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FERMENTATIVE PRODUCTS OF GENUS *CLOSTRIDIUM* FOR VARIOUS INDUSTRIAL APPLICATIONS

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ABSTRACT

Recently, there has been an important development in the fermentative industry observed. Bacteria belong to genus *Clostridium* has been observed and used as diversified chemical producers. Genus *Clostridium* produces many types of chemicals naturally like acetic acid, acetone, butyric acid, butanol, ethanol, 1,3-propanediol, isopropanol, and 2,3-butanediol from the fermentation of cellulose, glucose, glycerol, etc. Hydrogen production is also significant from this microorganism. To enhance the production of fermentative products many metabolic engineering pathways have been developed with the use of genetic engineering. In this review, we will discuss some important products from the genus *Clostridium* along with metabolic engineering strategies.

Keywords: *Clostridium,* Acetone-butanol-ethanol (ABE), Metabolic engineering

INTRODUCTION

Members of the genus *Clostridium* are rod shaped, gram-positive, spore-forming and strictly anaerobes (Akinosho *et al*., 2014). Many of them can infect humans and cause several serious illnesses, but a lot of them metabolize to form many important industrial products by the fermentation of assorted compounds and carbohydrates like cellulose, glycerol (Fig. 1.1) etc. Acetate, ethanol, butyrate, acetone, butanol, isopropanol, 2,3-butanediol, 1,3-propanediol, and hydrogen are principal fermentative products acquired by the various clostridia which are industrially highly considerable (Cho *et al*., 2015). Not only the production of new fermentative products has been noticed by clostridia but an increase of alcohol production is also observed by modifying the various pathways. Now few metabolic approaches have been grow to increase the production of products (Cho *et al*., 2015). Butanol production can be increased by the increase in metabolic flux toward the desired products with hot and cold channels route. Knocking out of many genes like *pta, ack, ptb* blocks the acid formation and increase in butanol production. Overexpression of *adhE1* also increases the butanol formation in acetone-butanol-ethanol (ABE) fermentation (Cho *et al*., 2015, Lütke-Eversloh, 2014). Genetically engineered strains of *C. thermocellum* by the knocking out of *hpt* and *idh* are involved in the increased production of ethanol (Akinosho *et al*., 2014). Isopropanol-butano-ethanol mixture production enhanced by the *sadh* gene introduction with the overexpression of *ctfA, ctfB* and *adc* gene (Cho *et al*., 2015). Isobutanol production is achieved by the introduction of *livCD, yqhD,* and *kivD* in *C. cellulolyticum* in batch culture by cellulose fermentation (Higashide *et al*., 2011). Hydrogen is also get from clostridia. Phenotypic screening methods are helpful in the isolation of industrially important clostridia. Butanol tolerance, suicidal substrate, colorimetric estimations, and flow cytometry are currently available for a selection of improved product producers' mutants. Enzyme engineering is under study, only a few studies have been performed. *Zymomonas mobilis* alcohol dehydrogenase-2 is many times effective on *adhE1* and the mutant library of *thlA* gene was generated. This enzyme engineering belongs to the explorative category of metabolic engineering (Lütke-Eversloh, 2014). Many microbes can produce acetone and butanol. Industrially ABE production is usually done by *Clostridium* strains like *C. beijerinkii, C. acetobutylicum, C. saccharobutylicum* and *C. saccharoperbutylicum.* Recent improvements in china in continuous fermentation from corn, breeding, and screening of greater butanol ratio *Clostridium* strains consumption of waste mash as animals feed, along with development in distillation designs have been done for ABE fermentation process (Ni and Sun, 2009).

