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Prospects on bio-based 2,3-butanediol and acetoin production: Recent progress and advances

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Abstract

The bio-based platform chemicals 2,3-butanediol (BDO) and acetoin have abundant applications in chemical, cosmetics, food, agriculture, and pharmaceutical industries, whereas the derivatives of BDO could be used as fuel additives, polymer and synthetic rubber production. This review summarizes the novel technological developments in adapting genetic and metabolic engineering strategies for selection and construction of chassis strains for BDO and acetoin production. The valorization of renewable feedstocks and bioprocess development for the upstream and downstream stages of bio-based BDO and acetoin production are discussed. The techno-economic aspects evaluating the viability and industrial potential of bio-based BDO production are presented. The commercialization of bio-based BDO and acetoin production requires the utilization of crude renewable resources, the chassis strains with high fermentation production efficiencies and development of sustainable purification or conversion technologies.

Keywords: 2,3-Butanediol; Acetoin; Metabolic Engineering; Bioprocess Development; Downstream Processing; Techno-economic Evaluation, Platform Chemicals

1. Introduction

2,3-Butanediol (BDO) is a C4 metabolite which is also known as 2,3-butylene glycol, 2,3-dihydroxybutane, dimethylene glycol, and dimethylethylene glycol. Its chemical formula is $C_4H_{10}O_2$ with a molecular weight of 90.1 (g mol^{-1}). BDO is a chiral compound that can exist in three isomeric forms: meso-BDO (2R, 3R-), L-BDO (*dextro*- or (2S, 3S)-(+)-) and D-BDO (*levo*- or (2R, 3R)-(-)-) (**Fig. 1**). The three stereoisomers of BDO have slightly different high boiling points, ranging from 177 to 182˚C, and low freezing point of -60˚C. It can be found as a colorless and odorless liquid or in crystalline form (Celińska and Grajek, 2009; Syu, 2001).

BDO has great potential in the manufacturing of printing inks, perfumes, synthetic rubber, antifreeze agents, fuel additives, foods and pharmaceuticals (**Fig. 2**). BDO can be converted into 1,3-butadiene that is subsequently used as intermediate chemical in the production of synthetic rubber and plastics. Diacetyl, derived from BDO dehydrogenation, can serve as a food additive and flavouring agent. Another application of BDO includes the production of methyl ethyl ketone (MEK) as effective fuel additive and solvent for resins and lacquers (Celinska and Grajek, 2009). The global market of BDO is expected to reach a value of $220 million by 2027, growing at a CAGR of 3% from 2019 to 2027 (Chemicals & Materials, Market Research Report) while the MEK market is expected to grow at a CAGR of 5.4% during the period of 2019-2024 (Research and Market, 2019).

Common practice for BDO production is the pyrolysis of diacetate. Nowadays, butenes from crack gases are the main raw material for BDO synthesis. A C4 hydrocarbon fraction is initially obtained after the removal of butadiene and isobutene. Chlorohydration of this fraction with an aqueous chloride solution and subsequent cyclization of chlorohydrins with sodium hydroxide leads to a butane oxide mixture with the following composition: 55% *trans*-2,3 butene oxide, 30% *cis*-2,3-butene oxide, 15% 1,2-butene oxide. Hydrolysis of the mixture (50 bar, 160-220 ˚C, enthalpy of reaction ΔH= - 42 kJ/mol) results in a mixture of BDO isomers which can be separated by vacuum fractionation (Gräfje et al., 2000; Ge et al., 2016). meso-BDO is obtained from *trans*-2-butene via *trans*-2,3-butene oxide, while racemic mixture of L-BDO and D-BDO is formed from *cis*-2-butene via *cis*-2,3-butene oxide (Gräfje et al., 2000). The high energy consumption and the expensive catalysts employed lead to high production cost ($1600/t), which is the major barrier for expanding the global BDO market (Bialkowska, 2016).

Bio-based BDO has successfully been produced by LanzaTEch from waste gas resources in an industrial setting (Simpson et al., 2014). INVISTA and LanzaTech have collaborated on the production of bio-based butadiene using LanzaTech's carbon monoxide-derived BDO and a direct-step process through a gas fermentation process (INVISTA and LanzaTech).

Acetoin, also known as 3-hydroxy-2-butanone or acetylmethylcarbinol, is a four-carbon hydroxyl-keto compound with molecular formula $C_4H_8O_2$ that exist in two chiral enantiomers, namely *dextro*- or S- and *levo*- or R- forms (**Fig. 1**). It has been included in the 30 most important sugar-derived platform chemicals by the US department of Energy. Acetoin is a pale to yellowish liquid with a yogurt odor and a fatty creamy butter taste with wide range of applications (Cui et al., 2018; Xiao and Lu, 2014).

Naturally, acetoin exists in fruits, vegetables flours or fermented foods such as cocoa, vinegar, butter and wine. Commercial acetoin is mainly used in foods as flavor enhancer and as ingredient in the formulation of strawberry, raspberry, vanilla, butter, coconut, coffee and fruit flavors. It can be also used in the cosmetic industry as fragrance agents and as a precursor for synthesis of platform compounds such as chelating agents. As bioactive compound, it has great potential in microbiology, botany and agriculture sectors (Xiao and Lu, 2014). Optically

pure acetoin is widely applied in the synthesis of optically active α-hydroxyketone derivatives, liquid crystal composites and pharmaceutical intermediates (Cui et al., 2018; Gao et al., 2013a).

Currently, the largest part of industrial acetoin production is conducted via chemical synthesis by partial reduction of diacetyl, selective oxidation of BDO, oxidation of butan-2-one (MEK) followed by basic hydrolysis and hydrogenation of acetaldehyde (**Fig. 3**) (Kochius et al., 2014). Due to the environmental impact, low conversion yield, high cost and low safety in food and cosmetic applications, alternative biotechnological methods have been developed for the production of natural and safe acetoin.

This review focuses on the presentation of recent advances on BDO and acetoin production. Acetoin and BDO are usually produced by the same bacterial strains and their alternating production could be easily controlled via manipulation of fermentation parameters (e.g. oxygen supply). Thus, considering their industrial significance as platform chemicals, this review addresses the production of either BDO or acetoin focusing on production efficiencies and metabolic pathways of wild type and engineered strains. Emphasis is given on metabolic pathways, genetic engineering approaches, utilization of renewable resources, fermentation efficiency, downstream separation and purification methods and bioprocess economics. Potential production of derivatives from BDO and acetoin is also covered.

2. Metabolic pathway and regulation mechanism of BDO and acetoin

Various microorganisms can metabolize several carbon sources for BDO production. In case of hexoses, the sugar enters the microbial metabolism through PTS (phosphortransferase system) and converts into 2 moles of pyruvate via Embden-Meyerhof pathway (glycolysis) with the generation of 2 moles of NADH and 2 moles of ATP. Pyruvate is then channeled for the generation of metabolites such as acetate, formate, lactate, succinate, ethanol and acetoin, besides BDO (Syu, 2001). Pyruvate production from C5 sugars involves combination of pentose phosphate and Embden-Meyerhof pathway (Jansen and Tsao, 1983). However, some strains exhibit extracellular glucose oxidation pathway coupled with respiration. In this pathway, glucose dehydrogenase and gluconate dehydrogenase sequentially oxidizes glucose to gluconate and 2-ketogluconate, respectively (Bouvet et al., 1989). The glucose dehydrogenase (*gcd*) enzyme exists in two forms such as soluble and membrane bound. This type of pathway occurs in some BDO producing strains such as *Enterobacter aerogenes* and *Klebsiella pneumoniae* (Jang et al., 2017a,b). The membrane bound glucose dehydrogenase and gluconate dehydrogenase belongs to the family of quinone protein containing pyrroloquinolinequinone. The quinoprotein basically oxidizes the substrate by transferring an electron to ubiquinone, which is an important component of bacterial respiratory system, further the electrons are transferred to oxygen in the respiratory system. The proton gradient generates membrane potential, which is subsequently used for generating energy using the ATPase system. In the case of *Klebsiella sp.*, glycerol uptake is observed at higher rate. Under aerobic conditions, glycerol is converted into dihydroxyacetone phosphate by a group of enzymes, such as glycerol kinase (glpk), glycerol-3-phosphate dehydrogenase (GPD) and glycerol dehydrogenase (dhaD). Under anerobic environment, the glycerol undergoes oxidoreductive pathway which is governed by dihydroxyacetone (*dha*) regulon (Rhie et al., 2019).

BDO and acetoin are produced from pyruvate, while various organic acids and ethanol are also produced depending on the microbial strains and fermentation conditions employed. Specifically, BDO and acetoin production includes initial condensation of pyruvate generated from the glycolysis to form one mole of α-acetolactate catalyzed by α-acetolactate synthase (ALS, EC 4.1.3.18). The formation of acetolactate will take place at low NADH availability. This step is immediately followed by conversion of α-acetolactate to R-acetoin by α-

acetolactate decarboxylase (ALDC, EC 4.1.1.5) under anaerobic condition. In aerobic environment, the α-acetolactate undergoes spontaneous decarboxylation to form diacetyl. The diacetyl reductase (DAR) converts diacetyl into S-acetoin. In the final step, the acetoin is reduced to BDO by BDO dehydrogenase (BDH, EC 1.1.1.76) also known as acetoin reductase (AR, EC 1.1.1.4), whereas the BDH assist in irreversible conversion of diacetyl to acetoin (Magee and Kosaric, 1987).

Depending on NADH availability different stereoisomers of acetoin are produced. R-acetoin is the product of anaerobic fermentation from acetolactate, while S-acetoin is produced under aerobic conditions from diacetyl. The existence of various BDHs in native BDO producers leads to different BDO isomers. R-acetoin can be reduced into either meso-BDO by R,S-BDH or D-BDO by R,R-BDH, whereas S-acetoin could be converted into either meso-BDO by R,S-BDH or L-BDO by S,S-BDH. BDO can be reversibly converted into acetoin to regenerate NADH to maintain a constant oxidation-reduction state (**Fig. 4**).

Most of the enzymes involved in BDO production are active under oxygen limiting conditions and tend to produce the product during late log phase or stationary phase. It is also reported that acetate acts as an effective inducer activating all three enzymes involved in the production of BDO from pyruvate (Ji et al., 2011a). BDO regulates $NADH/NAD^+$ by regenerating excess of reducing power associated with glycolysis and many microbes reutilize acetoin and BDO as carbon and energy source (Nakashimada et al., 2000; Xiao and Xy, 2007; Vivijs et al., 2014). It is quite evident that in some microbes, a single enzyme carries out two functions. For instance, S,S-BDH catalyzes the conversion of diacetyl to acetoin and acetoin to BDO, while in *Brevibacterium saccharolyticum* and in *K. pneumoniae* R,S-BDH converts the acetoin into BDO and it also displays strong DAR activity (Ui et al., 2014). Likewise, *E. aerogenes* converts diacetyl to BDO by diacetyl (acetoin) reductase or L-glycol dehydrogenase (Bryn et al., 1971; Carballo et al., 1991).

The genes for the key enzymes, such as ALS, ALDC and BDH/AR, are sequentially clustered to form the operon *budABC*, which are active in *K. pneumoniae*, *E. aerogenes* and *V. cholerae* (Blomqvist et al., 1993). The transcription of this operon is controlled by LysR family transcriptional activators. In anaerobic process, the genes at transcription level are activated by a putative fumarate nitrate reduction regulatory (FNR) protein.

As mentioned previously, the BDO is affected by acetate, which acts as an inducer and activates LysR-type transcription regulator. Also, in some microorganisms the presence of quorum sensing (QS) influence BDO production (Kovacikova et al., 2005; Moons et al., 2011). In *V. cholerae*, the transcription of BDO is regulated by AphA (triggers the entire virulence cascade upon host colonization and exhibits distinct modes of DNA binding and promoter regulation) and LysR (comprises of conserved N-terminal DNA-binding helix–turn–helix motif and a C-terminal co-inducer-binding domain and involved in virulence, metabolism, quorum sensing and motility) transcription activator, but AphA is majorly activated by multiple QS system. In *S. marcescens*, acetoin production is controlled by two transcriptional regulators SwrR and SlaR, which in turn control *slaA* (encoding ALDC) and *slaB* (encoding ALS) genes. Furthermore, the *slaA* and *slaB* genes are activated by the QS system (Kovacikova et al., 2005; Van Houdt et al., 2007). Microorganisms such as *B. subtilis*, *B. licheniformis* and *Alcaligenes eutrophus* have *aco* operon genes, which are responsible for acetoin catabolism and are controlled by signal dependent promoter (acoA). In this system, glucose represses the transcription and once the glucose is depleted the transcription is activated by acetoin (Thanh et al., 2010).

Most of the structural genes involved in acetoin formation are clustered in a single operon. Different microbes have different synonym of this operon. For example, *Bacillus sp.* have *als* and *bud* genes in the acetoin operon, whereas *S. marcescens* has *bud* and *sla* genes. The ALS, ALDC and BDH are located in the same operon in species such as *Klebsiella* and *Enterobacter*, but the *bdh* gene is not always clustered with ALS and ALDC. For instance, the BDH is present in the *aco* operon of *Pseudomonas putida* and are absent in *B. subtilis alsSD* operon. In few microorganisms, the acetoin biosynthesis operon is regulated by different regulatory proteins. One such protein is catabolite control protein A (CCPA) which is a global regulator of carbon metabolism and plays an important role of transcriptional activation and repression in gram positive bacteria. In *B. subtilis*, the CCPA protein involves in transcriptional activation of *alsSD* operon. In the case of *Klebsiella sp.*, FNR regulatory proteins are involved in transcriptional activation of *budABC* operon. In some species of *Serratia*, acetoin formation is regulated by N-acyl-L-homoserine lactone (AHL) which is quorum sensing based regulator. AHL deficient strain showed reduced acetoin biosynthesis and high acid accumulation.

3. Insight on stereoisomer forming mechanism in BDO producers

The exact mechanism of stereoisomer formation is still under investigation. Over the last few years, various hypothesis have been speculated on the formation of isomers in acetoin and BDO. Taylor and Juni (1960) carried out a preliminary experiment to demonstrate the possible mechanism for the formation of stereoisomers. These experiments were based on the assumption that there is an acetoin racemase and L- and D- type stereospecific BDO dehydrogenase, which are involved in the formation of stereoisomers. In late 80s, it was suspected that *K. pneumoniae*, forms S- and R-acetoin reductase and acetoin racemase, which are responsible for stereoisomer formation, but later it was confirmed that *K. pneumoniae* forms R-acetoin from pyruvate via α-acetolactate, also traces of acetoin racemase was detected in the cell free extract (Voloch et al., 1983). To further understand the mechanism of BDH in the formation of various isomers, Ui et al. (1986) established the method for the measurement of acetoin and BDO isomers. With this method, they were able to separate R,S-, R,R- and S,S-BDH from *B. polymyxa* and the activity was determined using pure isomers, but no band of BDO racemase was observed upon using disc electrophoresis and staining. The authors concluded that R- isomers of acetoin will be formed in the absence of NADH, while racemate form of acetoin will be formed in the presence of NADH. In *P. polymyxa*, the NADPH dependent diacetyl reductase (R-acetoin forming) and R,R-BDH were separated by blue Sepharose CL-B6B and identified with pure isomers, but BDO racemase was not identified. However, *P. polymyxa* was able to produce mainly the D- isomer and some quantity of the meso- form.

To get more insight on the mechanism involved in the stereoisomer formation, Ui et al. (1999) cloned and expressed the gene encoding R,S-BDH (R-acetoin forming) in *E. coli.* The enzyme was able to catalyze S-acetoin to L-BDO when diacetyl is used as substrate. The sequence analysis revealed that the *budC* can convert meso-BDO with R-acetoin as a substrate (Yan et al., 2009). It was reported that R-acetoin is spontaneously formed during aerobic fermentation in microbes, while D-BDO, meso-BDO, and diacetyl are used as potential substrates. In the presence of NADH, R,R-BDH exhibited reduction of diacetyl to R-acetoin. Further, the enzyme glycerol dehydrogenases (GDH) catalyzes the oxidation of D-BDO into R-acetoin using NAD^+ as a cofactor. The R-acetoin can be readily reduced to D-BDO by R,R-BDH as R-acetoin serves as an intermediate product and reduces the R-acetoin production. In the presence of NAD^+, this enzyme can act reversibly and produce R-acetoin using D-BDO (Ji et al., 2011a). Till date, limited research has focused on understanding acetoin racemase which is considered as the enzyme for the formation of BDO stereoisomers in bacteria.

