduction in the Sulfolobales. (A) Sulfur reduction; (B) sulfur oxidation. Solid arrows indicate participation in a reaction; dotted arrows represent transport of species; dashed lines show that the function is suspected but has not been demonstrated experimentally in the Sulfolobales. Enzyme colors indicate general grouping of function: coupled to electron transport chain (blue), implicated in transporting or trafficking sulfur species (yellow), transformation of sulfur species with no energy conservation (orange) and transformation of sulfur species directly coupled to energy-conserving biomolecules (green). Abbreviations: sulfur reductase (Sre), hydrogenase (Hyn), heterodisulfide reductase (Hdr), tetrathionate hydrolase (TetH), sulfide:quinone oxidoreductase (SQO), sulfite:acceptor oxidoreductase (SAOR), thiosulfate:quinone oxidoreductase (TQO), sulfur oxygenase reductase (SOR), adenosine-5 -phosphosulfate reductase (APSR), adenosine-5 -phosphosulfate (APS), adenylylsulfate:phosphate adenylyltransferase (APAT), ATP sulfurylase (ATPS), adenylate kinase (AK).

Table 2
Bioleaching capabilities of thermoacidophilic Archaea on various substrates.

Organism	Substrate	Metal/ion extracted	Reference
A. manzaensis YN25	$Cd(NO_3)_2{\cdot}4\ H_2O$ $CuSO_4{\cdot}5\ H_2OZn$ $(NO_3)_2{\cdot}6\ H_2ONi\ (NO_3)_2{\cdot}6\ H_2O$	Cd^{2+} Cu^{2+} Zn^{2+} Ni^{2+}	[8]
M. sedula DSM 5348	Chalcopyrite ($CuFeS_2$)	Cu^{2+}, Fe^{3+}	[7]
M. sedula	scheelite ($CaWO_4$)	Tungsten (W)	[79]
M. sedula	pyrite (FeS_2)	Fe^{3+}	[179]
S. metallicus	CuFeS2	Fe^{3+}, Cu^{2+}	[180]
S. metallicus	BOF-dust particles	Mn, Zn, Cu, & Fe	[9]
A. manzaensis	BOF-dust particles	Mn, Cd, Cr, Zn, Ni, & Cu	[9]
A. brierleyi	BOF-dust particles	Fe, Pb, & Mn	[9]
M. hakonensis	BOF-dust particles	Mn, Zn, Cu, Fe, Cd, & Ni	[9]
S. acidocaldarius	BOF-dust particles	Mn, Zn, Cu, Fe, Cd, Pb & Ni	[9]
M. sedula	V_2O_3, MoO_2	V, Mo	[82]
M. prunae	V_2O_3, MoO_2	V, Mo	[82]

detoxification and bioremediation [97,98]. *S. acidocaldarius* is capable of chemolithotrophic growth in the presence of a multimetallic waste product (e.g., BOF-basic oxygen furnace-dust) and solubilises Mn, Zn, and Fe into the leachate solution [9,99].

3.2.2. Metallosphaera

Metallosphaera strains could solubilise metals by attaching to their respective substrates and regenerating Fe^{2+} to Fe^{3+}, which is vital for chemical attack on minerals [9,80,100]. *Metallosphaera* are capable of catalysing both direct and indirect metal oxidation, possibly for bioenergetic benefit or to detoxify their microenvironment. The ferric iron regenerated in leachate by *Metallosphaera sedula* through its ferrous oxidation pathways was found to be essential for the dissolution of chalcopyrite via the 'indirect mechanism' [21,87]. Bioleaching acidophiles with ferrous iron oxidation capability would decrease the concentration of Fe^{2+} while simultaneously increasing the concentration of Fe^{3+} in leachate during bioleaching. *M. sedula* inocula propagated with typical energy substrates had different chalcopyrite bioleaching capabilities during subsequent chalcopyrite bioleaching, which was probably ascribed to their different capacities for ferrous iron and sulfur oxidation.

Mixotrophically propagated extreme thermoacidophile *M. sedula* with typical energy substrate sources (i.e., yeast extract, sulfur, pyrite or chalcopyrite) and subsequent adsorption to chalcopyrite and chalcopyrite bioleaching in shake flasks showed differences in the chalcopyrite bioleaching capabilities [7]. Furthermore, the acidity of the mixotrophic growth medium increased with the oxidation of elemental sulfur, pyrite or chalcopyrite by *M. sedula* DSM 5348. In contrast, the acidity of the heterotrophic growth medium with yeast extract was almost constant. The planktonic cell viabilities of *M. sedula* inocula were affected by different energy substrates (i.e., yeast extract, sulfur, pyrite or chalcopyrite). Cell density of heterotrophically grown cells was relatively higher than that of mixotrophically grown cells. Thus, the low growth rate observed in *M. sedula* inocula grown with chalcopyrite was probably connected to the mass of iron and sulfur elements contained in ores, which were much lower than that contained in sulfur and pyrite supplemented cultures with the same amount of substrates used [7]. The highly stable crystal structure of chalcopyrite limits the rate of dissolution of Fe^{2+} and intermediary reduced inorganic sulfur compounds (RISCs) during bioleaching [87], hence the lowest growth rate of inoculum propagated mixotrophically with chalcopyrite. Interestingly, *M. sedula* inoculum grown with chalcopyrite had the highest copper recovery yield throughout the bioleaching process [7]. This is because chalcopyrite is soluble under proton attack in acid [101].