CULTURING AND ISOLATION OF BACTERIA

The most important bacteria of interest isolated from different sources. The most common source of clostridium isolation is soil and human GIT tract. For pure culture, organisms are allowed to grow selective and then in non-selective media. For its growth batch and continuous enrichment is required. Growth on cheap media, Growth at a

higher temperature to reduce cooling costs and better resistance to contaminants are major important factors for pertinent and pure growth, isolation and industrial product enhancement (Stanbury *et al*., 2013). All clostridia are strictly anaerobes along with rod shaped morphology with gram positive appearance on microscopic examination. Spore formation is distinctive feature of *Clostridia.* Some of them expand through spores and others are industrially important (Levinson, 2016). Enrichment liquid culture is generally carried out in shake flasks. Isolation of pure organisms is done by the use of selective liquid media. Various solidified selective media used for the growth of bacteria. Batch culture is performed in a closed system with initially added limited amounts of nutrients. Due to the limited supply of nutrients, limited biomass and product formation happened in this process. Continuous culture is another modified way to obtain the product in exuberance amount as compared to batch culture. The system is not completely closed and exponential growth can be carried out with the continuous addition of nutrients until fermenting vessels become full of products, biomass, and effluents. So, the use of continuous culture with some other requirements is an industrially valuable process (Stanbury *et al*., 2013).

IMPORTANT SPECIES AND INDUSTRIAL PRODUCTS

Many Clostridium species have been discovered. Many of them like *Clostridium beijerinckii* (Ng *et al*., 1981), *Clostridium acetobutylicum* (Lee *et al*., 2012), *C. ljungdahlii* (Younesi *et al*., 2005), *Clostridium thermohydrosulfuricum, Clostridium thermocellum* (Papanek *et al*., 2015), etc. are used in the fermentation of different sources (Fig.1) to produce many important industrial products like ethanol, acetone, butanol, acetate, etc (Table 1).

Table 1. Some important industrial *Clostridium* species and their products.

Species	**Industrial Product**	**Reference**
Clostridium bifermentans	Ethanol, acetic acid	(Turton *et al*., 1983)
Clostridium sporogenes	Ethanol, butanol, butyric acid	(Turton *et al*., 1983)
Clostridium ramosum	Hydrogen	(Karadag and Puhakka, 2010)
Clostridium butyricum	Hydrogen, 1,3-propandiole	(Karadag and Puhakka, 2010; Papanikolaou *et al*., 2000)
Clostridium ljungdahlii	Ethanol, acetate	(Younesi *et al*., 2005)
Clostridium beijerinckii	Acetone, butanol, and ethanol	(Ezeji *et al*., 2003)
Clostridium ragsdalei	Ethanol, acetate, ethanol	(Kundiyana *et al*., 2011)
Clostridium cellulolyticum	Isobutanol	(Higashide *et al*., 2011)
Clostridium acetobutylicum	Isopropanol, butanol, ethanol, bacteriocin	(Lee *et al*., 2012)
Clostridium autoethanogenum	Acetate, ethanol	(Koepke *et al*., 2011)
Clostridium carboxidivorans	Acetate, buthanol, butyrate, ethanol	(Koepke *et al*., 2011)
Clostridium drakei	Acetate, ethanol, butyrate	(Koepke *et al*., 2011)
Clostridium scatologenes	Ethanol, Acetate, butyrate	(Koepke *et al*., 2011)
Clostridium pasteurianum	Propanediol, butyric and acetic acids and ethanol	(Biebl, 2001)
Clostridium cochlearium	Acetate, butyrate, carbon dioxide, ethanol, hydrogen	(Wilde *et al*., 1997)
Clostridium tetani	Acetate, butyrate, ethanol, hydrogen, carbon dioxide	(Wilde *et al*., 1997)
Clostridium malenominatum	Acetate, butyrate, ethanol, hydrogen, carbon dioxide	(Wilde *et al*., 1997)
Clostridium cellulovorans	Acetone, butanol, butyric acid, ethanol	(Wen *et al*., 2014)
Clostridium hathewayi	Acetate, ethanol, carbon dioxide, and hydrogen	(Steer *et al*., 2001)
Clostridium phytofermentans	Ethanol, acetate, carbon dioxide, hydrogen	(Jin *et al*., 2011)
Clostridium diolis	1,3-Propanediol	(Kaur *et al*., 2012)
Clostridium kluyveri	Butyrate, caproate, hydrogen	(Seedorf *et al*., 2008)
Clostridium fallax	Acetate, butyrate, and lactate	(Ueki *et al*., 1991)
Clostridium saccharolyticum	Methane, ethanol	(Khan, 2008; Murray *et al*., 1983)
Clostridium difficile	Lactate, acetate, butyrate, ethanol, butanol	(Janoir *et al*., 2013)
Clostridium perfringens	Butyrate, acetate, lactate, ethanol	(Ishimoto *et al*., 1974)