4. Biological function of BDO and acetoin

The metabolic pathway of BDO participates in the regulation of the intracellular NADH/NAD^+ ratio in the cell owing to the reversible transformation between acetoin and BDO coupled with the NAD^+/NADH conversion. The biosynthesis of BDO has a vital role in preventing the intracellular acidification environment. Acidification of culture medium due to organic acid formation is prevented by the conversion of pyruvate to neutral compounds (Celińska and Grajek, 2009; Ji et al., 2011a). When carbon and energy sources have been depleted, BDO is transformed to acetoin to regulate the carbon and energy balance. Acetoin biosynthesis is closely related to the branched-chain amino acid pathway and the regulation of cell life (Xiao and Xu, 2007).

5. Wild-type and genetically engineered microorganisms

Several anaerobic and facultative anaerobic microorganisms are able to accumulate BDO and acetoin using commercial substrates, industrial side streams and lignocellulosic materials. The most representative microorganisms that have been extensively investigated include the genera of *Enterobacter*, *Klebsiella, Serratia*, *Paenibacillus* and *Bacillus*. Highly efficient BDO producers are *K. pneumoniae*, *K. oxytoca* and *S. marcescens*, while the most well-known acetoin forming species belong to the genus *Bacillus* (Celińska and Grajek, 2009). *Neptunomonas concharum* has been recently reported as potential BDO and acetoin producer using acetate as carbon source (Li et al., 2019). **Tables 1** and **2** present BDO and acetoin production by different strains using various substrates.

Serratia, Klebsiella and *Enterobacter* are gram-negative, facultative anaerobic, rod-shaped, non-spore-forming bacteria belonging to the Enterobacteriaceae family. These genera have the potential for industrial BDO production due to their simple metabolic pathway, broad substrate consumption spectrum and cultural adaptability. The highest BDO production (150 g L^{-1}) has been achieved by wild-type *K. pneumoniae* strain isolated from orchard soil using glucose with yield and productivity of 0.44 g g^{-1} and 3.95 g L^{-1} h^{-1}, respectively (Ma et al., 2009). In this study, acetoin was considered as by-product with a final concentration of 10 g L^{-1}. Generally, acetoin production has not been reported as a main fermentation product by native bacteria of Enterobacteriaceae family. Engineered *E. coli*, *S. cerevisiae* and *Corynebacterium glutamicum* have been applied for acetoin production with promising results.

Bacillus, a genus of Gram-positive, endospore-forming, rod-shaped bacteria, have been used for both BDO and acetoin production via fermentation. The most interesting BDO producer of that genus is *B. licheniformis*, which produced 144.7 g L^{-1} of BDO with yield and productivity of 0.40 g g^{-1} and 1.14 g L^{-1} h^{-1}, respectively, in fed-batch culture using glucose (Jurchescu et al., 2013). The highest acetoin concentration (65.9 g L^{-1}) has been reported by a wild-type strain of *B. amyloliquefaciens* strain using bakery waste hydrolysate (Maina et al., 2021).

The BDO and acetoin isomers produced are dependent on the particular microorganism employed. Generally, a mixture of two BDO stereoisomers is formed by native strains. *Klebsiella* and *Enterobacter* produce L- and meso- stereoisomers of BDO, whereas a mixture of D- and meso- is generated by *Bacillus* species (Celińska and Grajek, 2009). Acetoin producing strains naturally accumulate the R-acetoin isomer (Xiao and Xu, 2007). The choice of the producing strain is largely reliant on the intended application of the end-product and the fermentation efficiency. Strains of Enterobacteriaceae family are considered pathogens or opportunistic pathogens and deletion of virulence genes should be conducted before large scale use. While *Bacillus* species have been granted GRAS (generally regarded as safe) status by the US Food and Drug Administration and are considered promising strains for commercial

operation, improvement of fermentation efficiency is required for the development of a cost-competitive process.

6. Genetic modification

Biological production of BDO and acetoin has gained attention due to environmentally friendly characteristics. However, major bottlenecks involve the utilisation of expensive fermentation media, by-product formation and high energy consumption in downstream separation and purification, which ultimately hinder the economic feasibility at industrial scale. To overcome these bottlenecks, considerable effort has been devoted over the last few years using strain engineering and bioprocess development strategies. GRAS microorganisms have been used to produce platform chemicals as their genetics are well characterized and the cultivation processes are well established (Prabhu et al., 2018). Extensive efforts have been devoted to produce high yield of optically pure forms of BDO and acetoin using genetically engineered strains (Yang and Zhang, 2018a).

6.1 Genetic manipulation using random mutagenesis approach

Henriksen and Nilsson (2001) isolated *Lactococcus lactis* with impaired pyruvate catabolism by adapting classical mutagenesis, such as chemical mutagenesis using ethyl methyl sulphone. When the lactate dehydrogenase (ldh) defective strain was cultivated under oxygenated condition using acetate as substrate and in the absence of lipoic acid, it tends to direct major proportion of the pyruvate flux towards α-acetolactate, acetoin and diacetyl production. Lipoic acid (coenzyme A and thiamine) acts as co factor for the NADH dependent enzyme and help regulating the formation of acetyl-coA during aerobic condition in *Lactococcus sp.* Further, the ldh and pyruvate formate-lyase (pfl) defective strain showed higher diacetyl reductase activities when the medium was supplemented with acetoin. Under oxygen limiting conditions, acetoin acts as more reduced substrate than glucose and aids in maintaining intracellular redox balance. Boumerdassi et al. (1997) performed N-methyl-N-nitro-N-nitrosoguanidine mutagenesis on *L. lactis* subsp. lactis biovar diacetyl lactis CNRZ 483 and developed a mutant 483L3 which was able to convert 78% of glucose to compounds such as diacetyl, acetoin and BDO in contrast to the wild-type strain which converted only 2% of glucose to C4 compounds under aerobic conditions. Under anerobic conditions, the mutant exhibited 25.8% conversion of carbon source to acetoin and BDO. The mutant renewed utmost of the cofactor NAD^+ via NADH oxidase under surplus oxygen environment and by ethanol production under oxygen deprived circumstances. Further, the ldh activity was strongly attenuated in the mutant strain, which increased the carbon flux towards acetoin and BDO formation.

6.2 Rational metabolic engineering of native acetoin and BDO producers

6.2.1 *Klebsiella sp.*

Various studies reported high production of BDO by *K. pneumoniae* and *K. oxytoca*. Optical purity was compromised due to the presence of various BDH. Attempts were made to enhance the carbon flux towards acetoin and BDO production by overexpressing enzymes involved in BDO biosynthesis. Guo et al. (2014b) developed recombinant *K. pneumoniae* by overexpressing *als, aldC* and *ar/bdh* genes individually and in combination, which improved the BDO production compared to the wild type strain. The recombinant strain *K. pneumoniae* KG-rs, which overexpressed both ALS and AR, exhibited the highest BDO conversion yield ($0.38\ g\ g^{-1}$) among the recombinant mutants. Kim et al. (2012) expressed the genes acetolactate decarboxylase (*budA*), acetolactate synthase (*budB*), and BDO dehydrogenase (*budC*) in different combinations under lac promoter in *K. pneumoniae* KCTC2242. The strain harboring

budA and *budB* genes produced 60% higher BDO than the parental strain. A BDO concentration of 101.53 g L^{-1} with a productivity of 2.54 g L^{-1} h^{-1} was reported in fed-batch culture.

Despite having higher productivity of BDO, *K. pneumoniae* exhibits potential pathogenesis. One potential solution for this hurdle is to remove the virulence factor that is the lipopolysaccharide coat of the strain. The mucoviscosity-associated gene A (*magA*) of *K. pneumoniae* is important factor for the biosynthesis of K1 capsular polysaccharide (CPS). Further, homology sequence showed that *magA* encodes Wzy-type CPS polymerase which have eight conserved residues G308, G310, G334, G337, R290, P305, H323, and N324. Mutation in these residues can alter CPS production. Alkaline substitution of R290, H323 and G334A severely impacted CPS production and consequently reduced the virulence factor of the *K. pneumoniae* NTUH-K2044 (Lin et al., 2012). In similar lines, Jung et al. (2013b) deleted the gene encoding glucosyltransferase (*wabG*), which is responsible for the synthesis of outer core lipopolysaccharides (LPS). The deletion of gene did not affect bacterial growth. However, BDO production was decreased from 31.3 g L^{-1} to 22.4 g L^{-1} in the mutant compared with the parental strain. Jung et al. (2014) studied the deletion of genes encoding glucosyltransferase (*wabG*), lactate dehydrogenase (*ldhA*), and pyruvate formate-lyase (*pflB*) in *K. pneumoniae* to reduce both its pathogenic characteristics and the production of several by-products. The NADH availability and the carbon flux towards BDO production were enhanced leading to BDO conversion yield of 0.46 g g^{-1}. Lee et al. (2015) developed a mutant *K. pneumoniae* strain by deleting the protein secreting outer core polysaccharides. Further, overexpression of the glycerol dehydrogenase gene (*gldA* and *dhaD*) and deletion of *ldhA* enhanced the production of D-BDO to 61 g L^{-1} in a fed-batch cultivation strategy using glycerol as carbon source.

Most of the bacteria exhibit mixed acid fermentation which generates substantial quantities of by-products as a part of overflow metabolism. The production of BDO and acetoin competes with other by-products such as ethanol, lactic acid, succinic acid, acetic acid and formic acid. Research has been focused on blocking the metabolic pathways leading to by-product formation, which ultimately channelize more carbon flux and redox co-factors towards acetoin and BDO production. Kim et al. (2014a) performed plasmid-based overexpression of *budC* (encoding BDH) and *budB* (encoding ALS) in *ldhA*$^{-}$ *K. pneumoniae* KCTC2242, which redistributed carbon flux towards BDO production and reduced lactic acid production. The mutant strain produced 90 g L^{-1} BDO with only 1.26 g L^{-1} lactic acid.

Guo et al. (2014a) created a mutant *K. pneumoniae* by deleting *ldhA*, *pta* (phosphotransacetylase) and *adhE* (alcohol dehydrogenase). The mutant strain showed reduced by-product formation, such as lactate, acetate and ethanol, and increased NADH/NAD^{+} pool ratio. It was observed that single deletion of these genes slightly affected the growth of the strain. The *ldhA* mutant strain showed lower growth rate at first 8 h as compared with the parental strain. The double mutant strain with *ldhA* and *adhE* deletion showed a yield of around 0.49 g g^{-1} with a BDO concentration of 116 g L^{-1} in fed-batch cultivation using glucose as carbon source. Bioconversion of starchy raw materials into BDO is also under investigation. Within this concept, Tsvetanova et al. (2014) cloned *amyl* gene encoding α-amylase in *B. licheniformis* under *lac* promoter control in the recombinant *K. pneumoniae* G31-A. The recombinant strain was capable of simultaneous saccharification and fermentation of 200 g L^{-1} potato starch for the production of 53.8 g L^{-1} BDO and 7.1 g L^{-1} acetoin.

K. oxytoca is a potential candidate to produce BDO. Cho et al. (2015a) overexpressed AR in *K. oxytoca* M1 strain and tested its ability for BDO production under optimized fermentation conditions using complex nitrogen source and NaOH as neutralizing agent. AR encoded by the *budC* gene is a key enzyme in the wild-type *K. oxytoca* M1 strain as it catalyzes both acetoin

reduction and BDO oxidation with the reduction activity being 8-fold higher than the oxidation activity. The engineered *K. oxytoca* M1 strain overexpressing the *budC* gene displayed 1.2-fold higher BDO production efficiency in fed-batch culture, corresponding to 142.5 g L^{-1} BDO concentration, 0.42 g g^{-1} yield and 1.47 g L^{-1} h^{-1} productivity, than the parent strain. Ji et al. (2010) showed that the deletion of *aldE* in *K. oxytoca* ME-UD-3 reduced ethanol formation and enhanced BDO production up to 130 g L^{-1} with a productivity of 1.63 g L^{-1} h^{-1}.

Glycerol is another potential feedstock for BDO production. *K. oxytoca* metabolizes glycerol through the oxidative pathway, but it also uses the reductive pathway to generate 1,3-propanediol (PDO), which is a major obstacle for industrially efficient BDO production. The deletion of *pduC* (encoding glycerol dehydratase large subunit) and *ldhA* in *K. oxytoca* M3 reduced the formation of PDO and lactic acid, respectively. The double mutant was able to produce 131.5 g L^{-1} BDO with productivity of 0.84 g L^{-1} h^{-1} and yield of 0.44 g g^{-1} in crude glycerol cultures (Cho et al., 2015b). Park et al. (2015) overexpressed D-BDH from *P. polymyxa* in the *K. oxytoca ΔldhA ΔpflB* strain. The strain produced 106.7 g L^{-1} D-BDO with 0.40 g g^{-1} yield and 3.1 g L^{-1} h^{-1} productivity. Jantama et al. (2015) deleted alcohol dehydrogenase E (*adhE*), acetate kinase A-phosphotransacetylase (*ackA-pta*), and *ldhA* in *K. oxytoca* KMS005. The strain produced 117 g L^{-1} BDO with a high yield of 0.49 g g^{-1} and reduced by-product formation such as alcohol, acetate and lactic acid.

Xylose utilization has been also under investigation due to its presence in hemicellulose hydrolysates that are produced in lignocellulose-based biorefineries. Many strains either display reduced xylose assimilation due to carbon catabolite repression (CCR) or cannot depend on xylose as the sole carbon source. Ji et al. (2011b) addressed the issue of CCR by overexpressing *crp(in)* gene encoding cyclic adenosine monophosphate (cAMP) receptor protein CRP(in), which can function devoid of cAMP requirement. The recombinant strain could metabolize glucose and xylose simultaneously for BDO production. The recombinant *K. oxytoca* ME-CRPin strain produced BDO (23.9 g L^{-1}) from a mixture of glucose (40 g L^{-1}) and xylose (20 g L^{-1}) in batch mode.

The studies presented above demonstrate that rational metabolic engineering is a useful tool for the elimination of the pathogenicity and the reduction of by-product formation, which in turn enhance the production of BDO by *Klebsiella sp.*

6.2.2 *Enterobacter sp.*

E. aerogenes is potential candidate for cost-competitive BDO production, but the production of by-products, such as lactic acid, hinders BDO production efficiency. Jung et al. (2012) adapted λ red recombination method to delete the *ldhA* gene in *E. aerogenes* and the resulting strain displayed BDO production of 118.05 g L^{-1} with productivity of 2.18 g L^{-1} h^{-1} in fed-batch culture using glucose as carbon source. Li et al. (2015a) attempted to advance the optical purity of BDO in *E. cloacae* via obliteration of the endogenous *bdh* encoding for meso- and L-BDO followed by overexpression of the *bdh* from *B. pumilus* encoding D-BDH. The bottleneck of by-product formation, such as lactic acid and succinic acid, was blocked by deleting *ldh* and *frdA* (fumarate reductase) genes, respectively. The CCR problem was addressed by inactivation of the gene encoding glucose transporter *ptsG* and consequent overexpression of *galP* gene encoding the galactose permease enzyme. The engineered strain produced 152 g L^{-1} D-BDO with 3.5 g L^{-1} h^{-1} productivity in fed-batch fermentation using a mixture of glucose and xylose. Cultivating the engineered *E. cloacae* in corn stover hydrolysate containing around 3:1 glucose to xylose ratio resulted in 119.4 g L^{-1} D-BDO with 2.3 g L^{-1} h^{-1} productivity. Thapa et al. (2019) metabolically engineered *E. aerogenes* ATCC 29007 by deleting *ldhA*, phosphate acetyltransferase (*pta*), malate dehydrogenase (*mdh*) and acetaldehyde

dehydrogenase (*acdh*) genes. The engineered *E. aerogenes* SUMI02 Δ*pta*Δ*ldh*A strain produced 38.2 g L^{-1} BDO with a productivity of 0.8 g L^{-1} h^{-1} in a batch bioreactor culture using glucose as carbon source.