However, to achieve the highest bioleaching rate and copper recovery yield, chalcopyrite-propagated *M. sedula* inoculum should be adopted to inoculate the heap of chalcopyrite. This would be extrapolated to other extremely thermoacidophilic organisms, ascribing to these highly conserved bioleaching-associated pathways [7]. However, *M. sedula* cells can biotransform calcium-tungstate mineral scheelite by leaching mineral-bound tungsten into the surrounding, resulting in tungsten carbide layers on the cell surface [79]. Precious metals from genuine extraterrestrial materials such as meteorites (ordinary chondrite NWA 1172; Martian breccia NWA 7034) are also solubilised by *M. sedula*, thus suggesting the possibilities for future asteroid biomining [102]. Wheaton et al. [82] assessed the capacity of *M. sedula* to solubilise various metal oxides (V_2O_3, Cu_2O, FeO, MnO, CoO, SnO, MoO_2, Cr_2O_3, Ti_2O_3, and Rh_2O_3) under chemolithoautotrophic growth conditions. They observed significant increases in V_2O_3 and MoO_2 solubilisation in the presence of *M. sedula* compared to the abiotic control, whereas no significant increase in solubilisation was observed for other metals. On the other hand, solubilisation of V_2O_3 occurred abiotically, but the presence of *M. sedula* remarkably accelerated the rate. Furthermore, both viable and whole-cell extracts of *M. sedula* oxidised V to V^{5+}, while no oxidation occurred with cell extracts, which demonstrates a key role of cell membranes in the oxidation process. The higher solubilisation of V compared to Mo indicates that MoO_2 is either more recalcitrant than V_2O_3 or its oxidation involves a different mechanism. Thus, the authors concluded that the presence of Fe impacted V and Mo solubilisation and stimulated *M. sedula* metabolism by increasing O_2 consumption rates.

Interestingly, *M. sedula* was previously reported as the most efficient metal mobiliser among *Metallosphaera* species [103,104]. *Metallosphaera hakonensis* has demonstrated great potential for industrial heap bioleaching [105] due to high Fe^{2+} oxidation rates when grown on inorganic sulfur compounds [9,100,106]. However, *M. hakonensis* demonstrated poor bioleaching performance on multimetallic waste products (basic oxygen furnace-dust particles), which could be due to the presence of silica precipitated by the acidic pH of the surrounding medium that could limit the release of metals from dust grains [107]. This phenomenon could apply to other thermoacidophilic strains [108].

3.2.3. Acidianus

Due to the exothermic nature of bioleaching processes, extreme thermophiles such as *Acidianus brierleyi* proved themselves intrinsically suitable for high-temperature operations [91]. *A. brierleyi* cells propagate chemolithotrophically by S^0 or Fe^{2+} redox chemistry [37] and provide a higher sphalerite (CuFeS2) leaching rate compared to a mesophilic bacterium, *Acidithiobacillus ferrooxidans* culture [9,109,110]. Moreover, *Acidianus brierleyi* is capable of oxidising arsenite [98]. *Acidianus manzaensis* YN25, an extremely thermo-acidophilic archaeon, has potential for chalcopyrite and pyrite bioleaching [9,111,112]. Li and co-workers [8] studied the effects of pH, temperature and metal ion concentration on biosorption behaviours of *A. manzaensis* YN25 towards metal ions of Cd^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+} in acidic solution imitating acid mine drainages (AMD). However, biosorption behaviors of metal ions (Cd^{2+}, Cu^{2+}, Zn^{2+} and Ni2 +) by *A. manzaensis* YN25 varied under different pH, temperature and metal ion concentration conditions [8]. *A. manzaensis* YN25 showed a four-fold adsorption capacity for Cu^{2+} and three-fold adsorption capacity for Ni^{2+}, Cd^{2+} and Zn^{2+} respectively, which were in good agreement with the order of adsorption capacity in the single system ($Cu^{2+} > Ni^{2+} > Cd^{2+} > Zn^{2+}$).

Moreover, the metal with the best biosorption ability in a single-metal system exhibited the most significant limiting effect for other metal ions in multiple-metal coexisting systems [8,113]. Thus, Cu^{2+} showed the highest adsorption affinity in the coexisting systems of multiple metals. Metals biosorption correlated with the protonation/deprotonation of functional groups on the cell surface. However, the cell surface of *A. manzaensis* YN25 exhibited electronegativity before adsorption, implying its ability to sequestrate positive metal ions due to effective electrostatic attraction [8]. Biosorption is identified as an

energy-dependent process and could be affected by changes in temperature [8]. The metal adsorption abilities of *A. manzaensis* increase with an increase in temperature, enhanced YN25 (15°C to 75°C), with 60°C being the optimum growth temperature [114]. In comparison, lower temperatures (below 45°C) generally result in low sorption capacity due to the decrease in the membrane fluidity [115] and the inactivation of various enzyme activities [116]. Conversely, high temperature can accelerate the solute's kinetic energy and surface activity, thereby increasing sorption efficiency [112,117].