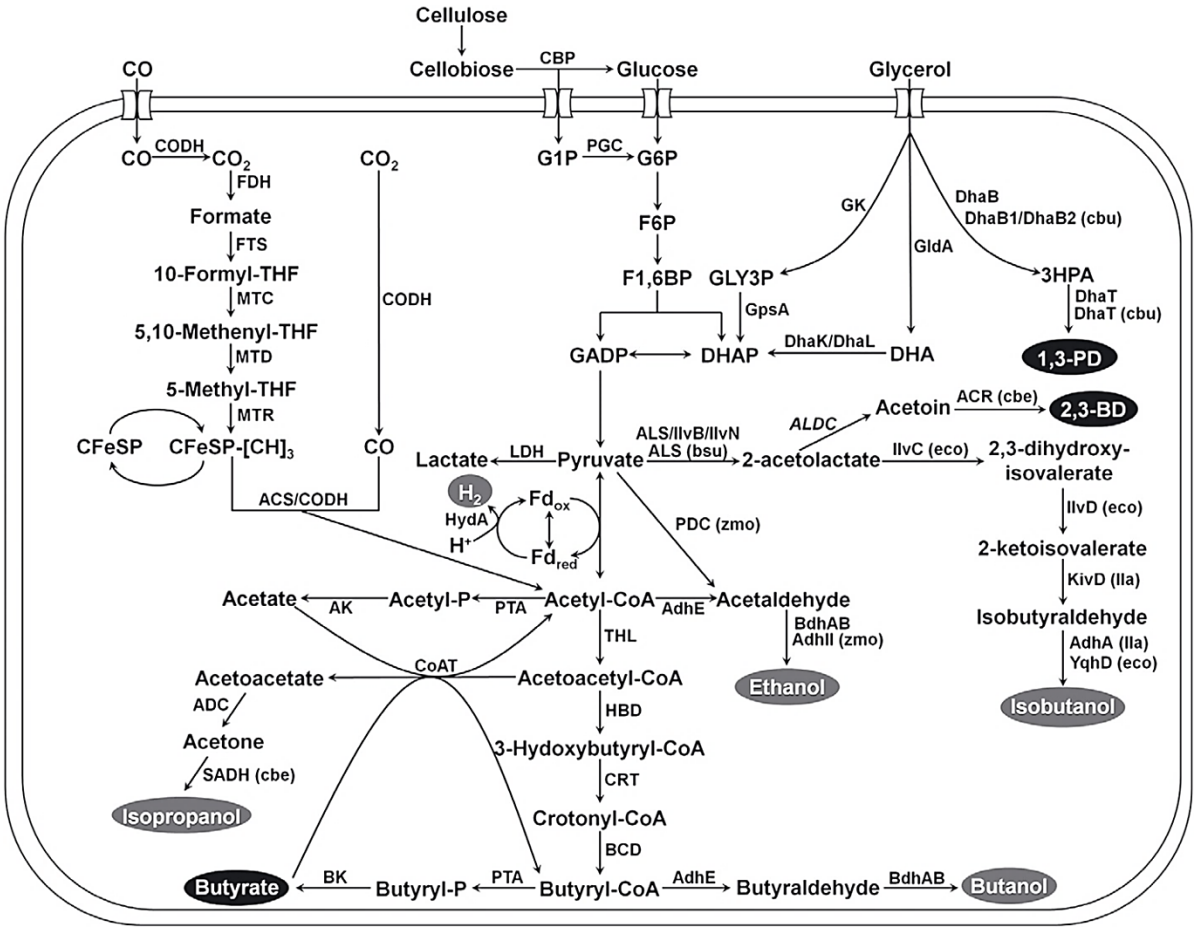

Fig. 1. Metabolic pathways for chemical productions in clostridia (Cho *et al*., 2015).