6.2.3 *Serratia sp.*

S. marcescens could be also used for efficient BDO and acetoin production. However, only few reports are available pertaining the metabolic engineering of this strain for efficient production of BDO and acetoin. Rao et al. (2012) cloned the genes *slaA, slaB, slaC* and *slaR* in *S. marcescens* and deleted the negative regulon *swrR* gene, which ultimately enhanced BDO production. Bai et al. (2015) identified that the *slaC* gene in *S. marcescens* MG1 encodes for meso-BDO and L- BDO synthesis. The deletion of *slaC* gene led to enhanced R-acetoin production (21.8 g L^{-1}) with reduced BDO production (6.1 g L^{-1}) as compared to the wild-type strain (32.2 g L^{-1} BDO and 1.4 g L^{-1} R-acetoin). The heterologous expression of *bdhA* gene from *B. subtilis* 168 in *slaC* deficient *S. marcescens* MG1 strain accelerated the conversion of R-acetoin to D-BDO. The mutant strain produced 89.8 g L^{-1} D-BDO and 2.1 g L^{-1} meso-BDO with productivity of 1.91 g L^{-1} h^{-1} in fed-batch mode.

Foaming is a major problem in BDO production which leads to microbial contamination. In *S. marcescens* fermentation, lipopeptide surfactants named serrawettins are responsible for foam production. Zhang et al. (2010a) preformed insertional inactivation of the *swrW* gene coding for serrawettin W1 synthase in *S. marcescens*. The mutant strain showed reduced foaming and produced higher BDO concentration (152 g L^{-1}) with a yield of 0.46 g g^{-1} using sucrose as carbon source.

6.2.4 *Bacillus sp.*

A plethora of genetic, molecular, metabolic and physiological information and databases for genetic, metabolic, transcriptomic and regulatory element information and resources/tools are available regarding *B. subtilis*, which is a non-pathogenic gram-positive bacterium. *Bacillus* produces both acetoin and BDO. It is well known that BDO production is likely governed by the quantities of the rate-limiting factor(s), namely the acetoin dehydrogenase complex AcoABCL and/or NADH. BDO production is NADH dependent, hence the availability of NADH and its proportion in reduced form plays a key role in BDO production. NADH oxidase catalyzes the oxidation of NADH to NAD^+. Yang et al. (2015b) developed a metabolic engineering strategy by manipulating the NADH level and re-distribution of carbon flux towards BDO production by overexpression of NADH regeneration system and disruption of NADH oxidase (YodC, encoded by *yodC*) by insertion of a formate dehydrogenase (*fdh*) gene in *B. subtilis.* The resulting strain showed higher efficiency (19.9%) in BDO production with 71.9% lower acetoin formation. The disruption of NADH oxidase increased the formation of BDO but there was still a trace amount of acetoin accumulation. Heterologous expression of *fdh* led to higher intracellular NADH pool, which improved the carbon flux towards BDO pathway. *B. subtilis* cloned with *E. coli* transhydrogenase (encoded by *udhA* that catalyzes the interconversion between NADH and NADPH) reduced the NADPH/$NADP^+$ ratio and increased the NADH availability, which in turn increased D-BDO production to 49.3 g L^{-1} (Fu et al., 2014). Fu et al. (2016) developed an engineered *B. subtilis* strain that can produce pure meso-BDO by deleting the native D-BDH and introducing the *budC* from *K. pneumoniae*. Furthermore, the BDO pathway involving *alsS* and *alsD* was overexpressed in the engineered strain. The mutant strain produced 103.7 g L^{-1} of meso-BDO with a yield of 0.49 g g^{-1} under optimized aeration and agitation in fed-batch cultivation using glucose.

B. amyloliquefaciens is a suitable and GRAS candidate for BDO production. Overexpression of NAD^+-dependent GAPDH and NADH-dependent BDH in *B. amyloliquefaciens* resulted in

lower by-product production, such as lactate, and 132 g L^{-1} of BDO in fed-batch cultivation, which was 22.7 % higher than the parental strain (Yang et al., 2013b). Wang et al. (2012b) performed a protoplast transformation and deleted the *ldh* gene using marker-less technique in *B. licheniformis*. Under optimized temperature of 50 °C and pH 5.0, the recombinant strain *B. licheniformis* produced 98% optically pure D-BDO using either glucose or xylose. The stereospecificity of *B. amyloliquefaciens* was investigated by deleting *gdh* (encoding R,R-BDH) and *budC* (encoding meso-BDH) (Ge et al., 2016). It was observed that the strain lacking *budC* accumulated 123.7 g L^{-1} D-BDO, while the strain with Δ*gdh* produced 90.1 g L^{-1} meso-BDO. Similar studies on the production of D-BDO and meso-BDO was done by deleting *budC* and *gdh*, respectively (Qi et al., 2014; Qui et al., 2016). Usually R-acetoin is produced through ALDC, whereas S-acetoin is synthesized through DAR. The *dudA* gene encoding DAR is responsible for the diversion of optically pure D-BDO to meso-BDO thus reducing the optical purity. Zhang et al. (2018) successfully knocked out the *dudA* gene in *P. polymyxa* ZJ-9 via single crossing over homologous recombination, which resulted in 99% pure D-BDO (25.88 g L^{-1}) in fed-batch cultivation.

It is evident from the literature-cited studies that engineering the NADH dependent oxidase pathway and deletion of by-product forming genes in the *Bacillus* strain results in substantial improvement in BDO production.

6.3 Metabolic engineering of heterologous host for the production of BDO

The production of acetoin and BDO depends on the expression of rate limiting enzymes such as ALS, ALDC and BDH. Overexpression of genes encoding these enzymes in the heterologous host has shown improved production of BDO and acetoin. *E. coli* has been extensively used for the production of heterologous proteins and metabolites. Ui et al. (1997) constructed a new BDO pathway in *E. coli* JM109 by overexpressing ALS, ALDC, and R,S-BDH from *K. pneumoniae* IAM 1063. The expression cassette was cloned in pUC118 under lac promoter and it was designated as pBDO118. The recombinant *E. coli* JM109/pBDO118 produced 17.7 g L^{-1} meso-BDO using 100 g L^{-1} initial glucose concentration. Ji et al. (2015) developed a recombinant *E. coli* strain for the production of optically pure D-BDO by cloning *budB* and *budA* genes from *K. pneumoniae* and *ydjL* gene (encoding R,R-BDH) from *B. subtilis*. D-BDO concentration of 115 g L^{-1} with 99% enantio-purity was obtained in fed-batch culture.

Tong et al. (2016) cloned the synthetic construct of *K. pneumoniae budB* and *budA* and *B. subtilis ydjL* under two different promoter strengths, AlperPLTet01 (P01) and AlperBB (PBB). The promoter P01 showed twice the transcriptional strength as compared to PBB. The recombinant strain showed higher glucose uptake and low acetic acid secretion profile. Under optimized fermentation conditions, the recombinant strain accumulated 30.5 g L^{-1} D-BDO and 3.2 g L^{-1} acetoin from 80 g L^{-1} glucose. Reshamwala et al. (2017) constructed a synthetic pathway with ALS and AR encoding genes from *Enterobacter* sp. and co-expressed these genes in *E. coli* under T7 promoter. The ALS and AR encoding genes were integrated in the *E. coli* genome under control of the constitutive ackA promoter. Under shake flask cultivation, the strain produced 1 g L^{-1} acetoin and 0.66 g L^{-1} BDO. The putative pathway for BDO was identified in *C. autoethanogenum* and overexpressed the pathway in *E. coli.* The reduction of acetoin to BDO was performed by NADPH dependent alcohol dehydrogenase (CaADH) (Köpke et al., 2014). Nakashima et al. (2014) addressed the issue related to the use of expensive inducers and plasmid loss by developing an engineered strain that produces BDO with xylose as inducer. The BDO cassette (*alsS, alsD* of *B. subtilis* and *butA* of *L. lactis*) was expressed under P_{xylF} (xylose inducible promoter) and it was integrated in the chromosomal DNA of *E. coli.* Since the P_{xylF} is a weak promoter, it was expressed under the strong T7 promoter using

xylose as inducer. The recombinant strain produced 54 g L^{-1} BDO from 99 g L^{-1} glucose and 11 g L^{-1} xylose with 99 % of the theoretical yield. This expression system was named BICES (biomass-inducible chromosome-based expression system) as it can utilize the xylose present in lignocellulosic biomass.

Many reports are available on the toxic effect of biofuel production in *E. coli,* but BDO showed no toxic effect in the growth of *E. coli*. The BDO production efficiency in *E. coli* cannot support industrial scale production. To overcome this barrier, Xu et al. (2014) screened the most efficient BDO pathway from native producers, such as *B. subtilis*, *B. licheniformis*, *K. pneumoniae*, *S. marcescens*, and *E. cloacae,* by cloning the gene cluster in pET28 vector under T7 RNA polymerase-based promoter. It was observed that *E. coli* BL21/pETP$_{T7}$-EcABC, carrying the BDO pathway gene cluster from *E. cloacae,* showed the best ability to produce BDO. The transcriptional efficiency of different promoters was identified. The gene cluster of *E. cloacae* was cloned under different promoters, including the IPTG inducible P_{tac} promoter, the constitutive promoter (P_c) and the native promoter of the BDO biosynthetic gene cluster of *E. cloacae* (P_{abc}). Among these, the native promoter P_{abc} showed the highest BDO production. Fed-batch fermentation under optimized fermentation conditions (400 rpm agitation rate and 1.5 vvm aeration rate) led to BDO production of 73.8 g L^{-1} (Xu et al., 2014). Co-expression of *budB* and *budC* gene of *E. cloacae* in *E. coli* with glucose as carbon source and addition of 10 mM iron chloride ($FeCl_3$) led to the formation of D-BDO (2.2 g L^{-1}), showing that the addition of $FeCl_3$ improves the conversion of α-acetolactate to diacetyl (Chu et al., 2015).

Most studies have engineered *E. coli* to produce BDO using monosaccharides, such as glucose. The utilisation of complex feedstocks has been also evaluated. Shin et al. (2012) engineered *E. coli* to utilize cellodextrin by introduction of a synthetic operon and periplasmic expression of a *Saccharophagus* cellodextrinase. The engineered strain converted 80% of cellodextrin to BDO. Mazumdar et al. (2013) used a similar approach to produce BDO from *E. coli* using seaweed as feedstock. Acetate is the main by-product that hinders the production of BDO in *E. coli.* Shen et al. (2012) cloned the BDO pathway into the wild-type *E. coli* BW25113 strain and observed acetate production of 3.0 g L^{-1} with 3.54 g L^{-1} of BDO production. The majority of the carbon flux was diverted towards acetate production, hence the authors deleted *ackA* or/and *poxB* genes that are responsible for acetate production. The mutant strain produced 9.54 g L^{-1} of BDO with no acetate formation. Li et al. (2010c) used a similar approach by cloning the BDO pathway in pEnBD (expression plasmid pMD-19 T derivative carrying *alsS*, *alsD* and *budC* genes) high copy number plasmid and deleting *ldhA, pta, adhE* and *poxB* genes in *E. coli* JM109. The mutant strain secreted low quantities of succinate, lactate, acetate and ethanol when grown on glucose-based media.

The entire genome sequence of *Saccharomyces cerevisiae* is available and the genetic tools are well established for engineering this strain. Several reports of BDO production in *S. cerevisiae* have been reported. Even though the BDO pathway is present in the genome, the strain produces low BDO concentrations. Ng et al. (2012) adapted genome scale metabolic modeling to enhance the carbon flux towards BDO. Three alcohol dehydrogenase genes (ADH1, ADH3, ADH5) were deleted, which increased the production of BDO by 55 folds under microaerobic condition. Deletion of glycerol-3-phosphate dehydrogenase (GDP2) reduced glycerol production but diverted the carbon flux towards ethanol production. With the deletion of ADH6, acetate accumulation was reduced but it also affected BDO production. The introduction of BDO pathway from *E. cloacae* and *B. subtilis* in ΔADH1, ΔADH3, ΔADH5 strain improved the production of BDO (2.29 g L^{-1}). Kim et al. (2013) constructed a pyruvate decarboxylase *Pdc*-deficient mutant, which can efficiently consume glucose and produce BDO without ethanol accumulation. Overexpression of *alsS* and *alsD* genes from *B. subtilis,* and

endogenous BDH1 in the *Pdc*-deficient mutant produced 96.2 g L^{-1} BDO with much higher glucose uptake rate compared with the parental strain under optimized aeration condition. Lian et al. (2014) developed a mutant strain by deleting pyruvate decarboxylase (*Pdc*) and overexpressing MTH1 with subsequent adaptive evolution resulting in faster uptake of glucose and no ethanol accumulation. Overexpression of biosynthetic pathway consisting of cytosolic acetolactate synthase (cytoILV2), along with *B. subtilis* ALSD and BDH1 resulted in generation of enantiopure D-BDO. The engineered strain produced 100 g L^{-1} of BDO using red algae extract containing glucose and galactose as carbon sources. Ishii et al. (2018) focused on tugging carbon flux across the pyruvate branch point by introducing codon optimized ALS from *Lactobacillus plantarum* (showing 27-fold higher activity) and deleting *Pdc*. The recombinant strain produced 81 g L^{-1} BDO using glucose as a sole carbon source in fed-batch cultivation.

In yeast, glycerol synthesis maintains the $NADH/NAD^+$ ratio in the cytoplasm. During glycerol biosynthesis, surplus amount of NADH is oxidized, hence excess NADH should be consumed via alternate pathway to mitigate the glycerol formation. To overcome this issue, Kim et al. (2015) cloned water-forming NADH oxidase gene (*noxE*) from *Lactococcus lactis* in *S. cerevisiae* leading to decreased $NADH/NAD^+$ ratio, 65.3 % lower glycerol production and 23.8 % higher BDO accumulation as compared to the parental strain. Kim et al. (2019) deleted GPD1 and GPD2 with overexpression of *noxE* from *L. lactis* in *pdc*-deficient strain, which resolved the redox imbalance issue related with BDO production. However, the mutant strain showed reduced growth rate due to unavailability of C2 compound, such as acetate and ethanol, during anaerobic fermentation condition, which is required for the biosynthesis of lysine and fatty acids by disruption of GPD. This is compensated by overexpression of PDC1 from *C. tropicalis*. The recombinant strain displayed a higher BDO concentration of 108.6 g L^{-1} corresponding to 92.4% of the theoretical yield with negligible glycerol production in a fed-batch cultivation. Kim and Hahn (2015) developed an engineered *S. cerevisiae* strain for efficient BDO production by deleting the pathway producing ethanol and glycerol (deletion of five alcohol dehydrogenases and two GPD) and introducing α-acetolactate synthase and α-acetolactate decarboxylase from *B. subtilis*, BDH from *S. cerevisiae* and *noxE* from *L. lactis.* The engineered *S. cerevisiae* strain produced 72.9 g L^{-1} BDO with a conversion yield of 0.41 g g^{-1}.

In order to utilize xylose as carbon source, the strain should possess xylose reductase (XR), xylose dehydrogenase (XDH) and xylulose kinase (XK) genes. Kim et al. (2014b) introduced *XYL1*, *XYL2* and *XYL3* genes coding for xylose assimilating enzymes derived from *Scheffersomyces stipitis* in *Pdc*-deficient *S. cerevisiae*. Furthermore, *alsS* and *alsD* genes from *B. subtilis* and the endogenous *BDH1* gene were overexpressed. The recombinant strain utilized xylose as carbon source for the production of 43.6 g L^{-1} BDO in fed-batch cultivation. Similarly, heterologous expression of the *S. stipitis* genes transaldolase, NADH-preferring xylose reductase and *noxE* in *S. cerevisiae* resulted in 96.8 g L^{-1} of BDO (Kim et al., 2017a).

Cellobiose consumption has been also evaluated. The introduction of the cellobiose utilization pathway gene cellodextrin transporter (cdt^{-1}) and β-glucosidase ($gh1^{-1}$) from *Neurospora crassa,* and overexpression of *alsS* and *alsD* genes from *B. subtilis* in *S. cerevisiae* resulted in BDO production of 5.29 g L^{-1} (Nan et al., 2014). To understand the effect of ALDC in the formation of BDO, Choi et al. (2016) cloned ALDC from two strains, namely *B. subtilis* and *E. aerogenes*, in recombinant *Pdc*-deficient *S. cerevisiae* strain containing two essential enzymes for BDO production (ALS and BDH). It was observed that the k_{cat}/K_m of ALDC for *E. aerogenes* was 1.7-fold higher than *B. subtilis*. The recombinant *S. cerevisiae* with *E. aerogenes* ALDC produced 132.4 g L^{-1} of BDO with a yield of 0.34 g g^{-1}. Ehsani et al. (2009) studied the importance of BDH1 and NADH cofactor for BDO production by overexpressing

BDH1 in a recombinant *S. cerevisiae* (Δ*gpd* and Δ*ald6*). Point mutation was done for the amino acids in the region of 221 EIA 223 to 221 SRS 223 of BDH. It was observed that engineered BDH1 redirected 90% of the flux from acetoin to BDO. Introduction of BDO pathway, redox balance and elimination of competing pathways, such as ethanol and glycerol, led to enhanced BDO formation. Yamada et al. (2017) screened a plasmid library of 15 different promoters for the efficient expression of *B. subtilis alsS, B. amyloliquefaciens alsD, bdh1* and *L. lactis noxE*, leading to a recombinant strain that produced 80 g L^{-1} BDO in fed-batch cultivation.