Kölbl et al. [9] studied bioleaching activity of *A. manzaensis* on basic oxygen furnace (BOF)-dust particles. Among the parameters (BOF-dust load, pH and temperature) assessed during *A. manzaensis* bioleaching operations, an increase in BOF-dust load led to high release of Fe, Zn, Mn, Mg, Cr and Cd, while the release of Ca, Al, Ni, Co, V, Ti, Pb and Mo were not affected by the increase in the load of BOF-dust particles. On the other hand, at low pH (1.5) *A. manzaensis* cells efficiently mobilised Fe, Ni, V, Ti, and most notably Cr, whereas a higher pH (2.5) was found to support the release of Zn, Mn, Cu, Cd, and Co. Slight temperature shifts (62°C/64°C/67°C) did not significantly affect bioleaching performance of *A. manzaensis*, which was in agreement with previous studies [118,119]. In addition, *A. manzaensis* efficiently leached important elements (Cu, Ni, Zn, Cr, Cd, and Mn) from BOF-dust particles. Moreover, the formation of a jarosite (KFe3(SO4)2(OH)6)passivation layer by *A. manzaensis* during leaching processes was similar to those previously reported from chalcopyrite bioleaching operations [119].

However, there are challenges to overcome to fully realise the biotechnological potential of thermoacidophilic Archaea as industrial microorganisms. For instance, central metabolic pathways (e.g., for lipid or glycerol degradation) are still not well understood. Thus, further work is needed to unravel the metabolic complexity of promising representatives of the Sulfolobales, for example, network regulation at the gene and protein levels requires further fundamental research [4]. Notably, only one patent (patent no.: WO2020187526A1) "Method for producing a composition comprising archaeal lipids from a *Sulfolobus* cell culture" is currently available on whole-cell application of *Sulfolobus*. Despite the vast potential of using extreme thermoacidophilic Archaea for biomining applications, challenges remain due to trace amounts of iron in the low-grade chalcopyrite ore heaps, making it difficult to differentiate between direct biological oxidation and Fe^{3+}-mediated abiotic oxidation. Additionally, the presence of silica precipitated by the acidic pH of the surrounding medium can limit the application of these organisms for biomining exploitation [9].

4. Genetic tool development

The first step towards developing a genetic system for a microorganism is to establish an effective means of introducing foreign DNA into the cell, a process called transformation [120]. In the study on the infectivity of SSV1 (the first Sulfolobus spindle virus), Schleper et al. [121] tested the introduction of viral DNA into the foreign host *S. solfataricus* P1 cells by electroporation. They found that electroporation introduces SSV1 DNA into *S. solfataricus* cells at high transformation efficiency (>106 plaque-forming units (pfu) per microgram of DNA) under certain conditions. These electroporation conditions were subsequently tested for plasmid transformation on a few strains belonging to *S. solfataricus*, *S. islandicus*, and *S. acidocaldarius*, and high transformation efficiency was obtained in all the tested strains except for *S. acidocaldarius* [122–125]. The use of methylated plasmid DNAs increases the transformation rates [122]. More recently, a restriction-deficient strain has been constructed and used as a host for genetic analysis, and thus, much higher transformation rates have been obtained for *S. acidocaldarius* [32,126]. Electroporation is an efficient method for incorporating foreign genetic elements (e.g., SSV1 DNA) into the host genome, thus paving the way for genetic manipulation and vector development in extreme thermoacidophilic Archaea.

The cryptic plasmids pRN1 and pRN2 were used to construct *Sulfolobus-E. coli* shuttle vectors coexist in the same strain; however, the two plasmids function independently because one of the cryptic plasmids is a derivative of REN1H1, hence each plasmid can be used as a *Sulfolobus* backbone for the construction of plasmid shuttle vectors [127]. A few pRN1-based shuttle vectors have been created, and they function in *S. acidocaldarius* DSM639 and *S. solfataricus* P1 and 98–2 strains [122]. These vectors have been used to study inducible and constitutive expression [128] and recombinant protein production in *S. acidocaldarius* [129]. Furthermore, the original pRN2-based shuttle vector pHZ2 [130] comprises the *E. coli* vector pGEM-3Z, the pyrEF marker gene, and the large Sph I fragment of pRN2 [31]. However, to increase the versatility of the *Sulfolobus* genetic toolbox, additional selectable markers for the *S. islandicus* 16.4 strain were explored and among them was the agmatine/argD system, which represented the second selection system based on auxotrophy, where agmatine auxotrophy was complemented by expression of argD coding for arginine decarboxylase from a vector [131]. This principle has been used for developing a gene knockout method for *S. islandicus* M.16.4, and mutants have been constructed for two of the genes showing UV-responsive expression by the newly developed method [132](Zhang et al., 2013). The apt/6-MP (adenine phosphoribosyltransferase gene/purine analog, 6-methylpurine) system is another counter-selection method developed for *Sulfolobus,* which has enabled the construction of unmarked gene knockout strains in *Sulfolobus* [133].