Abbreviations: (ACR,acetoinreductase) (ACS/CODH, acetyl-CoA synthase/CO dehydrogenase) (ADC/ ALDC, acetoacetate decarboxylase) (AdhA/ AdhE/ YqhD/ SADH, alcohol dehydrogenase) (ALS, acetolactate synthase) (AK, acetate kinase) (BCD, butyryl-CoA dehydrogenase) (BK, Butyrate kinase) (BdhAB, butanol dehydrogenase) (CoAT, Co-A transferase) (CBP, cellobiose phosphorylase) (CRT, crotonase) (DhaK, DHA kinase) (DhaB, glycerol dehydratase) (DhaT, 1,3-propanediol oxidoreductase) (DhaL, DHA kinase) (FDH, formate dehydrogenase) (FTS, formyl-THF synthase) (GldA, glycerol dehydrogenase) (GK, glycerol kinase) (GpsA, glycerol-3-phosphate dehydrogenase) (HBD, 3-hydroxybutyryl-CoA dehydrogenase) (HydA, hydrogenase) (llvB, llvC, llvN acetolactate synthase) (llvD, dihydroxyacid dehydratse) (KivD, ketoacid decarboxylase) (LDH, lactate dehydrogenase) (MTD, methylene-THF dehydrogenase) (MTR, methyl transferase) (MTC, methenyl-THF cyclohydrolase) (PTA, phophotransacetylase) (PTB, phosphotransbutyrylase) (PGC, phophoglucomutase) (PDC, pyruvate decarboxylase) (THL, thiolase) (bsu,*Bacillus subtilis) (cbu, Clostridium butyricum) (*cbe, *Clostridium beijerinckii) (*eco, *Escherichia coli) (*lla,*Lactococcus lactis*) (zmo, *Zymomonas mobilis)* (Cho *et al*., 2015).

Many species of the genus *Clostridium* involved in enhanced ethanol production from cellulosic substrates as a result of the dismissal of the type I glutamine synthetase (glnA) gene. Bacterial strains of interest include the genus of *Clostridium* including , but not limited to the following species:
C. absonum, C. aceticum, C. acetireducens, C. acetobutylicum, C. acidisoli, C. aciditolerans, C. acidurici, C. aerotolerans, C. aestuarii, C. akagii, C. aldenense, C. aldrichii, C. algidicarni, C. algidixylanolyticum, C. algifaecis, C. aurantibutyricum, C. algoriphilum, C. alkalicellulosi, C. aminophilum, C. aminovalericum, C. amygdalinum, C. amylolyticum, C. arbusti, C. argentinense, C. arcticum, C. asparagiforme, C. autoethanogenum, C. baratii, C. barkeri, C. bar tlettii, C. beijerinckii, C. bifermentans, C. bolteae, C. bornimense, C. botulinum, C. bowmanii, C. bryantii, C. butyricum, C. cadaveris, C. caenicola, C. caminithermale, C. car boxidivorans, C. carnis, C. cavendishii, C. celatum, C. celerecrescens, C. cellobioparum, C. cellulofermentans, C. cellulolyticum, C.