Pichia pastoris, a methylotrophic yeast, has been exploited for the production of various recombinant proteins and enzymes. The microorganism has the potential to utilize methanol as carbon and energy source due to the presence of alcohol oxidase gene. It is a GRAS microorganism with established genetic tools and high cell density cultivation. Yang and Zhang (2018b) integrated codon optimized *B. subtilis alsS* and *alsD*, and *bdh1* derived from *S. cerevisiae* in the genome of *Pichia pastoris* under the control of constitutive GAP (glyceraldehyde phosphate) promoter. Statistical-based medium optimization led to the production of 74.5 g L^{-1} D-BDO in fed-batch cultivation using glucose as carbon source.

Utilization of CO_2 for the biosynthesis of value-added products is gaining more attention in the area of sustainable process development. Photosynthetic organisms, such as cyanobacteria, can fix CO_2 and can be engineered to produce platform chemicals and biopolymers. *Synechococcus elongatus* PCC7942 was engineered by introducing the *alsSD* operon and sADH coding for NADPH-dependent secondary alcohol dehydrogenase, and optimized RBS (ribosomal binding site) from *B. subtilis* strain. Production of BDO from CO_2 reached 2.38 g L^{-1} with a productivity of 9.8 μg L^{-1} h^{-1} (Oliver et al., 2013). Even though the production is very low, this study shows a glimpse of hope for converting CO_2 into platform chemicals.

Corynebacterium glutamicum is a GRAS microorganism, which is used as cell factory for the commercial synthesis of amino acids and other value-added chemicals. A gene cluster of *als*, *alsB* and *butA* from *L. lactis* was introduced in *C. glutamicum* under IPTG inducible promoter with the deletion of genes encoding pyruvate dehydrogenase (*aceE*), *mdh, ldhA* and pyruvate:quinone oxidoreductase (*pqo*). The mutant strain was able to produce BDO (6.3 g L^{-1}) under optimized aeration condition (Radoš et al., 2015). Yang et al. (2015a) overexpressed *K. pneumoniae budB* and *budA* in *C. glutamicum* for the production of 18.9 g L^{-1} BDO using 80 g L^{-1} glucose in a batch cultivation. Zhang et al. (2019) developed a recombinant strain of *C. crenatum* by adapting a synthetic engineering approach. The authors introduced *alsS, alsD* and *bdh* genes from *B. subtilis* 168 and further to improve the selectivity of BDO they have eliminated *ldhA* gene which resulted in the production of 88.8 g L^{-1} with yield of 0.8 mol mol^{-1}. Nguyen et al. (2018) used in silico gene knockout method based on evolutionary algorithm to modify *Methylomicrobium alcaliphilum* 20Z. The *budA* of *K. pneumoniae* and *budB* of *B. subtilis* were cloned under Tac promoter and the developed Δ*ldh* Δ*ack* Δ*mdh* triple mutant produced 86.2 mg L^{-1} BDO using methane as carbon source under oxygen limited condition.

6.4 Pathway manipulation for enhanced acetoin biosynthesis

Most of the acetoin producing strains have *budC* gene which converts the acetoin to BDO with the consumption of 1 mole of NADH. Strains such as *Enterobacter, Klebsiella* and *Serratia* can produce more than 130 g L^{-1} of BDO (Ma et al., 2009a; Jung et al., 2014; Um et al., 2017). The preliminary strategy to enhance acetoin production is to simply delete the *budC* gene encoding BDH. Wang et al. (2015) reported higher accumulation of R-acetoin in the *budC* mutant *K. pneumoniae* in glucose-based medium. The strain having active *acoABCD* gene encoding acetoin dehydrogenase was able to consume acetoin as carbon source. Deletion of *acoABCD* gene resulted in loss of acetoin dissimilation ability. Providing high aeration with

mild acidic condition, R-acetoin production of 62.3 g L^{-1} was achieved in 57 h fed-batch cultivation with 28.7% substrate conversion efficiency. The deletion of *budC* gene causes redox imbalance towards NADH. This problem can be resolved by the overexpression of NADH oxidase (NOX) in the engineered strain. NADH co-factor plays a significant role in the interplay between acetoin and BDO. In *B. subtilis*, major by-products such as lactic acid, ethanol and BDO are produced in NADH dependent pathways. Manipulation of NADH levels in the strain can aid in redistribution of carbon flux towards acetoin. Overexpression of *yodC* gene encoding water dependent NADH oxidase in *budC* deleted *B. subtilis* strain displayed reduced by-product formation (Zhang et al., 2014). Sun et al. (2012) performed co-factor engineering by expressing NADH oxidase from *L. brevis* in *S. marcescens* H32, which tends to accumulate higher BDO. The NADH level increased by 1.5-fold in the engineered strain which significantly reduced the accumulation of BDO and enhanced acetoin production. Acetoin production of 75.2 g L^{-1} with productivity of 1.88 g L^{-1} h^{-1} was obtained in fed-batch fermentation mode. Similar approach was applied by Gao et al. (2014) by disrupting *budC* in *S. marcescens* G12 leading to higher acetoin production (47.5 g L^{-1}). Bao et al. (2014) developed an engineered *B. subtilis* 168 strain by overexpressing NOX protein which decreased the intracellular NADH concentration (1.6-fold) and NADH/NAD^+ ratio (2.2-fold) resulting in acetoin production of 91.8 g L^{-1}. Bao et al. (2015) developed a strategy to enhance acetoin production using a mixture of glucose and gluconate, which reduced the NADH/NAD^+ ratio. Overexpression of oxidative pentose pathway by increased expression of glucose-6-phosphate dehydrogenase resulted in a decrease in the intracellular NADH/NADPH ratio (1.9-fold) and NADH/NAD^+ ratio (1.7-fold). The mutant strain was able to produce 43.3 g L^{-1} acetoin in fed-batch cultivation.

Promoter plays a crucial role in the expression of genes as stronger promoter aid in efficient binding of RNA polymerase and translation mechanism. Zhang et al. (2013a) generated an engineered *B. subtilis* strain by expressing the transcription regulator *alsR* gene (control the transcription of *alsSD* operon) under *pbdh* promoter, which increased acetoin production (41.5 g L^{-1}). Li et al. (2014) adapted in silico method to channelize the pyruvate flux toward the production of acetoin. A synthetic metabolic pathway involving ALS and ALDC was constructed to produce acetoin in *C. glabrata*. With the addition of nicotinic acid to regulate NADPH level, *C. glabrata* produced 3.67 g L^{-1} acetoin. The metabolic and regulatory pathways of *C. glabrata* were subsequently studied and the carboligase activity reaction (CAR) was identified. The pathway involving genes such as alcohol dehydrogenase, acetaldehyde dehydrogenase, pyruvate decarboxylase and butanediol dehydrogenase was crucial for acetoin production. With rational engineering, the strain produced 2.24 g L^{-1} acetoin. With in silico simulation and redox balancing, NADH was identified as the limiting factor and *noxE* overexpression led to increased acetoin production (7.33 g L^{-1}). Subcellular compartmentalization plays an important role in acetoin biosynthesis as the α-acetolactate synthesized in the mitochondria is transported across the membrane to form acetoin. This process resulted in low yield of acetoin due to BDH and oxidized decarboxylation. This hurdle can be reduced by introducing the heterologous acetoin pathway in mitochondria and increasing the availability of mitochondrion pyruvate by coupling acetoin pathway with mitochondrial pyruvate carrier. This engineering approach improved acetoin production (3.26 g L^{-1}), which is 59.8 % higher than acetoin production achieved in the cytoplasmic pathway (Li et al., 2015b).

E. aerogenes is a well known producer of acetoin. However, the diversion of carbon flux towards by-product formation hinders the accumulation of acetoin. Jang et al. (2017a) eliminated the production of lactate and BDO by elimination of *ldhA, budC* and *dhaD* genes to redirect the carbon flux towards acetoin production. Under aerobic condition, the engineered *E. aerogenes* strain accumulated 2-ketogluconate due to the activation of a glucose oxidative

pathway leading to reduced acetoin production. The 2-ketogluconate accumulation was significantly reduced by deleting the gene *gcd* encoding glucose dehydrogenase leading to increased acetoin production (71.7 g L^{-1}) with 2.87 g L^{-1} h^{-1} productivity.

Bacillus strains can also produce acetoin. However, the presence of *bdhA* gene converts acetoin into BDO, which in combination with other by-product synthesis reduce acetoin production. Zhang et al. (2017) adapted Cre-lox system to disrupt the *bdhA* gene from *B. subtilis* 168, which completely blocked the production of BDO and improved the acetoin production 1.5 times as compared with the parental strain. Overexpression of genes in the genome generates a metabolic stress and channelizes the carbon flux towards recombinant protein generation, ultimately leading to reduced supply of energy currencies such as ATP. Genome reduction approach is best strategy to overcome the hurdle of metabolic burden. Yan et al. (2018) endowed the genome reduction by deleting *bdhA* and *acoA* encoding acetoin dehydrogenase E1 component, which converts acetoin to acetaldehyde and acetyl-coA. Further with incorporation of xyl operon comprising of xylose isomerase (XI) and xylulose kinase (XK) the strain showed ability to uptake the pentose sugar. The uptake of xylose is further increased by deleting *araR* transcriptional repressor of the arabinose operon. The mutant strain showed 39 % higher acetoin production compared to the wild type strain with xylose to acetoin yield of 0.46 g g^{-1}. Li et al. (2018) demonstrated the consolidated bioprocessing concept by incorporation of endo 1,4-beta-xylanase and β-xylanase in *B. subtilis* strain with Δ*acoA* and Δ*bdhA*. The recombinant strain produced 6.9 g L^{-1} acetoin with conversion yield of 0.345 g g^{-1}. Overexpression of acetolactate synthase (AlsA) in *B. licheniformis* WX-02 showed the K_m and K_{cat} of 3.96 mM and 514/s for pyruvate respectively and were able to produce 57.06 g L^{-1} acetoin and BDO (Huo et al., 2018). Fan et al. (2018) designed and constructed the metabolic pathway for the simultaneous production of uridine and acetoin by deleting the *bdhA* gene in the pathway of *B. subtilis*. The mutant strain exhibited positive correlation with the production of both products, but the main product was slightly disturbed by the production of acetate. This hurdle was addressed by overexpressing the alsSD operon which boosted acetoin production and abolished acetate accumulation. With the integration of medium optimization, the recombinant strain produced 60.5 g L^{-1} acetoin and 40.6 g L^{-1} uridine using glycerol as carbon source. Yuan et al. (2019) demonstrated the acetoin toxicity on the growth of *B. licheniformis* and the resistance mechanism in the strain. The metabolic profiles of the strain were tested at 20, 40 and 60 g L^{-1} of acetoin. The transcriptional analysis revealed that 119 metabolites are involved in the mechanism. The TCA and pentose pathway were negatively affected, while the glyoxylate pathway was active to supply malic acid. The acetoin stress resulted in build up of amino acids such as glycine, leucine, proline and valine and the saturated fatty acids ratio was elevated. It was known that acetoin could inhibit cell growth by inducing the concentration of intracellular reactive oxygen species and damage to mitochondria there by resulting in cell apoptosis. The amino acid metabolism and osmoregulation are the key active mechanisms against the inhibitory effects of alcohols and aldehydes in the microbial cells, hence build up of these mechanisms substantiate the cell response towards acetoin accumulation (Yuan et al., 2019).

K. pneumoniae may produce acetoin from glycerol as it bears *budABC* gene cluster. Deletion of two BDO genes namely *budC*, *dhaD* and transcriptional regulator *acoK* gene and overexpression of *noxE* gene led to 51 g L^{-1} acetoin with a yield of 0.34 g g^{-1} (Jang et al., 2017b). Furthermore, the *K. pneumoniae* KP-9 overexpressed with *glpK* and *noxE* and Δ*budC*, Δ*dhaD*, and Δ*gldA* showed acetoin production of 32.2 g L^{-1} in fed-batch cultivation (Wang et al., 2017). The concept of whole cell catalysis was introduced to produce S-acetoin and L-BDO from meso-BDO in *E. coli* (**Table 3**). Heterologous R,R-BDH and NADH oxidase genes are co-

expressed in *E. coli* to produce S-acetoin. The recombinant strain was able to produce S- acetoin (72.4 g L^{-1}) with the stereoisomeric purity of 94.65%.

7. Feedstock selection and prospects

The economic viability of industrial BDO and acetoin production is highly dependent on the cost of raw material (Jang et al., 2018; Koutinas et al., 2016). The carbon sources employed for BDO and acetoin fermentation are mainly commercial refined sugars. Renewable resources have been recently used as alternative, abundant, and cheap substrates for BDO and acetoin production (**Tables 1 and 3**). The substrates mainly used so far for the production of BDO and acetoin can be classified into three categories: a) agricultural products and lignocellulosic residues, b) food industry side streams, and c) biofuel industry by-products (**Fig. 5**). Lignocellulosic biomass requires thermochemical pretreatment procedures (e.g. dilute acid, steam explosion, hydrothermal) followed by enzymatic hydrolysis (e.g. cellulase, hemicellulase) to produce hydrolysates rich in C5 and C6 sugars (Khare et al., 2015). Starch and sugar crops have been also used for BDO production (Božić et al., 2011). Simultaneous saccharification and fermentation has also been reported using a microbial strain capable of secreting hydrolytic enzymes, while at the same time it can convert various sugars into acetoin and BDO (Li et al., 2014; John et al., 2007). Food industry side streams and crude glycerol from the biodiesel industry could be also used for BDO production (Salakkam and Webb, 2018).

Corn stover hydrolysate has been considered as an alternative substrate for BDO and acetoin production. Li et al. (2014b) reported BDO concentration of 74 g L^{-1} with productivity of 2.1 g L^{-1} h^{-1} by *B. liqueniforms* using corn stover hydrolysate. The strain showed great tolerance to inhibitors present in lignocellulosic hydrolysate such as furfural, vanillin, formic acid and acetic acid. Ma et al. (2018) studied the valorization of corn stover hydrolysate in three fermentation modes using *P. polymyxa* for D-BDO production. Fed-batch fermentation led to 46.12 g L^{-1} D-BDO concentration, but productivity was relatively low (0.57 g L^{-1} h^{-1}). Significant improvement of productivity was achieved in continuous fermentation (1.13 g L^{-1} h^{-1}). A metabolically engineered *E. cloacae* SDM could utilize glucose and xylose simultaneously for the production of D-BDO (119.4 g L^{-1}) from corn stover hydrolysate with a productivity of 2.3 g L^{-1} h^{-1} (Li et al., 2015a). Zhang et al. (2016b) reported the efficient valorization of corn stover hydrolysate for acetoin production (45.6 g L^{-1}) in fed-batch cultures of *E. cloacae.*

Apple pomace, a by-product generated after juice separation, containing cellulose (43.2 %) and hemicellulose (20.3 %), has been applied for BDO production. Enzymatic hydrolysis of apple pomace using a mixture of cellulase, xylanase, pectinase and invertase led to 105.9 g L^{-1} reducing sugar. The subsequent fermentation with *B. licheniformis* NCIMB 8059 produced 77.6 g L^{-1} BDO in fed-batch culture (Białkowska et al., 2015).

Sweet sorghum stalk juice containing fermentable sugars and other nutrients has been utilized for BDO production by *S. marcescens* (Yuan et al., 2017). Under optimized conditions, 109.4 g L^{-1} of BDO was produced with a productivity of 1.4 g L^{-1} h^{-1} in fed-batch fermentation. Sorghum stalks, witchgrass poplar and oil palm front biomass has been evaluated for BDO production, however low production has been achieved (Guragain and Vadlani, 2017; Hazeena et al., 2020).

Fruit and vegetable wastes from open markets have also been used as carbon sources in BDO production via fermentation (Liakou et al., 2017). Dilute acid hydrolysis was initially evaluated for the production of sugar-rich hydrolysate that was subsequently used as carbon source for BDO production (50 g L^{-1}) by *E. ludwigii* at a conversion yield of 0.40 g g^{-1}.