Eventually, a mutant-independent selection marker has been developed for *Sulfolobus*, which was based on the hmg gene coding for the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [31]. The selection system was first established as a selection marker for euryarchaea *Thermococcus kodakaraensis* and *Pyrococcus furiosus* in Archaea [134] and was implemented in *Sulfolobus* genetics for selecting transformants containing expression shuttle plasmids [135]. Moreover, the hmg selection marker has been successfully applied for genetic manipulation of *S. islandicus* 16.4 [136], thus providing a general protocol for a gene knockout in any *Sulfolobus* strains [31]. Consequently, the apt/6-MP (6-methylpurine) counter-selection system developed by Zhang et al. [133] is suitable for studying mutation rates in *Sulfolobus* and allows the mutants based on ΔpyrEF hosts to be directly assessed for forward mutation rates. A reporter gene is an assay for testing in vivo promoter activity to identify proper promoters for protein overexpression and for genetic analysis in *Sulfolobus* [31]. Since β-glycosidase encoded by lacS has been well characterised, its activity can readily be assayed by the standard assay for the *E. coli* β-galactosidase [137]. Thus, the lacS gene has been chosen for the development of a reporter gene assay for all *Sulfolobus* models [31]. It was first tested in *S. solfataricus* using pMJ03 (SSV1-based vector) [138].

Moreover, promoter of the *Sulfolobus* chaperonin (thermosome) (tf55α) has been analysed for its ability to confer heat-shock inducible expression, and it causes > 10-fold enhancement of reporter gene expression after heat shock [138]. The reporter gene plasmid pRp contains the *Sulfolobus-E. coli* plasmid shuttle vector pZC1 [139], a fusion gene of the araS promoter, and the coding sequence of the lacS gene from *S. solfataricus*; however, the reporter gene assay has revealed several functional elements in the promoter of the *S. solfataricus* araS gene coding for an arabinose-binding protein [120]. Upon the binding of the ara-box-specific binding protein, the protein recruits TFB to the BRE element or stabilises the interaction between TFB and BRE on the araS promoter and strongly promotes the gene expression in the presence of D-arabinose [120,140].

Peng et al. [140] designed a synthetic araS promoter in which a ribosome-binding site was inserted before the transcription start site. The resulting promoter, designated araS-SD promoter, was used to construct *Sulfolobus* expression vector pSeSD. The reporter gene assay has tested the activity of the new promoter. The result revealed that the synthetic promoter drives the arabinose-inducible expression and was unexpectedly higher than the activities of the well-known strong

promoters of *S. solfataricus* alba and sso7d genes coding for crenarchaeal chromatin proteins. Currently, pSeSD is a vector widely used for genetic complementation of gene deletion mutants and protein expression in *Sulfolobus* [31]. Two gene promoters, particularly the promoter of the mal gene coding for a maltose-binding protein and that of Sac7d (coding for one of the *Sulfolobus* main chromatin proteins), have been tested using the *S. solfataricus* lacS gene as the reporter and the *Sulfolobus-E. coli* vector pC as the replication backbone [31]. This method generated two reporter plasmids, pCmalLacS and pCSac7dLacS, which revealed dextrin-inducible and constitutive expression of the reporter gene in *S. acidocaldarius*, respectively [128]. Moreover, several *S. solfataricus* proteins have been expressed in *S. acidocaldarius* using this expression plasmid constructed from the pC vector [31].

Recently, the *S. acidocaldarius* expression system was further improved upon by combining the previously established vector system with the newly identified pentose inducible promoters as well as FX cloning to construct a whole cassette of different vectors from which genes can be expressed in *S. acidocaldarius* [17]. Nowadays, *Sulfolobus* genetics has advanced to a post-CRISPR era such that gene deletion and mutated genes can be readily generated by means of endogenous CRISPR-Cas systems of DNA interference; also, CRISPR-Cas systems of RNA interference have successfully been applied to gene silencing in *S. islandicus* REY15A [4]. The same strategy can be used in another model easily because all known *Sulfolobus* organisms carry CRISPR-Cas systems of DNA and RNA interference, as other archaeal organisms do [31]. Thus, the current *Sulfolobus* genetic toolkit enables sophisticated studies on genes. Nevertheless, there is an apparent lack of a proper inducible promoter in the current genetic toolbox of *Sulfolobus* [31]. An ideal inducible promoter is a very stringent one that turns gene expression on upon induction, with little or no detectable background activity of gene expression. Future development of genetic tools should focus on identifying or generating such a promoter, as was done for constructing the synthetic araS-SD promoter, which causes powerful gene expression.

5. Biorefinery applications of thermoacidophilic Archaea

5.1. Feedstock conversion capabilities

The *Sulfolobus* genus harbours various well-known sugar transport and degradation pathways and lacks catabolite repression; hence, it can utilise hexose and pentose sugars simultaneously [2,12]. In this organism, D-glucose and D-galactose are degraded via a modified promiscuous branched Entner-Doudoroff (ED) pathway in which phosphorylation is omitted in initial enzymatic conversions, leading to 2-keto-3-deoxygluconate (KDG) as a key intermediate (Fig. 5) [2,10]. However, in *S. solfataricus* and *S. acidocaldarius*, pentoses (D-arabinose and D-xylose) degradation occurs via oxidative Weimberg and Dahms pathway leading to direct formation of the citric acid cycle intermediate a-ketoglutarate or of glycolaldehyde and pyruvate, respectively [2,141]. In contrast, D-xylose degradation in *S. acidocaldarius* strain MW001 occur only via the Weimberg pathway [65,142].