cellulosi, C. cellulovorans, C. chartatabidum, C. chauvoei, C. chromiireducens, C. citroniae, C. clariflavum, C. clostridioforme, C. coccoides, C. cochle arium, C. colletant, C. colicanis, C. colinum, C. collagenovorans, C. cylindrosporum, C. difficile, C. diolis, C. disporicum, C. drakei, C. durum, C. estertheticum, C. aramiense, C. fallax, C. felsineum, C. fervidum, C. fimetarium, C. formicaceticum, C. frigidicarnis, C. frigoris, C. ganghwense, C. gasigenes, C. ghonii, C. glycolicum, C. glycyrrhizinilyticum, C. grantii, C. haemolyticum, C. halophilum, C. hastiforme, C. hathewayi, C. herbivorans, C. hiranonis, C. histolyticum, C. homopropionicum, C. huakuii, C. hungatei, C. hydrogeniformans, C. hydroxybenzoicum, C. hylemonae, C. jejuense, C. indolis, C. innocuum, C. intestinale, C. irregulare, C. isatidis, C. josui, C. kluyveri, C. lactatifermentans, C. lacusfryxellense, C. laramiense, C. lavalense, C. lentocellum, C. leptum, C. lentoputrescens, C. limosum, C. lituseburense, C. litorale, C. lortetii, C. ljungdahlii, C. lundense, C. magnum, C. malenominatum, C. mangenotii, C. mayombei, C. methylpentosum, C. methoxybenzovorans, C. neopropionicum, C. novyi, C. nitrophenolicum, C. orbiscindens, C. oroticum, C. oceanicum, C. oxalicum, C. papyrosolvens, C. paradoxum, C. paraperfringens, C. paraputrificum, C. pascui, C. pasteurianum, C. peptidivorans, C. perenne, C. perfringens, C. pfennigii, C. phytofermentans, C. piliforme, C. polysaccharolyticum, C. populeti, C. propionicum, C. proteoclasticum, C. proteolyticum, C. psychrophilum, C. puniceum, C. purinilyticum, C. putrefaciens, C. putrificum, C. quercicolum, C. quinii, C. ramosum, C. rectum, C. roseum, C. saccharobutylicum, C. saccharogumia, C. saccharolyticum, C. saccharoperbutylacetonicum, C. sardiniense, C. sartagoforme, C. scatologenes, C. schirmacherense, C. scindens, C. septicum, C. sordellii, C. sphenoides, C. spiroforme, C. sporogenes, C. sporosphaeroides, C. stercorarium, C. stercorarium leptospartum, C. stercorarium, C. stercorarium thermolacticum, C. sticklandii, C. straminisolvens, C. subterminale, C. sufflavum, C. sulfidigenes, C. symbiosum, C. tagluense, C. tepidiprofundi, C. termitidis, C. tertium, C. tetani, C. tetanomorphum, C. thermaceticum, C. thermautotrophicum, C. thermoalcaliphilum, C. thermobutyricum, C. thermocellum, C. thermocopriae, C. thermohydrosulfuricum, C. thermolacticum, C. thermopalmarium, C. thermopapyrolyticum, C. thermosaccharolyticum, C. thermosuccinogenes, C. thermosulfurigenes, C. thiosulfatireducens, C. tyrobutyricum, C. uliginosum, C. ultunense, C. villosum, C. vincentii, C. viride, C. xylanolyticum, C. xylanovorans (Rydzak and Guss, 2018).