Jerusalem artichoke contains cellulose and hemicellulose in stalk and inulin in the tuber. Utilization of inulinase or microbes secreting inulinase can efficiently hydrolyse inulin into

glucose and fructose. Sun et al. (2009) performed separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) of Jerusalem artichoke tubers by *K. pneumoniae*. BDO and acetoin production of 81.6 g L^{-1} and 91.6 g L^{-1} was obtained in SHF and SSF respectively in fed-batch fermentation. Li et al. (2010a) evaluated Jerusalem artichoke stalk hydrolysate in fed-batch fermentation by *K. pneumoniae.* The highest BDO concentration (67.4 g L^{-1}) was obtained using in a stage-shift aeration mode.

Molasses, produced after crystallization of sucrose in sugar production facilities, contains around 50% fermentable sugars along with salts, proteins and vitamins. It is a suitable feedstock for BDO production. Bailkowska et al. (2016) cultivated *B. subtilis* in fed-batch cultures for BDO production using molasses as feedstock. The highest BDO concentration (75.7 g L^{-1}) was achieved using molasses as carbon source and glucose as feeding medium (Baikowska et al., 2016). Jung et al. (2013a) reported BDO production (98.7 g L^{-1}) from sugarcane molasses by *E. aerogenes*. The optimization of media composition using molasses as carbon source has been studied by Dai et al. (2015b) leading to increased BDO production. In fed-batch fermentation mode and under optimized conditions, 99.5 g L^{-1} of BDO plus acetoin were produced with a productivity of 1.66 g L^{-1} h^{-1}.

Cheese whey, a side stream from cheese production, is another renewable resource of interest for BDO and acetoin production. Guo et al. (2017a) optimized the media composition using cheese whey as carbon source with *K. pneumoniae* leading to 57.6 g L^{-1} of BDO with conversion yield of 0.40 g g^{-1}. *Lactococcus lactis* was able to produce acetoin (27 g L^{-1}) and BDO (83 g L^{-1}) in shake flask fermentation using whey permeate as substrate (Kandasamy et al., 2016).

The valorisation of crude glycerol generated as by-product from the biodiesel industry has been also employed for BDO production employing various wild and metabolically engineered strains. *B. amyloliquefaciens* was reported to produce 102.3 g L^{-1} of BDO without by-product formation in fed-batch fermentation mode using crude glycerol supplemented with molasses as carbon source (Yang et al., 2015c). The most efficient BDO producers are metabolically engineered strains by simultaneous expression of the genes involved in BDO production and inactivation of competitive pathways. Kim et al. (2017b) demonstrated BDO production of 65.4 g L^{-1} without 1,3-propanediol formation from crude glycerol using a *bud*ABC overexpression mutant *R. ornithinolytica*. In the same study, pretreatment of crude glycerol improved BDO production and yield by 19% and 7.7%, respectively. The highest BDO production (131.5 g L^{-1}) from crude glycerol was achieved by a metabolically engineered *K. oxytoca* strain. Deletion of *pdu*C and *ldh*A genes improved BDO production and reduced by-product formation (1,3-propanediol and lactic acid). A mixture of L-BDO and meso-BDO in ratio of 0.92:1 was produced by *K. oxytoca* M3 (Cho et al., 2015b).

8. Bioprocess development

8.1 Oxygen supply

Oxygen supply during fermentation is considered one of the most critical factors in BDO production affecting the distribution of metabolites produced, yield and productivity (Celińska and Grajek, 2009; Ji et al., 2011a). BDO is produced under limited oxygen supply to maintain an internal redox balance of pyridine nucleotide pairs (NAD^+/NADH and $NADP^+$/NADPH) during glycolysis and biosynthesis. Under low oxygen supply, the respiratory chain cannot effectively regenerate the excess reducing power associated to glycolysis and NADH-consuming pathways are activated leading to the formation of by-products (Converti et al., 2003). At high oxygen supply, NADH from glycolysis is generated via respiration thus bacterial growth and CO_2 formation are favored. Controlling the oxygen supply could modify the ratio

of acetoin to BDO. The formation of metabolites is strongly associated with oxygen availability and the NAD^+/NADH balance. In general, relatively low oxygen supply favour BDO production, while high dissolved oxygen concentrations favour acetoin production. Therefore, a suitable oxygen supply by manipulation of dissolved oxygen concentration is crucial for efficient BDO and/or acetoin production.

Several control parameters including oxygen transfer rate, OTR (Beronio and Tsao, 1993), volumetric oxygen transfer coefficient, k_La (Häßler et al., 2012; Maina et al., 2019b; Rebecchi et al., 2018), oxygen uptake rate, OUR (Converti et al., 2003) and respiratory quotient, RQ (Zeng et al., 1994; Zhang et al., 2010b) have been applied to control BDO production during fermentation.

RQ has been used as oxygen control parameter for BDO production by *E. aerogenes* (Zeng et al., 1994). A detailed study was carried out in batch, fed-batch and continuous mode in various bench-top and pilot plant bioreactors. High BDO concentration (96 g L^{-1}) with additional acetoin production of 6.9 g L^{-1} was achieved by controlling RQ value between 4 to 4.5 in fed-batch fermentation mode. RQ was further used as control strategy in different bioreactors for scale-up of BDO production showing that bioreactor hydrodynamics and initial OTR affect BDO synthesis (Zeng et al., 1994). The same strategy has been applied in fed-batch fermentation by Zhang et al. (2010b). By controlling RQ value within the range of 1 to 1.5 in the growth phase and between 1.8 to 2 in BDO production stage, improved BDO and acetoin concentration (146.6 g L^{-1}) was produced by *S. marcescens* H30 (Zhang et al., 2010b).

Häßler et al. (2012) studied the effect of oxygen supply on BDO production by *P. polymyxa* using k_La as control parameter. Various k_La values within the range of 19.7 to 194 h^{-1} were evaluated. Increasing oxygen availability caused reduction in D-BDO formation with simultaneous increased meso-BDO and acetoin formation. Low oxygen availability favored lactate and ethanol production. The highest D-BDO production of 46.1 g L^{-1} was achieved at k_La value of 30 h^{-1}. Agitation cascade with k_La value within the range of 46-77 h^{-1} led to the highest productivity (3.95 g L^{-1} h^{-1}) in fed-batch fermentation of *E. ludwigii* (Maina et al., 2019b).

A simple oxygen supply method based on constant agitation speed has been extensively used for BDO production. High agitation rates stimulate bacterial growth, acetoin and acetate accumulation leading to decreased BDO yields, whereas low agitation rate favor BDO and by-product (namely succinate, lactate and ethanol) formation (Priya et al., 2016; Maina et al., 2019a). Cho et al. (2015) reported BDO and acetoin production of 118.5 g L^{-1} and 42.1 g L^{-1}, respectively at 400 rpm with low by-product accumulation. Similar results have been reported by Kim et al. (2016b) using *Raoutella ornithinolytica*. BDO concentration of 68.3 g L^{-1} was obtained at 400 rpm accompanied by low formation of NADH-dependent by-products. Increasing agitation rate at 500 rpm, bacterial growth and acetic acid production was enhanced resulting in low BDO yield (0.19 g g^{-1}). However, BDO production by *B. licheniformis* was enhanced at low agitation rate (200 rpm) with high formation of formic acid, though higher agitation rates (400 rpm) resulted in equal amounts of BDO and acetoin (Li et al., 2013).

The evaluation of two-stage agitation and aeration rates has been proposed for BDO production. High oxygen supply at the growth phase followed by low oxygen availability in the stationary phase has been evaluated (Ji et al., 2009; Yang et al., 2015; Priya et al., 2016). BDO concentration of 115.7 g L^{-1} with productivity of 2.4 g L^{-1} h^{-1} has been achieved by *B. licheniformic* using two-stage agitation speed (Li et al., 2013).

8.2 pH

pH value is another important parameter that regulates the intracellular enzyme activity and hence affects the composition of metabolites by altering the metabolic pathway. The optimal pH value of key enzymes for BDO synthesis (ALS, ALDC and BDHs) vary for different microorganisms. The activity of BDH is decreased with increasing pH values from 5 to 7.3 for *E. aerogenes* (Nakashimada et al., 2000). Moreover, the highest specific activity of BDH was at pH 6 in cultivation with *P. polymyxa* (Gao et al., 2013b). ALS, which is responsible for the first step in acetoin-BDO pathway, is inactivated at pH values above 6 in *K. pneumoniae* (Stormer, 1986; Chan et al., 2016).

The metabolic function of acetoin and BDO biosynthesis is physiologically important to the microorganisms to prevent intracellular acidification. Generally, pH value above 6.5 favours the production of organic acids, causing reduced BDO formation. Organic acid production results in culture acidification with increasing concentrations of toxic undissociated forms of acids, resulting in inhibitory effects on both bacterial growth and BDO and acetoin biosynthesis. On the other hand, pH value equal or below 6.5 leads to reduction of organic acid synthesis corresponding to enhanced BDO and acetoin formation (Celińska and Grajek, 2009).

Lee et al. (2017) reported maximum BDO production at pH value of 6.5 using *E. aerogenes*. Controlling pH value at higher levels resulted in accumulation of succinate, acetate and ethanol, whereas pH value lower than 6.5 led to reduced bacterial growth and substrate consumption rate (Lee et al., 2017). Similar optimal pH value has been demonstrated by Wong et al. (2014) using *Klebsiella sp*. In general, the optimum pH value for BDO production by *K. oxytoca* has been reported to be in the range of 5 to 6 (Xiao and Lu, 2014), while high pH values (6.8 to 7) are beneficial for enhanced bacterial growth. A two-stage pH control strategy maintaining pH at 6.0 after a natural pH drop from (pH value of around 7) neutral culture media has been also applied for BDO and acetoin production leading to increased concentration and yield (Sun et al., 2012; Kim et al., 2016c).

8.3 Temperature

Enzyme activity, cellular maintenance and product formation are strictly temperature dependent processes. Mesophilic species have optimum bacterial growth at temperature within the range of 25 to 45 ˚C. Acceleration of enzymatic processes has been observed with increasing temperature (Xiao and Lu, 2014).

Perego et al. (2003) reported a progressive elevation of BDO production and productivity from 34 to 37 ˚C by *B. licheniformis*. Similarly, optimal temperature for growth and BDO formation by *B. amyloliquefaciens* was at 37 ˚C. The reverse behaviour has been reported for acetoin production when temperature was increased from 25 to 40 ˚C (Yang et al., 2011). The effect of temperature on BDO production by *K. pneumoniae* and *E. aerogenes* has been evaluated by Barret et al. (1983). A temperature of 33 ˚C was optimal for *K. pneumoniae*, while changes in temperature within the range of 30 to 37 ˚C had little effect on BDO formation by *E. aerogenes*. In general, the optimum temperature for BDO production by *Bacilliaceae* strains is in the range of 34 to 37 ˚C (Li et al., 2013), whereas *Klebsiella* and *Enterobacter* strains have optimal temperature from 30 to 37 ˚C (Celińska and Grajek, 2009). However, the optimal temperature for growth and BDO formation by a newly isolated *R. ornithinolytica* strain was 25 ˚C (Kim et al., 2016b). Different strains may have diverse optimal temperature, therefore the optimal value should be evaluated individually for each strain and substrate used.

Thermophilic strains (*Geobacillus sp.* and *Bacillus licheniformis*) have been applied for BDO production (Xiao et al., 2012; Li et al., 2013; Ge et al., 2016). Thermophilic fermentation

process could reduce the risk of bacterial contamination and can be operated without sterilization making the process more efficient and cost-effective. Thermophilic strains have been successfully applied for simultaneous saccharification and fermentation resulting in a more cost-effective process (Li et al., 2014a). Typically, the optimum temperature of thermophilic strains is in the range of 50 to 60 ˚C (Xiao et al., 2012).

8.4 Nitrogen source

Nitrogen source is necessary for bacterial growth and accumulation of the desired metabolite. Complex organic nitrogen sources including yeast extract, peptone, casamino acid and corn steep liquor (CSL) could accelerate BDO and acetoin production (Tables 1 and 2). Yeast extract containing the essential growth micronutrients has been mainly used for BDO and acetoin production.

Zhang et al. (2010b) reported BDO production of 139.9 g L^{-1} with productivity of 3.49 g L^{-1} h^{-1} using 33.4 g L^{-1} yeast extract. However, the high concentration of yeast extract leads to increased production cost (Koutinas et al., 2016). CSL has been applied as an alternative and inexpensive organic nitrogen source (Ma et al., 2009; Sun et al., 2012; Yang et al., 2015c). A mixture of yeast extract and CSL has been applied for enhanced BDO and acetoin production by *Bacillaceae* species (Yang et al., 2013a). However, high concentrations are needed for enhanced production. The usage of inorganic nitrogen sources including urea and ammonium sulfate have been also evaluated. Efficient BDO production has been achieved using inorganic media composition by *E. ludwigii* (Maina et al., 2019).

8.5 Media composition

Mineral supplements are important for bacterial growth and product formation. Different media compositions could lead to alterations in the intracellular metabolic activities resulting in different fermentation products. Recent studies have indicated the important role of phosphate, acetate, Fe^{2+}, Mn^{2+}, K^{+} and Mg^{2+} salts on cell metabolism, substrate consumption rate and BDO production (Ma et al., 2009a; Adlakha and Yazdani, 2015). Specifically, phosphate ions improve the formation and yield of diol (Laube et al., 1984), Mn^{2+} activates ALDC in the BDO pathway (Laube et al., 1984), Fe^{2+} stimulates the growth and increase the consumption rate (Deshmukh et al., 2015). K^{+} ions having essential role in bacterial physiology, including the osmoregulation and maintenance of cellular pH, are important in the structure and function of various enzymes (Kinsinger et al., 2005), EDTA improves the cell permeability which may enhance the mass transfer (Song et al., 2012). High concentrations of each supplement could result in osmotic stress resulting in inhibitory effect upon enzymatic activity and hence product formation.

The distribution of acetoin and BDO stereoisomers is also affected by the metal ions composition. It has been demonstrated that the BDHs activity from *Corynebacterium crenatum* was increased by Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} ions, though Cu^{2+} inhibited BDHs activity (Zhao et al., 2015). Yu et al. (2015) reported and characterized the D-BDH from *Rhodococcus erythropolis*. The BHDs activity was enhanced by K^{+} cations, slightly decreased with Na^{+}, Mg^{2+} and Co^{2+}, and inhibited by Ag^{+}, Cu^{2+} and Fe^{2+}. In the study with *Serratia marcescens*, it was found that the reaction of 3S-acetoin to meso-BDO could be strongly inhibited by Fe^{2+} and Fe^{3+}, while the presence of Mn^{2+} could increase the efficiency of the reaction (Zhang et al., 2014).

Acetate supplementation for enhanced BDO production has been widely evaluated. Ernest and Saddler (1982) showed that growth and BDO yield by *K. pneumoniae* was increased in the presence of acetic acid (1-5 g L^{-1}). Acetic acid in concentration of more than 10 g L^{-1} inhibits

the growth and BDO formation. It has been reported that the undissociated form of acetic acid can penetrate into the cytoplasm of the cell resulting in acidification and depletion of ATP (Zeng et al., 1990). Recently, sodium and potassium acetate in the range of 0-0.2 M were evaluated as sources of acetate. The optimal source for BDO production by *E. aerogenes* was potassium acetate at concentration of 0.1 M (Lee et al., 2017). On the other hand, sodium acetate at concentration of 2.02 g L^{-1} was found to induce acetoin production by *P. polymyxa* (Zhang et al., 2012).

8.6 Substrate and product inhibition on BDO and acetoin

Initial substrate concentration along with products formation affect cell growth, BDO and acetoin production. High concentration of sugars causes osmotic stress resulting in cell membrane disruption and cell death, while organic acids may inhibit cell growth by altering intracellular pH and deactivating enzymes. Generally, BDO producers tolerate synthetic sugar concentrations up to 200 g L^{-1}. High specific growth rates and yields are normally obtained at lower concentration of sugars (Kim et al., 2016a). Increased initial concentration of substrate influence the metabolic pathway leading to enhanced by-products formation (1,3-propanediol, acetic acid and ethanol) (Priya et al., 2016).

The inhibitory effect of fermentation products on growth of BDO producers has not been extensively studied. Kim et al. (2016a) proposed a model for substrate and product inhibition for BDO production by *K. oxytoca*. Formic acid and acetic acid had the strongest inhibition effect, whereas high tolerance to succinic acid was observed. *K. oxytoca* showed high tolerance to BDO and ethanol (74 and 36 g L^{-1} respectively), though acetoin strongly inhibited and completely ceased the growth at concentration of 23.6 g L^{-1}. A reduction of specific growth rate by 50% has been observed in *E. aerogenes* with 20 g L^{-1} of ethanol (Zeng et al., 1991a). The growth of *P. polymyxa* was inhibited by 60 g L^{-1} of D-BDO (Okonkwo et al., 2017).