The recent developments in metabolic engineering and synthetic biology approaches enabling a powerful combination of archaeal, bacterial and eukaryotic metabolic and regulatory components have opened the way for establishing *Sulfolobus* as a platform organism for bioconversion of sustainable, inexpensive feedstocks like lignocellulosic biomass into value-added products, such as volatile products (e.g., bioalcohol) under harsh pretreatment conditions [2]. However, due to the recalcitrant nature of lignocellulosic biomass, pretreatment is a key

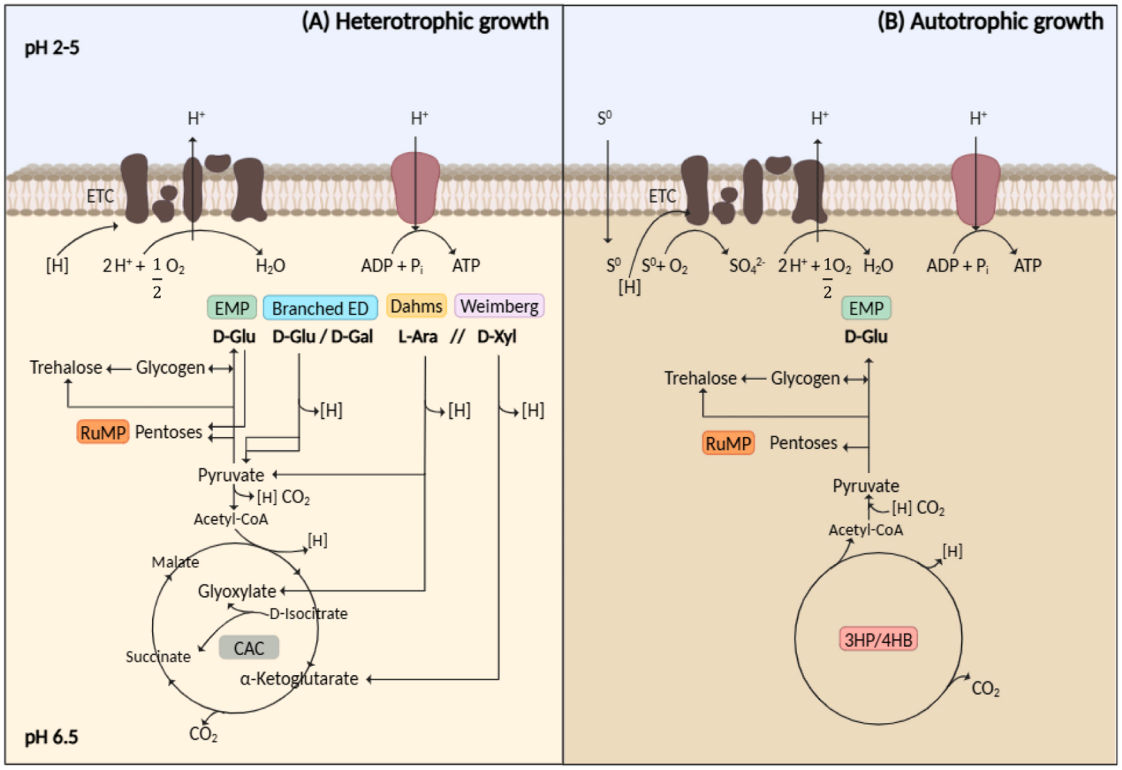

Fig. 5. Central metabolism of *Sulfolobus spp*. under heterotrophic and autotrophic growth conditions. (A) For heterotrophic growth the promiscuous, branched Entner-Doudoroff (ED) pathway is utilized for D-glucose (D-Glu) and D-galactose (D-Gal) degradation. The Embden-Meyerhof-Parnass (EMP) pathway is solely needed for gluconeogenesis. The upper catabolic part of the EMP pathway (glucose to fructose-6-phophate) supplies sugar phosphates for glycogen, trehalose, and pentose formation via the reversed ribulosemonophosphate pathway (RuMP). Pyruvate is fully oxidized to CO_2 via the citric acid cycle (CAC). Pentoses (i.e. D-xylose (D-Xyl) and L-arabinose (L-Ara)) are degraded via the Weimberg and/or Dahms pathway. Reduction analogs [H] are channeled into the aerobic electron transport chain (ETC) and the proton motive force generated is used for ATP synthesis via the archaeal type ATP; (B) Under autotrophic growth conditions, CO_2 is fixed via the 3-hydroxyproprionate/4-hydroxybutyrate cycle (3HP/4HB) and the EMP pathway is used for gluconeogenesis.

step to segregate biomass components and facilitate polysaccharide accessibility to microbial enzymes [143–145]. Several pretreatment methods (physical, physicochemical, chemical and biological) have been applied to lignocellulosic biomass [146,147]. Thus, the integration of biomass pretreatment with processes using thermoacidophilic Archaea (e.g., *S. solfataricus* and *S. acidocaldarius*) in the form of consolidated bioprocessing will further improve the production of high-value biochemicals from lignocellulosic feedstocks.

Although thermoacidophilic Archaea have not yet been engineered to the point of being primary producers of alcohols, specifically the broad substrate range of the aldehyde:ferredoxin oxidoreductase-alcohol dehydrogenase (AOR-Adh) pathway for acid reduction unfolds new possibilities to produce different alcohols [148]. Additionally, the high cultivation temperatures of archaeal thermophiles enable alcohol removal from culture supernatant [1]. As a result, the expensive distillation steps and inhibition by toxic products can be minimised, thus enabling novel designs of 'one-pot' strategies for lignocellulosic biomass processing [4,52]. Although industrial production processes using *Sulfolobus* are yet to be realised, its potential applications have already been demonstrated in several lab-scale processes [3,14,19,65].