Metabolic engineering strategies

Rational metabolic engineering strategies

A major advancement in butanol formulation by *C. acetobutylicum* and other solventogenic strains was attained by minimizing the redox potential to enhance butanol fermentation. Significant metabolic engineering strategies increased to obtain significant products from C. *acetobutylicum.* One of the metabolic strategies is to enhance the metabolic flux toward the desired product, usually achieved by limiting the byproduct formulation (Lütke-Eversloh and Bahl, 2011). Targeting the removal of the acid forming pathways and overexpression of product enhancer's genes *adhE1* (aad) in unalike host strains for improved alcohol production was analyzed too (Sillers *et al*., 2009). Mutants showed a great decrease in products like reduction in butanol production which can be restored by combination like the *ctfB* 'knock-down' (Tummala *et al*., 2003) strain with *adhE1* overexpression. Hence, ethanol and butanol production increased. Later on, optimization of expression and overexpression of *ctfb* and *adhE1* was achieved by using a unique type of promoters like *atc and ptb* promoters respectively. The *adc* KO mutants showed a small amount of acetone but notably lower butanol production. Butanol production was enhanced by the use of buffers like calcium carbonate and the addition of methyl viologen (Tummala *et al*., 2003). Solventogensis is naturally occur by the process of sporulation, which eventually stops butanol production. The use of asporogenous strains of *Clostridium* might present a great starting point for metabolic engineering. Most popular asporogenous strain *C. acetobutylicum* M5 due to lost megaplasmid *pSOL1* with *adhE1* restored butanol production which was lost during sporulation (Dürre, 2008, Lütke-Eversloh and Bahl, 2011). Another simple idea from an economical side was the production of Vitamin B_2 by the over expression of *ribGBAH* in *C. acetobutylicum* (Cai and Bennett, 2011).

Solventogenic clostridia undergo ABE fermentation and butanol is produced along with acetate and butyrate. Acetate and butyrate production is due to two unique characteristics of bacteria known as solventogenic and acidogenic also known as biphasic fermentation. Complex metabolic pathways have been reviewed with respect to butanol production. Hot and cold channels for butanol formation has been decided by mass balance analyses and metabolic flux in *C. acetobutylicum* (Jang *et al*., 2012). Production of butanol due to uptake of butyrate and acetate by CoA transferase is cold channel flux of butanol production. *pta* and *buk* gene knocked out while cold channel flux decreasing and the hot channel flux become active. NADH and NADPH used as co factor by mutant aldehyde alcohol dehydrogenase (Jang *et al*., 2012, Cooksley *et al*., 2012). Mutant aldehyde dehydrogenase over-expressed. Blocking of acid formation pathway flux and acid re-assimilation flux can cause decrease in cold flux. To increase butanol production acid formation is inhibited by knocking out of *pta* encoding for phosphotransacetylase, *ptb* encoding for phophotransburyrylase, *ack* for acetate kinase and *buk* encoding for butyrate kinase (Sillers *et al*.,

2008). *Clostridium beijerinckii* strains also follows above mentioned mechanisms. Knocking out of *ctfAB* encoding for CoA transferase and *adc* encoding for acetoacetate decarboxylase block the re-assimilation of acid. But significant increase in butanol production was observed. Overexpression of *adhE1* is also significant in butanol production as well as ethanol production. Ethanol production also enhanced by elimination of *hbd* gene encoding for 3-hydroxybutyryl-CoA dehydrogenase. Simultaneous knocking out of *hpt, idh* and *pta* gene enhanced the ethanol production (Sillers *et al*., 2008, Lee *et al*., 2009). So, solventogenic clostridia like *Clostridium acetobutylicum, C. beijerinckii, C. saccharobutylicum, C. phytofermentants, C. thermocellum,* and *Clostridium cellulyticum* are involved in the formation of ethanol by starch, sugar, and cellulose (Papoutsakis, 2008). Isopropanol, isopropanol-butanol-ethanol mixture production also obtained by many clostridia. Production was enhanced by the introduction of *the sadh* gene while the *ctfB, ctfA* and *adc* genes over-expressed in two different *buk* mutant strains. *Sadh* and *hydG* genes under the control of *thl* promotor in *C. acetobutylicum* produced IBE mixture (Dai *et al*., 2012, Jang *et al*., 2013). *alsS* gene from *Bacillus subtilis* and *livCd* and *yqhD* gene from *E. coli* inserted into the *C. acetobutylicum,* Isobutanol production was observed (Higashide *et al*., 2011). Hydrogen is also produced by any type of clostridia.