8.7 Fermentation mode

The bioreactor operation mode is an important factor for optimal process design. Various bioreactor operation modes, including batch, fed-batch, continuous cultures, cell recycle and immobilized cell systems, have been implemented for BDO production (Tables 2 and 3). Batch and fed-batch operation modes have been widely studied. Fed-batch fermentation mode has been used to overcome the effect of initial substrate inhibition and it is considered the most favourable operation mode for industrial scale. Using a balanced feeding strategy could lead to high BDO concentration. Ma et al. (2009) compared different fed-batch strategies for BDO production, including constant feed rate, pulse, constant residual glucose concentration and exponential feeding. Maximum BDO and acetoin production of 160 g L^{-1} with productivity of 4.21 g L^{-1} h^{-1} was obtained by constant residual glucose concentration feeding method. A similar method was applied in BDO fermentation by *S. marcescens* (Zhang et al., 2010b). Generally, constant feeding of substrate provides suitable environment for cell metabolism and BDO production.

Continuous fermentation with cell recycle by maintaining high biomass density allows high and stable process efficiency with high values of productivity. Contamination is still the limiting factor of this technique. Improved economic viability can be achieved by continuous fermentation (Ji et al., 2011). Zeng et al. (1991b) evaluated continuous culture with cell recycle system resulting in high productivity (14.6 g L^{-1} h^{-1}), though the final production of BDO and acetoin were lower than in fed-batch mode (54.2 and 110 g L^{-1}, respectively).

A promising system employing immobilized cells has been also evaluated to increase BDO efficiency. Maximum BDO concentration of 118.3 g L^{-1} was achieved by *B. licheniformis*

immobilized cells. Compared to free cells, the immobilized system showed lower BDO concentration. However, using proper amount of nutrients could enhance the efficiency of the bioprocess (Jurchescu et al., 2013).

8.8 Downstream separation and purification

Downstream separation and purification (DSP) of BDO should be achieved at low energy consumption and high recovery efficiency. The recovery of BDO is difficult due to the high boiling point, the hydrophilic nature and the presence of dissolved and solid components (Xiao and Lu, 2014).

Until now, a promising commercial technique for BDO recovery is the simulated moving bed (SMB) implemented in LanzaTech fermentation plant for the production of anhydrous ethanol and BDO (Schultz et al., 2015). However, the drawbacks of SBM are the existence of strong non-linear behavior during design and optimization and the expensive unit operations employed.

Several separation methods including distillation (Kawamura et al., 2014), pervaporation (Qureshi et al., 1994), steam stripping (Xiu and Zeng, 2008), solvent extraction (Eiteman and Gainer, 1989), salting-out (Dai et al., 2017) and reverse osmosis (Xiu and Zeng, 2008; Kawamura et al., 2014) have been reported for BDO recovery. These methods could also be applied in the acetoin DSP process due to their similar properties. Though, these techniques have some drawbacks and limitation for industrial application. The conventional steam stripping and distillation require high energy consumption. Salting-out extraction (SOE) using hydrophilic solvents is mainly used for the separation and purification of BDO and acetoin. The employment of K_2HPO_4/ethanol system resulted in 99% BDO recovery, 98.7% acetoin and 89% protein removal from fermentation broth (Jianying et al., 2011; Dai et al., 2017). Due to the hydrophilic nature of solvent used in SOE, large amount of water existed in the top phase requiring high energy demand for the recovery. Solvent extraction methods require utilization of high solvent volumes and the recovery efficiency of BDO is relatively low (Shao and Kumar, 2009). The development of an efficient process to fulfil the requirement of separation is necessary to be integrated with other methods.

A combination of liquid-liquid extraction with distillation known as hybrid extraction distillation has been proposed (Harvianto et al., 2018). Solvent is used to extract the BDO. The organic phase is then inserted into a distillation column where the solvent is recycled back to the extractor and BDO is recovered. Harvianto et al. (2018) evaluated various solvents including ethyl acetate, butyl acetate, 1-butanol, isobutanol, 2-ethyl-1-hexanol and oleyl alcohol for BDO recovery. High distribution coefficient was obtained with oley alcohol.

A novel method combing solvent extraction and salting-out known as aqueous two-phase extraction has been applied for BDO recovery. Salting-out increases the extraction efficiency and decreases the quantity of solvent needed for extraction. Ethanol/ammonium sulfate (Li et al., 2010b), ethanol/phosphate (Jiang et al., 2009), isopropanol/ammonium (Sun et al., 2009a) and acetone/phosphate (Sun et al., 2012) systems have been evaluated for BDO and acetoin purification achieving high recovery efficiency.

Another recovery technique named as fermentation-derivatization-recovery was studied (Xiao and Lu, 2014). This method relies on the derivatization of BDO and acetoin to other valuable compounds, which subsequently are recovered and purified from a complex mash. 2,3,5,6-tetramethylpyrazine (TTMP) can be generated by the condensation of acetoin and ammonia in aqueous solution under mild conditions (Rizzi, 1988). The TTMP can be recovered and purified by crystallization. Extraction of BDO by cyclic acetal formation using

formaldehyde or acetaldehyde for acetalization has been reported by Hao et al. (2006). The acetals formed are recovered by aromatic solvent extraction or distillation. This technique requires less operating time and small amount of reactant and extractant.

9. Techno-economic evaluation of BDO production

Koutinas et al. (2016) estimated the minimum selling price of bio-based BDO production (MSP_{BDO}) using glycerol, sucrose and sugarcane molasses as carbon sources using literature-cited experimental data. The annual BDO production capacity, the raw materials used, the fermentation efficiency (expressed as carbon source to BDO conversion yield, productivity and final BDO concentration) and the DSP employed are the main factors that influence the BDO production cost. The MSP is reduced significantly up to 50 kt/year BDO production capacity where economies of scale have been attained. The production capacity should be always considered in techno-economic evaluation studies as this is also related to feedstock requirements. In the case of renewable resources, feedstock requirement is an important aspect as logistics of feedstock collection and transportation should be considered in biorefinery development.

The cost of carbon source and nutrient supplements used in fermentation media formulation influence significantly the MSP of BDO. Reduced cost due to the carbon source utilisation could be achieved by increasing the conversion yield during fermentation achieving values close to the theoretical conversion yield. Including complex nitrogen sources (e.g. yeast extract) in fermentation media increase significantly the cost of raw materials. Koutinas et al. (2016) mentioned that the utilisation of casamino acids in molasses-based fermentation media contributed \$0.7/kg to the MSP_{BDO}, whereas the utilisation of yeast extract in sucrose-based fermentation media contributed \$0.5/kg to the MSP_{BDO}. Thus, using fermentation media containing inorganic nutrient sources leads to reduced MSP_{BDO}.

Fermentation efficiency is one of the most decisive factors in achieving process profitability. Maina et al. (2019b) used the fermentation efficiency (86.6 g/L BDO concentration, 0.37 g/g yield and 3.95 g/L/h productivity) achieved with *E. ludwigii* cultivated in very high polarity (VHP) cane sugar-based fermentation media for the estimation of MSP_{BDO}. At 50 kt BDO production per year and VHP cane sugar market price in the range of \$0.05-0.4/kg, the MSP_{BDO} varied from \$1.56/kg to \$2.67/kg. Considering that the petrochemically-derived BDO market price is on average ca \$1.6/kg, the BDO production efficiency during fermentation should be further increased in order to develop a cost-competitive process.

Utilities consumption and the capital cost of the DSP section in BDO production processes are major impediments towards process sustainability. Maina et al. (2019) estimated a cost of utilities of \$0.29 per kg BDO only for the DSP section based on treatment with n-butyraldehyde to produce the acetal 2-propyl-4,5-dimethyl-1,3-dioxolane followed by one reactive distillation and two distillation columns to recycle the n-butyraldehyde and purify the BDO. For comparison purposes, the utilities cost of the fermentation section in the process evaluated by Maina et al. (2019b) was \$0.034 per kg BDO. Harvianto et al. (2018) showed that hybrid extraction distillation required 9.5% higher capital cost than conventional distillation. However, the hybrid extraction distillation process improved the overall process economics. Haider et al. (2018) estimated the cost of four different DSP for the purification of BDO. Dual distillation columns and vacuum flash distillation integrated with a vapor compression system for heat recovery resulted in 55% and 61.2% reductions in the total annual costs, respectively.

10. Derivatives of BDO and acetoin in the bio-economy era

BDO could be used as precursor for the production of bio-based chemicals and fuels. One such compound is methyl ethyl ketone (MEK, 2-butanone), which is conventionally produced using C4-raffinates (Penner et al., 2017). MEK displays promising fuel additive properties for spark ignition engines with similar engine efficiency as ethanol. MEK has special features such as lower hydrocarbon combustion, high energy density (31.5 MJ/kg), a low heat of vaporization (0.45 MJ/Kg), superior cold start properties and less oil dilution (Hoppe et al., 2016). Yoneda et al. (2014) attempted direct production of MEK from glucose via fermentation by overexpressing B12-dependent glycerol dehydratase from *K. pneumoniae* in *E. coli* but the strain produced only 0.004 $g_{MEK}/g_{glucose}$. Dehydration of BDO has been extensively studied for MEK production. MEK production via direct aqueous BDO dehydration using sulphuric acid as catalyst has been reported using either synthetic aqueous BDO solutions with MEK conversion yield higher than 90 mol% or cell-free fermentation broths with lower MEK conversion yields due to the presence of remaining salts and unconsumed sugars (Emerson et al., 1982). Multer et al. (2012) used a solid acid catalyst, the proton form of ZSM-5, for the conversion of BDO into MEK at conversion yield higher than 90% when synthetic aqueous BDO media were used and low conversion yield (13%) when fermentation broths were used. The BDO to MEK conversion yields achieved depends on the catalyst used (e.g. sulphuric acid, p-toluenesulfonic acid, phosphoric acid), the reaction temperatures and the mode of operation and unit operations used (e.g. batch or continuous processing, distillation) (Bai et al., 2020).

Conversion of BDO into olefins (butenes and butadiene) has been studied using various catalysts including aluminosilicates, gamma-Al_2O_3 and Cu/ZSM-5 (Zheng et al., 2017; Dagles et al., 2019). The primary reaction involves dehydration of BDO into MEK, hydrogenation of MEK into butanol and dehydration of butanol into hydrocarbons (Lilga et al., 2016). A hybrid pathway for the production of biojet fuel involving BDO conversion into C3 olefins, oligomerization and hydrogenation was studied by Adhikari et al. (2020). Long chain hydrocarbons with more than 70% (w/w) jet hydrocarbons (iso-olefins and iso-paraffins) was obtained using BDO over Amerlyst-36 catalyst. Butanol production could be also achieved via whole cell biotransformation using *Lactobacillus brevis* involving diol dehydratase (EC 4.2.1.28) catalyzing the conversion of diol into butan-2-one, which is then reduced to the secondary alcohol by dehydrogenases (Manitto et al., 1998).

1,3-Butadiene (BD) is used in the synthesis of polymer resins, elastomers and synthetic rubber (Makshina et al., 2014). More than 95 % of BD is produced as the by-product of ethylene production through naphtha steam cracking at temperatures around 800 °C (Duan et al., 2016). BD production could be also achieved via BDO dehydration using various catalysts. Liu et al. (2016) reported one step production of BD via 10% BDO dehydration using alumina catalyst in a fixed bed reactor with more than 80% selectivity to BD and 3-buten-2-ol. Nguyen et al. (2019) used rare-earth orthophosphates-based catalyst for the dehydration of BDO at 300 °C but achieved only 58% selectivity to BD. It is quite tedious to convert BDO into BD through dehydration process due to by-products formation, such as MEK. The formation of MEK has led to the development on alternative processes based on BDO esterification with organic acids followed by pyrolysis of the diester to produce BD at high purity and recycle the organic acid (Baek et al., 2014; Pacheco et al., 2017). Song et al. (2018a, 2018b) reported a conceptual techno-economic model showing that economic viability of BDO dehydration into BD using a hydroxyapatite-alumina catalyst, involving also a detailed separation process for BD and MEK, can be achieved at BDO purchase prices in the range of $0.991-1.3/kg depending on the BD market price.

BDO could be also dehydrated via a solvent-free process using a heterogeneous acid catalyst Amberlyst-15 into a dioxolane mixture that could be used as a gasoline blending component, diesel oxygenate and industrial solvent (Harvey et al., 2016). The production of BDO esters has been also reported with potential applications as additive in food, cosmetics and drug manufacture.

Acetoin can be converted to BDO by hydrogenation or electroreduction using decorated cathodes with subsequently dehydration of BDO into MEK (Gomez et al., 2018a; Gomez et al., 2018b).

11. Conclusions

Significant improvement has been achieved on microbial production of BDO and acetoin using wild type and metabolically engineered strains. With the primary motivation of providing the key advancements in genetic engineering strategies, process advancements, and detailed information on metabolic pathways for BDO and acetoin production, limitations to be addressed for the successful commercialization of the process have been presented in this review. Further process development is required to reduce the MSP of bio-based BDO lower than the petrochemical market price (ca. $1.6/kg). The catalytic conversion of BDO and acetoin into bio-based chemicals and fuels directly from fermentation broths should be further developed and optimised.

12. References

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Figure captions

Fig. 1 Stereoisomers of BDO and acetoin. *Source: https://pubchem.ncbi.nlm.nih.gov*

Fig. 2 Derivatives of BDO

Fig. 3 Industrial chemical synthesis of acetoin *(adapted from Xiao and Lu, 2014)*

Fig. 4 Metabolic pathway and regulation mechanism of BDO

Fig. 5 Renewable feedstocks for BDO and acetoin production

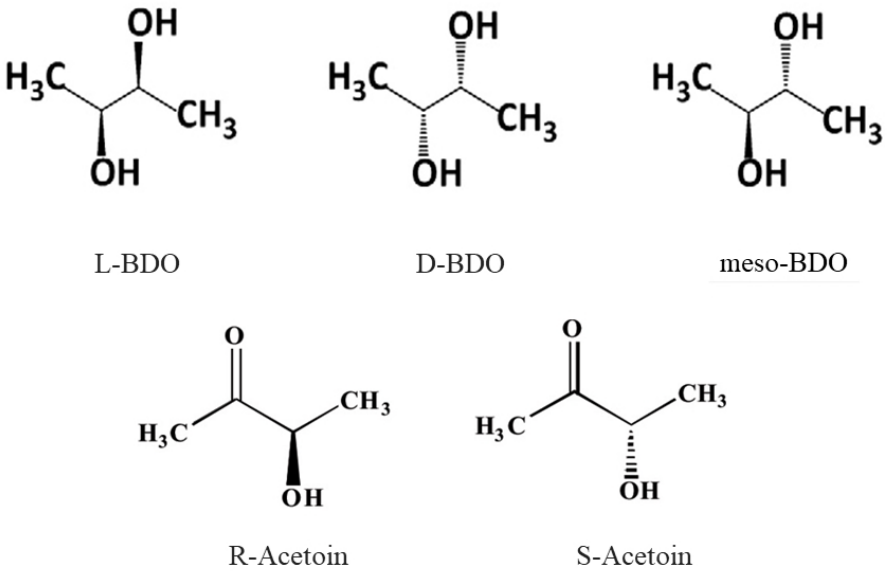
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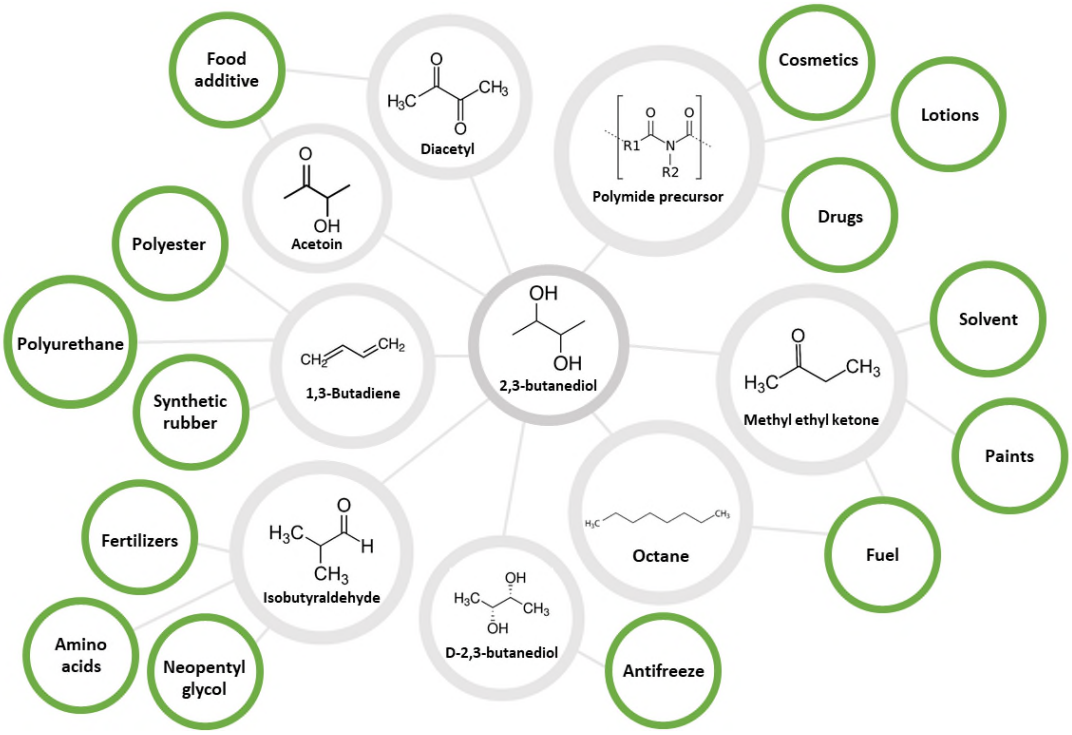
Fig. 1