S. acidocaldarius offers the advantage of engineering microbial metabolic pathways for efficient decomposition of hemicellulose because of its genetic stability, coupled with a well-developed genetic manipulation system without a mobile element [65]. Previous studies identified the ability of *S. acidocaldarius* to transport pentose sugars, such as xylose and arabinose, using ABC transporters, and utilising these sugars via the Weimberg and Dahms pathways [141,142]. Nevertheless, *S. acidocaldarius* cannot utilise xylan or xylooligosaccharide (XOS), whereas its closest related strain (*S. solfataricus*) can utilise both xylan and XOS using xylanase and β-xylosidase [149]. However, Lee et al. [65] introduced the genes, sso1354 and sso3032, encoding for xylanase and β-xylosidase from *S. solfataricus* P2 into the S. acidocaldarius strain. The constructed strain with heterologous genes degraded the hemicellulose component of lignocellulosic biomass and utilised the sugars as a carbon source. The enzymes had maximum activity at a temperature of 90°C and a pH of 4.0, respectively. However, the enzymes' activities were slightly different from those obtained from *Escherichia coli* (80–85°C and pH 6.5) [149] due to the difference in expression host.

However, heterologously expressed SSO1354 in *S. acidocaldarius* harbours both xylanase and cellulase activities but has inactive β-glucosidase, unlike *S. solfataricus*, which has an active β-glucosidase, hence cannot convert cellooligosaccharide (COS) into glucose [65]. Xylanase can degrade xylan to xylooligosaccharide (XOS), while β-xylosidase degrade XOS into xylose; hence, both recombinant enzymes must work synergistically to degrade xylan into xylose effectively. Thus, for *S. acidocaldarius* with the sso1354 gene to simultaneously utilise cellulosic and hemicellulosic biomasses, additional enzymes that can convert COS into glucose need to be expressed [65].

Several studies have demonstrated the potential for biorefinery application of thermoacidophilic Archaea (e.g., *Sulfolobus*) [65]. Despite the inability of *S. acidocaldarius* to simultaneously metabolise both pentose and hexose sugars, heterologous metabolic pathways have been successfully incorporated into these organisms at the lab scale, enabling them to use these sugars as energy sources. However, for industrial-scale applications utilising cheap feedstocks such as lignocellulosic biomass, maintaining these metabolic pathways in these organisms without compromising efficiency and product yield could be challenging.

5.2. Bioprocess development

Sulfolobus acidocaldarius - a thermoacidophile capable of growing optimally at pH 2.0–3.5 and 75°C - 80°C in conjunction with its obligate aerobic lifestyle is considered a promising platform organism for biotechnological applications [1–3,16,18]. However, *S. acidocaldarius* was initially described as sulfur oxidising autotroph [35], but the currently available commercial strains are strictly heterotrophs that thrive best on protein hydrolysates, sugars and single amino acids [11, 150]. *S. acidocaldarius* is genetically tractable, thus enabling metabolic engineering for potential applications in industrial processes [1,3]. Furthermore, the biofilm lifestyle of *S. acidocaldarius* allows enhanced tolerance against adverse environmental conditions such as toxic reactants or products during biotechnological processes [151]. Thus, biofilm formation generally seems to improve microbial tolerance to suboptimal environmental factors due to diverse protective mechanisms, commonly with a significant contribution of the extracellular polymeric substances (EPS) matrix [152].

Benninghoff et al. [3] investigated the ability of *S. acidocaldarius* to tolerate process-related stress conditions toward industrially relevant organic solvents. This study revealed the capacity of *S. acidocaldarius* to tolerate organic solvents (1–1.5 % v/v 1-butanol, 1–4 % v/v ethanol, 0.5–2.5 % v/v propanol, and 0.5–1.0 % v/v isobutanol). Cell aggregation and biofilm formation were reported to enhance tolerance to 1-butanol in *S. acidocaldarius*, which correlated with previous studies on *Clostridium acetobutylicum* [153]. Nevertheless, archaeal extremophiles offer advantages over mesophilic organisms concerning organic solvent tolerance, due to their intrinsic robustness and adaptation to hostile environments [3,18,154]. According to the trends in Fig. 6, most genes encoding the archaellum for motility were significantly downregulated during 1-butanol stress in the *S. acidocaldarius* biofilm cells. In contrast, the genes encoding endosomal sorting complexes required for transport (ESCRT III) machinery were upregulated, while the genes encoding the CRISPR-Cas system were downregulated during the 1-butanol stress response. Consequently, 1-butanol stress enhanced biofilm formation and EPS composition in *S. acidocaldarius* [3].