Combinatorial and other explorative metabolic engineering strategies

The selection of branched fermentation pathways along with solventogenic clostridia has peculiar advantages for the organisms. Some biotechnologists considered this technique as an inverse metabolic engineering technique. For proper implementation of this technique, a suitable screening method is required. The conventional screening method is mimicking nature which is the selection of cells according to various environmental conditions. This screening method is very successful so far for the production of butanol. By the implementation of chemical mutants like in *C. acetobutylicum and in C. beijerinckii* fermentative products obtained in high value. Another screening method was the selection of solvent negative mutants for biochemical and genetic analysis. Solvent negative mutants and suicidal substrates like allyl alcohol and bromobutyrate were selected along with the use of special technique "colorimetric alcohol assay. Overexpression of (CAC1869) encoded transcriptional regulators was confirmed to increase butanol tolerance. After that this combinatorial strategy also helped in the pointing out of RNA mediated carboxylic acid tolerance improvement (Cho *et al*., 2015).

Random mutagenesis

Physiological, biochemical and genetic knowledge helps us to manipulate the genetic combination at the molecular level. Random mutagenesis helps us to isolate useful strains or mutants with enhanced phenotype. For the generation of such mutant populations, UV radiations were used, but some chemicals like *N-methyl-N-nitro-n-nitrosoguanidine* was more effective in clostridia. Ionized gas beams, nitrogen ion beams and atmospheric and room temperature plasma have replaced the use of highly toxic chemicals for the isolation of mutants (Lütke-Eversloh, 2014).

Rapid phenotype also improved by the use of an evolutionary method known as Genome shuffling. Selection of appropriate parent and chemical mutagenesis were two steps involved in the above-mentioned techniques and were enhanced the ABE production in clostridia.

Another more complex system was used in which chromosomal mismatch repair *operon mutSL* was inactivated and replaced with plasma bearing fused mutSL alleles with anhydrotetracycline-inducible Pcm-2tetO1 promoter along with two copies of repressor gene TetR (Lütke-Eversloh, 2014).

Phenotypic screening

Many methods are available to create mutant collections in *Clostridia.* Single or multiple genomic mutations, overexpression of collections of heterologous and homologous genomic DNA fragments, mutated global regulators and other specific proteins are the foundation of such methods. Explorative metabolic engineering strategies are limited due to screening methods (Borden and Papoutsakis, 2007). Phenotypic methods must be organized in high throughput manners and must be feasible concerning handling, costs and time. Detection of biofuels is not easy but they can be observed with the fluorescent and other calorimetric and growth-dependent assays (Borden and Papoutsakis, 2007). Increased production of ABE as compare to its parental strains is phenotypically associated with improved tolerance (Mao *et al*., 2010). Many explorative approaches were reported in association with the high butanol production by *C. acetobutylicum* (Mao *et al*., 2010). In the genomic era, modification of metabolic pathways was included the use of mimicking nature substrates and the suicidal substrates as described previously. The use of suicidal substrates only cleavage products remain toxic, which allowed the positive selection of such clones which did not have degradative nature and then various mutants with enzymes defect isolated. Halogenated carboxylic acid analogs affected on many clostridia and mutants isolated with altered metabolites like in *C. acetobutylicum.* The first

example of the combined systematic and explorative metabolic strategy was the use of highly toxic fluoroacetyl-CoA formed by the uptake and the conversion of fluoroacetate by the clones for their growth. It simply called as mutations acetate metabolic pathway.

Another strategy for phenotypic selection was directly based on analyzing alcohol synthesis. A high-value screening method was developed with the use of nitroblue tetrazolium derivatization in a colorimetric assay for the observation of butanol and ethanol production in microtiter cultures (Lütke-Eversloh, 2014).

Flow cytometry is another screening strategy to analyze at a single cellular level with the use of several stains and staining methods for observation of cell morphologies. Cell morphologies are related to metabolic states, especially in sporulation. Flow cytometry is also helping the full tool in ABE fermentation mapping (Patakova *et al*., 2013). In nutshell four screening principles are present for the selection of the desired phenotype, especially for clostridia. First, is tolerance based on the second principle is based on suicidal substrates, the next one is the use of colorimeter for alcohol detection and the last high-value principal based on Flow cytometry. Scientists are in a way of expanding the other techniques like transcriptional factor engineering toward the clostridia (Lütke-Eversloh, 2014).