Food additive
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Cosmetics
Lotions
Polymide precursor
Drugs
Acetoin
Polyester
Polyurethane
1,3-Butadiene
2,3-butanediol
Solvent
Methyl ethyl ketone
Paints
Synthetic rubber
Fertilizers
Isobutyraldehyde
Octane
Fuel
Amino acids
Neopentyl glycol
D-2,3-butanediol
Antifreeze

Fig. 2

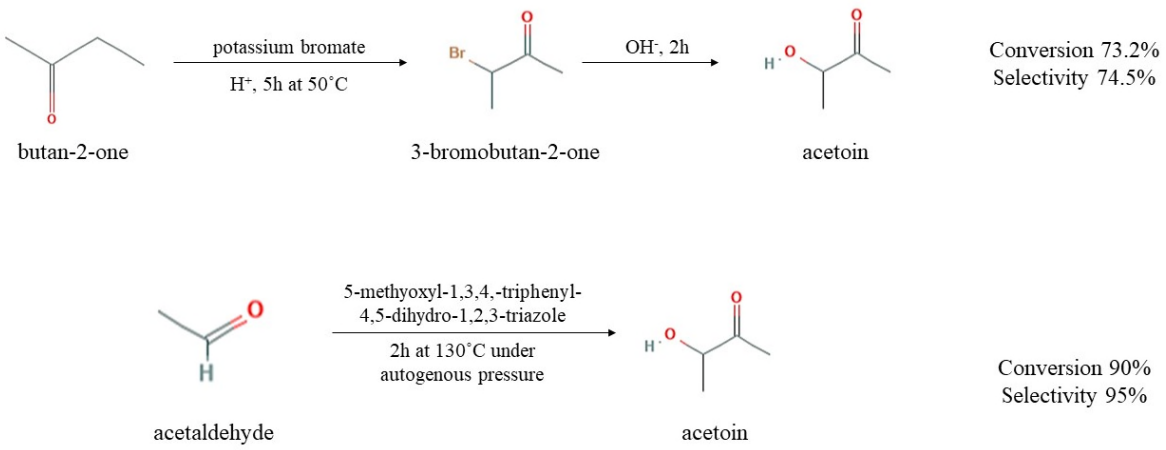
potassium bromate
H+, 5h at 50˚C
OH-, 2h
Conversion 73.2%
Selectivity 74.5%
butan-2-one
3-bromobutan-2-one
acetoin
5-methyoxyl-1,3,4,-triphenyl-
4,5-dihydro-1,2,3-triazole
2h at 130˚C under
autogenous pressure
Conversion 90%
Selectivity 95%
acetaldehyde
acetoin

Fig. 3

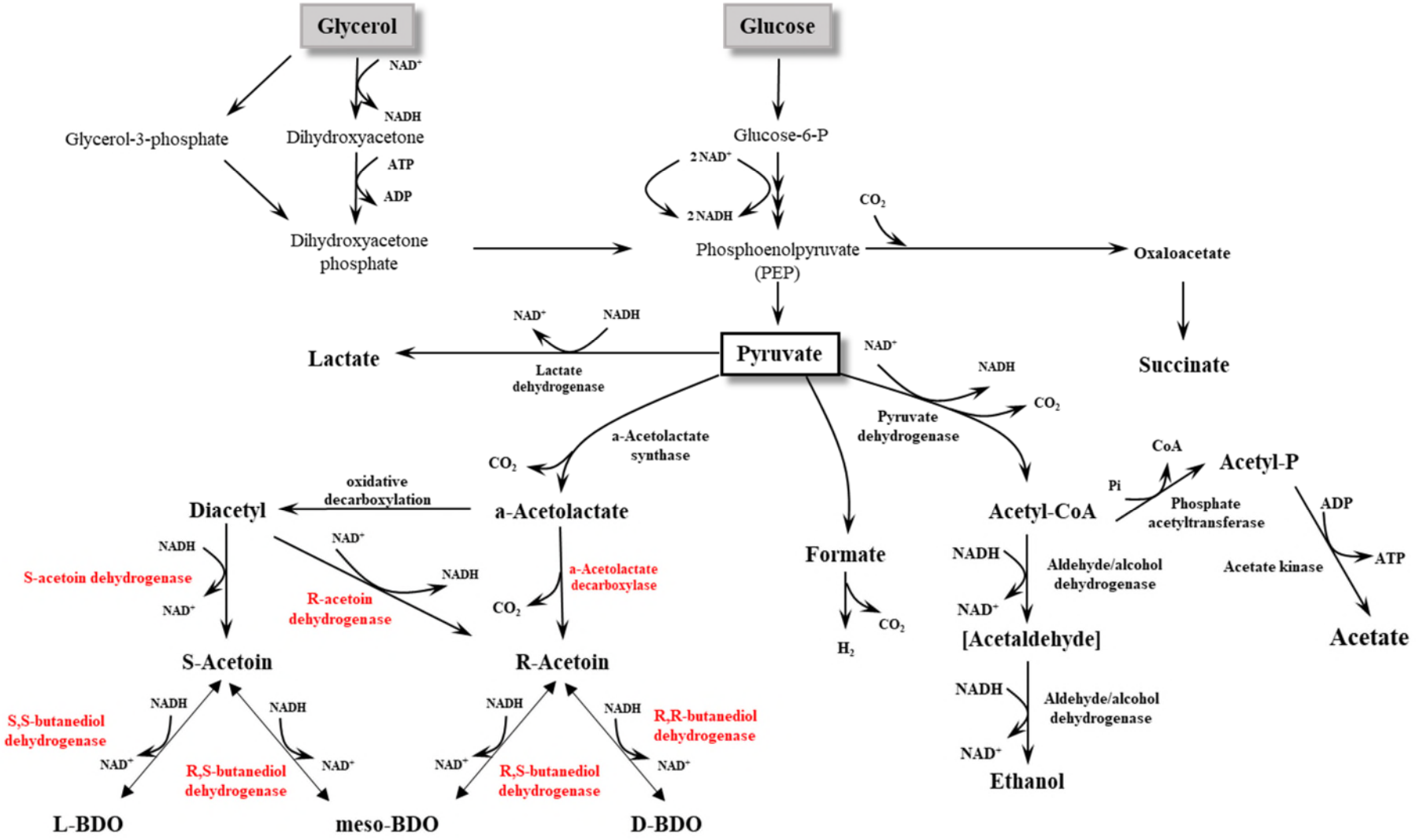
Glycerol
Glucose
NAD+
NADH
Glycerol-3-phosphate
Dihydroxyacetone
ATP
ADP
Dihydroxyacetone phosphate
Glucose-6-P
2 NAD+
2 NADH
CO2
Phosphoenolpyruvate (PEP)
Oxaloacetate
Succinate
Lactate
Lactate dehydrogenase
Pyruvate
Pyruvate dehydrogenase
a-Acetolactate synthase
oxidative decarboxylation
Diacetyl
a-Acetolactate
Formate
H2
Acetyl-CoA
CoA
Pi
Phosphate acetyltransferase
Acetyl-P
ADP
ATP
Acetate kinase
Acetate
S-acetoin dehydrogenase
R-acetoin dehydrogenase
a-Acetolactate decarboxylase
S-Acetoin
R-Acetoin
Aldehyde/alcohol dehydrogenase
[Acetaldehyde]
Ethanol
S,S-butanediol dehydrogenase
R,S-butanediol dehydrogenase
R,R-butanediol dehydrogenase
L-BDO
meso-BDO
D-BDO

Fig. 4

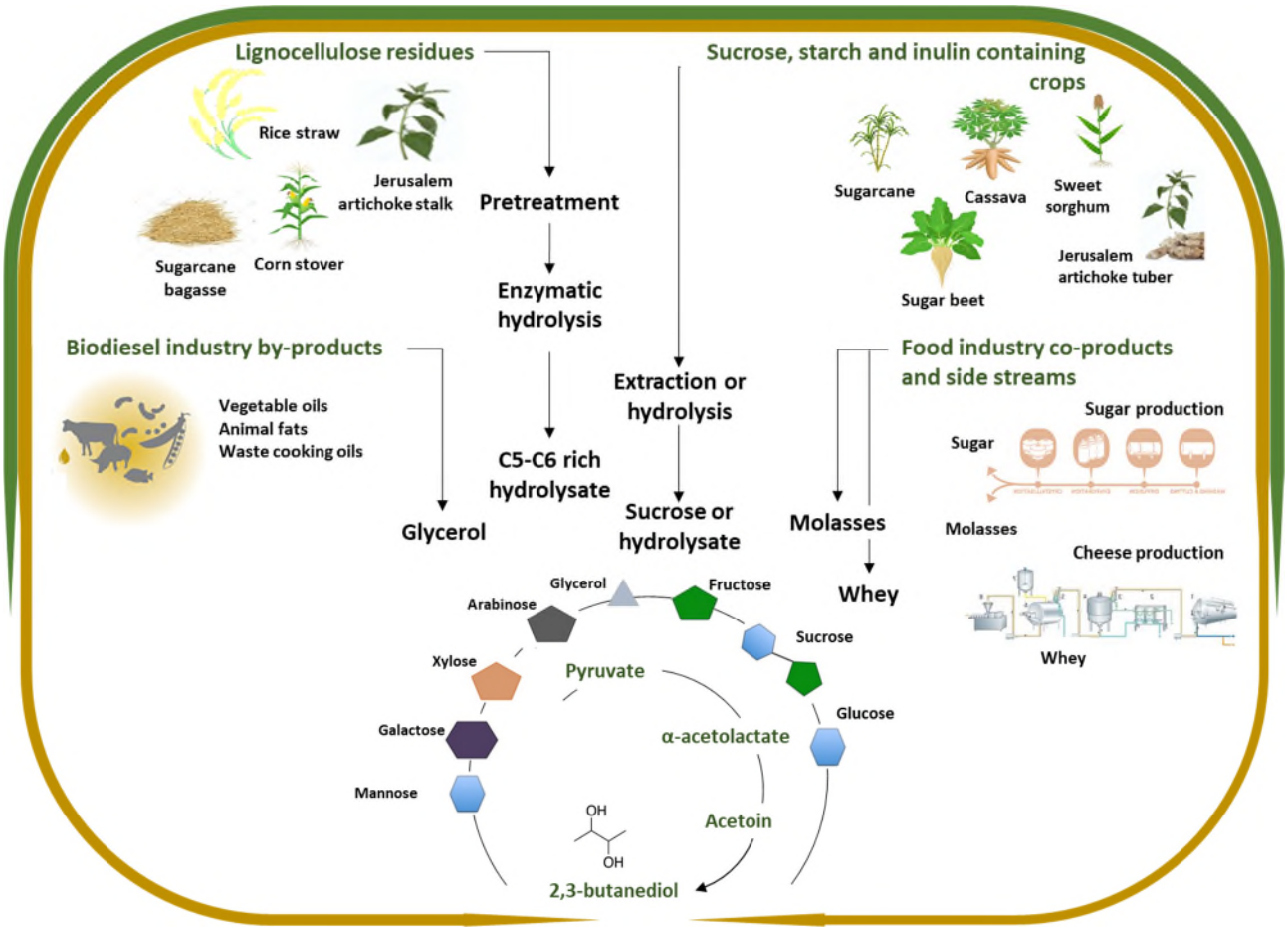
Lignocellulose residues
Rice straw
Jerusalem artichoke stalk
Sugarcane bagasse
Corn stover
Pretreatment
Enzymatic hydrolysis
Sucrose, starch and inulin containing crops
Sugarcane
Cassava
Sweet sorghum
Sugar beet
Jerusalem artichoke tuber
Biodiesel industry by-products
Vegetable oils
Animal fats
Waste cooking oils
Glycerol
C5-C6 rich hydrolysate
Extraction or hydrolysis
Sucrose or hydrolysate
Molasses
Whey
Food industry co-products and side streams
Sugar production
Sugar
Molasses
Cheese production
Whey
Glycerol
Fructose
Arabinose
Sucrose
Xylose
Pyruvate
Glucose
Galactose
α-acetolactate
Mannose
OH
OH
Acetoin
2,3-butanediol

Fig 5

Table 1 Acetoin production by native strains, genetically engineered strains and whole cell biocatalysis

Substrate	Microorganism	Acetoin (g/L)	Isomer	Yield (g/g)	Productivity (g/L/h)	By-products (g/L)	Nitrogen source (g/L)	Type of fermentation, aeration	References
Native strains									
Glucose	*B. subtilis* JNA 3-10	42.2			0.32	BDO (15.8)	CSL (6), U (2), BE (5)	b, 160 rpm	Zhang et al., 2011
Glucose	*B. liqueniformis* MEL09	41.3		0.42		BDO (12), AA (2)	YE (12), P (1), $(NH_4)_2SO_4$ (8)	b	Liu et al., 2011a
Glucose	*P. polymyxa* CS107	55.3		0.38	1.32	BDO (10)	YE (15.93)	fb, 500 rpm 0.5 vvm	Zhang et al., 2012
BDO	*Gluconobacter oxydans* DSM 2003	89.2		0.91 mol/mol	1.24		YE (20), $(NH_4)_2SO_4$ (1.5)	fb in sf	Wang et al., 2013
Glucose	*B. amyloliquefaciens* FMME044	51.2		0.43	1.42		YE (20)	b, 350 rpm (0-24h) and then 500 rpm 0.4 L/min	Zhang et al., 2013c
Glucose	*B. subtilis* SF4-3	48.9		0.39	0.56	BDO (5.5)	YE (10), CSL (5), U (2)	b, 300 rpm 0.5 vvm	Tian et al., 2016
Bakery waste hydrolysate	*B. amyloliquefaciens*	65.9		0.31	1.57	BDO (5.6)	YE (15)	fb, 1 vvm, (k_La 200 h^{-1})	Maina et al., 2021
Sugarcane molasses	*B. subtilis* CICC10025	35.4		0.41	0.63		SMH (22% v/v)	b, 700 rpm 1 vvm	Xiao et al., 2007
Sugarcane molasses	*B. subtilis* DL01	61.2	R-	0.34	0.807		$(NH_4)_2HPO_4$ (10)	b, 200 rpm (0-6h) 300 rpm (6-24h) 400 rpm 0.4 vvm	Dai et al., 2015a
Engineered stains									
Glucose	*K. pneumoniae* CICC 10011	56.7	R-			BDO (12.5)	$(NH_4)_2HPO_4$ (15), P (0.5), YE (1.5)	sf, 120 rpm	Liu et al., 2011b
Glucose	*K. pneumoniae* XZF-308	25.9			0.32	BDO (42.8)	$(NH_4)_2SO_4$ (6.6), $(NH_4)_2HPO_4$ (3.3)	fb, 400 rpm 1 vvm	Ji et al., 2013
Glucose	*B. subtilis* JNA-UD-6	53.9		0.36	0.37	BDO (6.5)	BE (5), CSL (6), U (2)	b, 160 rpm	Zhang et al., 2013b
Glucose	*B. amyloliquefaciens* E-11	71.5		0.41	1.63	BDO (11)	YE (12.5), P (12.5)	b, 350 rpm (0-32h) 500 rpm (32-48h) 4L/min	Luo et al., 2014
Glucose	*K. pneumoniae* CGMCC 1.6366	62.3	R-	0.14	1.09	BDO (6.7)	$(NH_4)_2SO_4$ (5), YE (2), CSL (4)	fb, 500 rpm 4L/min	Wang et al., 2015
Glucose	*S. cerevisiae* JHY617-SDN	100.1		0.44	1.82	BDO (0.39)	P (20), YE (10)	fb, 500 rpm 1 vvm	Bae et al., 2016
Glucose	*E. aerogenes* EJW-03	71.7		0.32	2.87	BDO (2)	$(NH_4)_2HPO_4$ (6.8, $(NH_4)_2SO_4$ (5.35), YE (5), C (10)	fb, 600 rpm 2.5 vvm	Jang et al., 2017a
Glucose	*B. subtilis* BS168D	24.6			0.34	BDO (2.4)	P (10), YE (5)	sf, 100 r/min	Zhang et al., 2017
Glucose	*Corynebacterium glutamicum* CGR7	96.2	R-	0.36	1.3		$(NH_4)_2SO_4$ (10), U (5)	fb, 400 rpm 1 vvm	Mao et al., 2017
Glucose	*B. licheniformis*	78.79		0.31	0.58	BDO (20)	CSL (33), $(NH_4)_2SO_4$ (9)	fb, 350 rpm (0-16h) 250 rpm (16-96h) 350 rpm (96-136h) 1vvm	Li et al., 2017