In addition, Sedlmayr et al. [155] studied the physiological responses of *S. acidocaldarius* upon excess nutrient concentrations. It was reported that the cell's ability to deplete intermediates of the saturated tricarboxylic acid cycle was affected by high nutrient concentrations. Furthermore, cells of *S. acidocaldarius* could not secrete common fermentation products (e.g., organic acids) despite the elevated glucose consumption, suggesting intracellular energy conservation under nutrient stress. Thus, the ability of *S. acidocaldarius* to adapt its respiratory enzymes under nutrient stress shows high metabolic flexibility

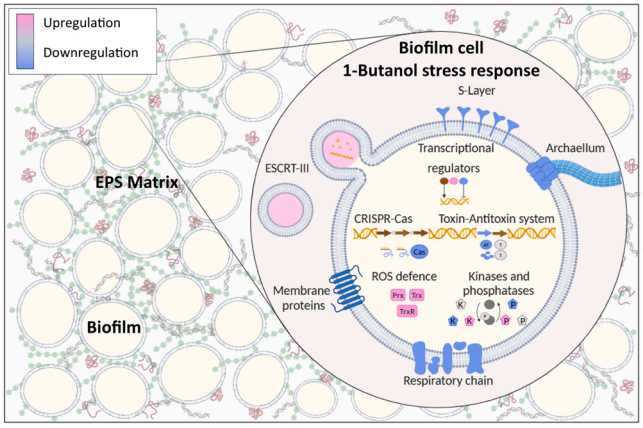

Fig. 6. Model of 1-butanol stress response in *S. acidocaldarius* biofilm cells. Increased and decreased transcription of genes in cellular structures and processes are represented by green and red color, respectively. Major EPS matrix components (polysaccharides, proteins, and eDNA) are distributed between the cells. Genes encoding membrane proteins, the archaellum for motility, the adaptive immune system (CRISPR-Cas), the dormancy- or cell death-inducing defense (toxin-antitoxin) system, and components of the respiratory chain are downregulated (red). Genes encoding proteins of the ROS defense system and the ESCRT-III system for vesicle formation and/or cytokinesis are upregulated (green). Several transcriptional regulators, as well as protein kinases (K) and protein phosphatases (P) for reversible protein phosphorylation, were differentially expressed.

and robust regulatory mechanisms.

However, the ease of cultivation of *Sulfolobus* on a multitude of different substrates (D-glucose, D-xylose, L-arabinose, starch, dextrin, saccharose, tryptone, NZ amine and single amino acids) [11] is another key factor for its application as a platform organism coupled with relatively low oxygen requirement during cultivation [156]. Another promising aspect is the potential for high-cell-density cultivation by adapting sophisticated bioreactor setups commonly used in workhorse bacteria such as *E. coli* [157], thus making them highly attractive for large-scale industrial processes [18]. Complex and minimal media have been described, e.g. for S. acidocaldarius, and high cell density cultivation has been established for *S. shibatae* and *S. acidocaldarius* [4,18,158]. The Brock medium developed by Brock et al. [35] is the most widely adopted medium for cultivating the extreme-thermoacidophilic genus *Sulfolobus*.

This medium has proven its applicability many times and can be considered the gold standard in biologic research with *Sulfolobus*; however, its major drawbacks include a lack of reproducible cultivation and physiological strain characterisation in a controlled environment and high cost [19]. Quehenberger et al. [19] developed Vienna Defined Medium (VD Medium) to overcome these issues by modifying Brock Medium. Then, it was used to shake flask cultivations of *Sulfolobus* with Monosodium L-glutamate (MSG), D-glucose, and citric acid as carbon sources. Fe(III)-citrate was added to VD medium to prevent precipitation and ensure medium homogeneity. However, adding L-glutamic acid and L -aspartic acid to VD medium increased the growth rate of *S. acidocaldarius* compared to a medium with D-glucose as the sole carbon source. L-glutamic acid had the highest growth-promoting effect on *S. acidocaldarius* among the tested amino acids, and correlated with the findings of Park and Lee [159], who investigated the growth-promoting effect of amino acids on *S. solfataricus*.

Furthermore, comparison between VD Medium and complex Brock Medium on the growth of *S. acidocaldarius* DSM 639 showed maximum specific growth rates (μmax) of 0.08 h^{-1} and 0.12 h^{-1}, respectively. Thus, it was concluded that the reduced maximal growth rate on the VD Medium was due to a prolonged lag phase. Additionally, all three carbon sources were taken up simultaneously, indicating the absence of carbon catabolite repression. VD Medium allowed a biomass yield of 0.33 $g_{DCW}/g_{substrate}$ to be achieved from shake flask cultivation of *S. acidocaldarius* DSM 639 after 95 h. The temperature of 75°C has been widely accepted as the optimal growth temperature for *S. acidocaldarius* [14,158,160,161], but the optimal cultivation pH still varies [158,160,162]. In the study conducted by Rastädter et al. [14], an optimum pH of 3.0 was determined for S. *acidocaldarius* with respect to cell density during continuous cultivation using Vienna Defined (VD) medium, but this finding negated the previous report of Cobban et al. [163]. Thus, the differences in cultivation conditions (temperatures, medium and the mode of cultivation) were mainly responsible for the inconsistency. However, changes in pH affected specific glucose uptake rate and specific trehalose production rate, and higher pH resulted in increased trehalose production. Furthermore, increased pHs have been previously reported to affect biofilm formation and upregulation of sugar transport for biofilm formation [164].

On the other hand, substrate uptake rates correlated with the obtained affinity constants (Ks) values, demonstrating D-glucose transportation into the cell via an ATP-binding cassette (ABC) transporter [14,142,165]. It was possible to achieve a maximum growth rate of 0.097 h^{-1} in both the batch and chemostat cultivation of *S. acidocaldarius* using VD Medium. These values of maximum growth rate correlated with those of previous reports in shake flask cultivation within the genus *Sulfolobus* [19,166]. Continuous cultivation of *S. acidocaldarius* in stirred-tank bioreactor setups showed a typical stress response similar to those obtainable in biorefineries, hence it can be employed for medium optimisation based on substrate affinity, control of biomass and metabolite production as well as optimisation of time-space-yields in production processes in industrial settings [14].

5.3. Targeted products

Extreme thermoacidophilic Archaea via synthetic biology and metabolic engineering offers great potential as platform organisms to produce high-value and value-added products (e.g., ethanol, butanol, 1,2,4-butanetriol, 1,4-butanediol, ethylene glycol, glycolate, polyhydroxyalkanoates, tetraether lipids and 3-hydroxypropionate) from cheap feedstocks (e.g. lignocellulosic biomass) by integrating fermentation conditions with downstream processing. Ethanol is an important renewable fuel and an attractive alternative to fossil-derived fuels. It is the most important biotechnological product by production volume, with an annual global production of about 100 billion litres [167]. Butanol is a promising biofuel alternative based on several advantages compared to the more established biofuels, ethanol and methanol [168]. It can also be utilised as a chemical platform, e.g., for producing coatings, paints, pharmaceuticals, paint additives, or plasticisers [148,169].

Ethylene glycol (EG) is a commodity chemical that can be employed in multiple conventional applications. The two most prominent EG uses are automotive antifreeze and one of the precursors for poly(ethylene terephthalate) [170]. Besides, EG can be converted into acetaldehyde with the help of diol dehydratase and later transformed into ethanol, acetate, or acetyl-CoA [171]. Glycolate is an organic acid containing both carboxyl and alcohol groups, which has a wide range of applications, such as a precursor for biopolymer synthesis, a rinsing agent in the tanning and dyeing industry, and a skincare product in the cosmetics industry [172]. Glycolate polymer is also a new packaging material with superior performance because of its gas-barrier and mechanical properties [172].

1,2,4-Butanetriol (BT) is an important fine chemical with many applications in many fields. For example, polyurethane foam made from BT has compression-bending properties similar to natural rubber [173]. Additionally, BT, as a potential building block, is used for synthesising various pharmaceuticals and as a direct precursor for BT trinitrate (a great energetic plasticiser) [174]. 1,4-Butanediol (BDO) is a valuable chemical commodity, and as a chemical intermediate, BDO goes into a vast spectrum of products, including automotive parts, electronics, and apparel [175].

Polyhydroxyalkanoates (PHA) are biodegradable, water-insoluble, non-toxic, and biocompatible. These present promising alternatives to petroleum-derived plastics [150]. Their eco-friendly properties position PHA as a leading candidate for sustainable plastic production in packaging and biomedical applications [176]. Tetraether lipids (TELs), the major membrane constituents of *S. acidocaldarius*, are gaining rapidly increasing attention as unique biological material primarily used in medical applications to form drug delivery vehicles [177]. 3-hydroxypropionate (3-HP) has received increased attention after it was identified as one of the top 12 building blocks and a potential alternative to petroleum-based technologies on the path to sustainability [148]. 3-HP is a platform chemical used to synthesise various high-value chemicals such as bioplastics, 1,3 1,3-propanediol, acrylic acid or acrylamide [178].

6. Concluding remarks

The recent developments in synthetic biology and metabolic engineering approaches, as well as bioprocesses allowing continuous cultivations in stirred tank bioreactors, have opened the way for using *Sulfolobus* as microbial cell factories. Through these developments, it is possible to convert inexpensive feedstocks such as lignocellulosic biomass into industrially relevant compounds. Currently, mesophilic organisms (e.g., bacterial, fungal and algae) are utilised to produce a vast majority of value-added and high-value compounds on an industrial scale. Still, these processes are often associated with a high risk of contamination, lower substrate solubility, and high energy and process costs. Thus, the use of thermoacidophilic Archaea as microbial cell factories will offer many benefits for industrial processes due to easy

cultivation on cheap feedstocks (e.g., agro-residues, forest residues and agro-industrial waste) under non-sterile and harsh conditions, thereby reducing production cost.

Several industrial and laboratory-scale operations have been established using thermoacidophilic Archaea (e.g., *Acidianus*, *Metallosphaera* and *Sulfolobus*) for the mobilisation of base, precious, and strategic metals from mineral ores during biomining operations. On the other hand, the members of *Sulfolobus* (e.g. *S. solfataricus* and *S. acidocaldarius*) are promising novel platform organisms for the conversion of lignocellulosic biomasses into platform chemicals (Ethanol, Butanol, BT, BDO, 3-HP, PHA, TELs, etc.), which are potential alternatives to fossil-based fuels and products. The current advances in developing genetic tools and cultivation systems for thermoacidophilic Archaea and a better understanding of the cultured strains offer opportunities for their applications in biotechnology, biorefinery and biomining operations.

Despite the progress in using thermoacidophilic Archaea as microbial cell factories at laboratory and pilot scales, their commercial-scale use remained challenging. Thus, future studies focusing on consolidated bioprocessing using whole cells of thermoacidophilic Archaea and cheaper substrates such as lignocellulosic biomass may improve process efficiency and lower production cost.

Submission declaration

All authors declare that this review article has not been published before and is not under consideration for publication elsewhere.

CRediT authorship contribution statement

Alfred Fernandez-Castane: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition. **Alan D. Goddard:** Writing – review & editing, Visualization, Validation. **Marta Maso Martinez:** Writing – review & editing, Visualization, Validation, Data curation. **Longinus Ifeanyi Igbojionu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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