Sucrose	*S. marcescens* H32	60.5			1.44	BDO (38.9)	CSLP (9.8)	fb, 700 rpm (0-8h) and then 600 rpm 1 vvm	Sun et al., 2012
Sucrose	*S. marcescens* H32	75.2		0.36	1.88	BDO (20)	CSLP (9.8)	fb, 600 rpm 1.25 vvm	Sun et al., 2012
Oil palm mesocarp fiber hydrolysate	*E. coli* mlc-XT7-LAFC-YSD	15.5		0.49	0.64		YE (5)	b, 500 rpm4 mL/min	Yusoff et al., 2017
Lignocellulosic hydrolysate	*E. cloacae* SDM-45	45.6		0.29	1.52	BDO (20), AA (4), FA (2.4)	BE (5), CSL (6), U (2)	fb, 500 rpm 1 vvm	Zhang et al., 2016b
XGA[a]	*B. subtilis* ZB02	62.2		0.29	0.86		CSL (30)	fb, 400 rpm 1 vvm	Zhang et al., 2016a
Lignocellulosic hydrolysate		11.2		0.34				sf, 220 rpm	
Whey permeate	*Lactococcus lactis*	27.0	R-	0.42		BDO (83)	YE (20)	sf, 200 rpm	Kandasamy et al., 2016
Whole cell biocatalysis									
Diacetyl	*E. coli*	39.4			1.97			fb, 80rpm	Gao et al., 2013a
BDO	*B. subtilis*	91.8		0.77	2.30			rb, 200rpm	Bao et al., 2014
meso-BDO	*E. coli*	86.7	R-		3.61				Guo et al., 2017b
BDO	*S. marcescens* MG1ABC	44.9			3.74			sf, 220 rpm	Ben et al., 2017
BDO	*G. oxydans* NL71	165.9			4.61			COS-SSTR[b], 500 rpm 0.02-0.05 Mpa	Zhou et al., 2018
meso-BDO	*E. coli*	72.4			3.02			b	He et al., 2018

By-products: BDO: 2,3 butanediol, AA: acetic acid, FA: formic acid

Nitrogen source: CSL: corn steep liquor, YE: yeast extract, U: urea, BE: beef extract, P: peptone, SMH: soybean meal hydrolysate, CSLP: corn steep liquor powder, C: casein

Type of fermentation: fb: fed-batch, b: batch, sf: shake flask, rb: repeated batch, fb in sf: fed-batch in shake flask mode

[a]XGA: xylose glucose and arabinose

[b] COS-SSTR: sealed aerated stirred-tank reactor

Table 2 BDO production using commercial carbon sources

Carbon source	Microorganism	BDO	Isomer	Yield (g/g)	Productivity (g/L/h)	By-products (g/L)	Nitrogen source (g/L)	Type of fermentation, aeration	References
Glucose	*E. cloacae* DSM 30053	110* (BDO&Ac)		0.49	5.4	AA (8)	$(NH_4)_2SO_4$ (12)	fb, OUR[a] (100-30 mmol/L)	Zeng et al., 1991b
Glucose	*K. pneumoniae* CICC 10011	92.4	meso-, L-	0.39	1.85	Ac (13.1)	$(NH_4)_2HPO_4$ (24)	fb, 300 rpm 1vvm	Jiayang et al., 2006
Glucose	*K. pneumoniae* SDM	150	meso-, L-	0.44	3.95	Ac (10)	CSLP (8), $(NH_4)_2HPO_4$ (4.64l)	fb, 500 rpm 1.5 vvm	Ma et al., 2009
Glucose	*B. amyloliquefaciens* B10-127	92.3			0.96	Ac (42.5)	CSL (10), SM (10)	fb, 300 rpm 0.3 vvm	Yang et al., 2011
Glucose	*B. amyloliquefaciens* pBG*	132.9			2.95	Ac (7.0)	CSL (31), SM (20)	fb, 350 rpm, 0.33 vvm	Yang et al., 2013b
Glucose	*K. pneumoniae* SGJSB04*	101.5		0.34	2.54	Ac (11)	YE (5)	fb, 300 rpm 0.75 vvm	Kim et al., 2012
Glucose	*B. licheniformis* 10-1-A	115.7	meso-, D-	0.47	2.4	AA (4.8), EtOH (1.1), FA (28.3)	CSL (10), YE (5)	fb, 400 rpm (0-10h) and then 200 rpm	Li et al., 2013
Glucose	*K. pneumoniae* KG1*	116		0.49	2.23	Ac (10.5), AA (7)	YE (10)	fb, agitation cascade (DO[b] 15%) 1 vvm	Guo et al., 2014a
Glucose	*K. oxytoca* M1*	142.5		0.42	1.47	Ac (18), AA (11), SA (3.4), EtOH (1.1)	$(NH_4)_2HPO_4$ (3.3), $(NH_4)_2SO_4$ (6.6)	fb, 400 rpm 1 vvm	Cho et al., 2015a
Glucose	*Vibrio natriegens* DSM 759	36.7		0.22	3.88	Ac (10.4)	T (10), YE (5)	fb, agitation cascade (DO[b] 0-1%) 1 vvm	Eria et al., 2020
Inulin	*B. licheniformis* ATTC 14580	103	meso-, D-		3.4	-	YE (5.8), CSL (14.7)	fb-SSF, 200 rpm 1vvm	Li et al., 2014a
Sucrose	*S. marcescens* H30*	139.9		0.47	3.33	Ac (6.7)	YE (33.36)	fb, RQ[c] (1-1.5 from 0-15h and then 1.8-2.0)	Zhang et al., 2010b
Sucrose	*S. marcescens* swrW*	152		0.46	2.67	Ac (0.3)	YE (33.36)	fb, 500 rpm 1 vvm (0-15h) and then 300 rpm 0.5 vvm	Zhang et al., 2010a
Sucrose	*S. marcescens* MG1*	91.9	meso-, D-	0.36	1.92	Ac (2.0)	YE (33.36)	fb, 500 rpm 1 vvm (0-15h) and then 300 rpm 0.5 vvm	Bai et al., 2015
Glycerol	*K. pneumoniae G31*	70		0.39	0.47	AA (3.1), PDO (16.3)	$(NH_4)_2SO_4$ (5.35), YE (2)	fb, 1.1-2.2 vvm	Petrov and Petrova, 2010
Glycerol	*R. ornithinolytica* B6	89.5		0.41	0.75	Ac (9.1)	$(NH_4)_2SO_4$ (3.3), YE (5), CA (10)	fb, 400 rpm 1 vvm	Kim et al., 2017b

* Genetically engineered strains

[a]OUR: oxygen uptake rate

[b]DO: dissolved oxygen concentration

[c] RQ: respiratory quotient

By-products: Ac: acetoin, AA: acetic acid, SA: succinic acid, FA: formic acid, EtOH: ethanol

Nitrogen source: CSL: corn steep liquor, YE: yeast extract, T: tryptone, SMH: soybean meal hydrolysate, CSLP: corn steep liquor powder, CA: casamino acid

Type of fermentation: fb: fed-batch.

Table 3 BDO production in fermentations utilizing crude renewable feedstocks

Carbon source	Microorganism	BDO	Isomer	Yield (g/g)	Productivity (g/L/h)	By-products (g/L)	Nitrogen source (g/L)	Type of fermentation, aeration	References
Lignocellulosic feedstocks						-			
Rice straw hydrolysate	*Klebsiella sp.* Zmd30	24.6			2.41	-	Urea (2.28)	b	Wong et al., 2012
Bagasse hydrolysate		8.3			0.66	-		b	
Apple pomace hydrolysate	*B. licheniformis* NCIMB 8059	77.6		0.32	0.42	-	YE (3.2)	fb, 250 rpm 1.2 vvm	Bialkowska et al., 2015
Apple pomace hydrolysate	*B. subtilis* LOCK 1086*	51.5		0.29	0.43	-	YE (10), P (20)	fb in sf, 130 rpm	Bialkowska et al., 2016
Sweet sorghum stalk	*S. marcescens* H30	109.4		0.42	1.4	-	YE (10)	fb, 300r rpm (0-12h) and then 200 rpm	Yuan et al., 2017
Corn stover hydrolysate	*B. liqueniforms* X10	74	meso-, D-	0.47	2.1		YE (5), CSLP (14.5)	fb, 400 rpm (0-12h) and then 200 rpm, 1 vvm	Li et al., 2014b
Corn stover hydrolysate	*E. cloacae* SDM 09*	119.4	D-	0.48	2.3	Ac (6)	CSLP (2)	fb, 500 rpm (0-36h) and then 300 rpm 1 vvm	Li et al., 2015a
Corn stover hydrolysate	*P. polymyxa* ATCC 12321	18.8	D-	0.31	1.13	-		c, 500 rpm 0.2 vvm	Ma et al., 2018
Soybean hull hydrolysate	*K. pneumoniae* BLh-1	20.1		0.5	0.28	-	YE (5)	sf	Cortivo et al., 2019
	Pantoea agglomerans BL1	19.9			0.28	-			
Sugarcane bagasse hydrolysate	*E. aerogenes* EMY-22_M1Gb*	114.3		0.44	1.49	SA (8)	YE (5), CA (10)	fb, 200 rpm 2vvm	Kim et al., 2020
Fruit and vegetable hydrolysate	*E. ludwigii* FMCC 204	50		0.4	0.41	SA (16.5), LA (10.8), EtOH (5.8)	YE (2.5), P (5), ME (5)	fb, agitation cascade (150-250 rpm) 1 vvm	Liakou et al., 2017
Jerusalem artichoke tuber	*K. pneumoniae* CICC 10011	84		0.29	2.1	Ac (7.6)	$(NH_4)_2SO_4$ (6.61)	fb-SSF, 300 rpm 0.15 L/min	Sun et al., 2009
Jerusalem artichoke stalk and tuber	*K. pneumoniae* CICC 10011	67.4		0.26	0.99	Ac (13.4), LA (0.4)		SSF, stage shift aeration	Li et al., 2010a
Cassava powder	*E. cloacae* subsp. *dissolvens* SDM	93.9	meso-, D-, L-	0.42	2	Ac (5.3)	CSL (10)	fb-SSF, 500 rpm 1vvm	Wang et al., 2012a
Jerusalem artichoke tuber extract & inulin	*K. pneumoniae* H3	80.4		0.43	2.23	-	$(NH_4)_2SO_4$ (2), YE (2), P (2)	b, 250 rpm 0.2 vvm	Dai et al., 2020
Food industry side streams and co-products									
Cheese whey powder	*K. pneumoniae* CICC 10781	57.6		0.4	0.96	-	YE (11.35)	fb, 500 rpm 1 vvm	Guo et al., 2017a
Bakery waste hydrolysate	*B. amyloliquefaciens*	55.2		0.42	1.19		YE (15)	b, 300 rpm, 1 vvm (k_La 64 h^{-1})	Maina et al., 2021
Whey powder	*K. oxytoca* PDL-K5*	65.5		0.44	2.73	-	CSLP (8.27)	fb, 400 rpm 1 vvm	Meng et al., 2020
Molasses	*K. oxytoca* DSM3539	118		0.42	2.35	Ac (2.3)	YE (5), T (5)	rb, 200 rpm 0.5 vvm (0-15h) and then 150 rpm 0.3 vvm	Afschar et al., 1991

VHP[a] cane sugar	*E. ludwigii*	86.8	meso-, L-	0.37	3.95	Ac (7.6), SA (6.7), LA (1.6), EtOH (3.3)	$(NH_4)_2SO_4$ (6)	fb, agitation cascade (150-400 rpm) 1 vvm	Maina et al., 2019b
Sugarcane molasses	*E. aerogenes* EMY-68*	98.7		0.37	2.74	-	$(NH_4)_2SO_4$ (5.35), YE (5), CA (10)	fb, 280 rpm 1.5 vvm	Jung et al., 2013a
Sugarcane molasses	*E. cloacae* EMY-70S*	140		0.39	2.59	-	$(NH_4)_2SO_4$ (5.35), YE (5), CA (10)	fb, 280 rpm 1.5 vvm	Jung et al., 2015
Sugarbeet molasses	*B. subtilis* LOCK 1086*	75.7		0.31	0.66	-	YE (10), P (20)	fb in sf, 130 rpm	Bialkowska et al., 2016
Sugarcane molasses	*E. ludwigii*	50.6	meso-, L-	0.31	2.66	Ac (9.5), SA (11), LA (1.9), AA (7.8), EtOH (5)	$(NH_4)_2SO_4$ (6.2), YE (10)	fb, agitation cascade (150-400 rpm) 1 vvm	Psaki et al., 2019
Sugarcane molasses	*B. amyloliquefaciens* 18025	48.7	meso-, D-	0.4	0.83	Ac (6.7)	YE (15)	fb, 400 rpm 1 vvm	Maina et al., 2019a
Corncob molasses	*K. pneumoniae* SDM	78.9	meso-, L-	0.41	1.29	-	CSLP	fb, 400-500 rpm 0.5-1 vvm	Wang et al., 2010
Biofuel industries by-products									
Crude glycerol	*K. oxytoca* M3*	131.5	meso-, L-	0.44	0.84	LA (0.8), EtOH (1.7)	YE (5), CA (10)	fb, 400 rpm 1 vvm	Cho et al., 2015b
Crude glycerol	*K. variicola* SW3 mutant	64.9		0.63	0.39	Ac (6.32), PDO (2.9)	YE (2.5), P (5)	fb in sf, 200 rpm	Rahman et al., 2017
Crude glycerol pretreated	*R. ornithinolytica* B6*	78.1		0.42	0.62	-	YE (5), CA (10)	fb, 400 rpm	Kim et al., 2017b
Crude glycerol: molasses (5:1)	*B. amyloliquefaciens* B10-127	83.3		0.42	0.87	-	CSL (30), SM (20)	fb, 0.66 vvm	Yang et al., 2013c
Crude glycerol: molasses (5:1)	*B. amyloliquefaciens* GAR*	102.3		0.44	1.16	Ac (5)	CSL (30), SM (20)	fb, 350 rpm (0-5h) 400 rpm (5-22h) 350 rpm (22-88h) 0.66 vvm	Yang et al., 2015c

* Genetically engineered strains

By-products: Ac: acetoin, AA: acetic acid, PDO: 1,3 propanediol, LA: lactic acid, SA: succinic acid, EtOH: ethanol

Nitrogen sources: YE: yeast extract, P: peptone, CA: casamino acid, T: tryptone, SM: soybean meal, CSL: corn steep liquor, CSLP: corn steep liquor powder

Type of fermentation: fb: fed-batch, fb-SSF: fed-batch simultaneous saccharification and fermentation, b: batch, c: continuous, sf: shake flask, SSF: simultaneous saccharification and fermentation, rb: repeated batch, fb in sf: fed-batch in shake flask mode.

[a] VHP: very high polarity

Prospects on bio-based 2,3-butanediol and acetoin production: recent progress and advances

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2021-06-29

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