Enzyme engineering

Specific enzyme engineering needs more concentration because only a small work has been done on enzymes but except for cellulose-degrading enzymes. Many chief fermentative enzymes have been purified and characterized biochemically. Butyryl –CoA helps in the enhancement of carbon flux in butanol from acetyl CoA and it has been done by optimizing the activity of *AdhE1* (Jang *et al*., 2012). Thiolase enzyme was engineered to decrease its respond towards inhibitors like CoA-SA. First of all thiolase activity and CoA-SA activity observed without the gene mutation. Then the activity of thiolase enzymes was observed after the mutation in *thlA* gene. 18 % higher butanol formation was observed after overexpression of the engineered thlA gene (Mann and Lütke-Eversloh, 2013, Lütke-Eversloh, 2014).

Conclusions and future directions

As compared to other microorganisms like *Escherichia coli* and *Bacillus subtilis, Clostridia* have limited industrial applications due to confined manipulative tools for *clostridia*, but ABE fermentation from *Clostridium* has significant importance which can be obtained from the fermentation of cellulose, glucose, and glycerol. Many countries like China have been developed new highly advantageous industries for ABE fermentation and especially to enhance the higher butanol ratio based on continuous fermentation. Many alcohols used as gasoline, drinking and other purposes are produced by *clostridia* nowadays. *Clostridia* also enhance alcohol production by adopting different pathways. Several metabolic engineering strategies consist of rational, combinatorial and explorative metabolic strategies that have been developed. Metabolic flux and mass balance analysis and the selection of mutant strains by many phenotypic screening methods are also helpful in the enhancement of fermentative products from different *Clostridium species.* Different genetic manipulative tools are using against other microorganisms, researchers are trying to shift modified genetic manipulative techniques for *Clostridium* to enhance the industrially important products from it.

Acetone-butanol-ethanol fermentation from different species of *Clostridium* is the most common fermentative process used in the industry for product formation. Butanol production from *Clostridium acetobutylicum* is under observation. Ethanol production from *Clostridium thermocellum* has great importance but many other additional products are formed during the ethanol production pathway. The optimization of production pathways can help the *clostridia* to become a key industrial organism. Hydrogen production optimization can help us in various energy sources. Scientists are discovering new pathways for biofuel production. By the discovery of new pathways for the production of ethanol, butanol, and hydrogen and their optimization will help the industry to shift toward natural important products isolation from *Clostridium* species.

REFERENCES

Akinosho, H., K. Yee, D. Close and A. Ragauskas (2014). The emergence of *Clostridium thermocellum* as a high utility candidate for consolidated bioprocessing applications. *Frontiers in Chemistry*, 2**:** 66.

Biebl, H. (2001). Fermentation of glycerol by *Clostridium pasteurianum*—batch and continuous culture studies. *Journal of Industrial Microbiology and Biotechnology*, 27**:** 18-26.

Borden, J. R. and E.T. Papoutsakis (2007). Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.,* 73**:** 3061-3068.

Cai, X. and G.N. Bennett (2011). Improving the *Clostridium acetobutylicum* butanol fermentation by engineering the strain for co-production of riboflavin. *Journal of Industrial Microbiology & Biotechnology*, 38: 1013-1025.

Cho, C., Y.S. Jang, H. G. Moon, J. Lee and S.Y. Lee (2015). Metabolic engineering of clostridia for the production of chemicals. *Biofuels, Bioproducts and Biorefining,* 9: 211-225.

Cooksley, C. M., Y. Zhang, H. Wang, S. Redl, K. Winzer and N.P. Minton (2012). Targeted mutagenesis of the *Clostridium acetobutylicum* acetone–butanol–ethanol fermentation pathway. *Metabolic engineering,* 14: 630-641.

Dai, Z., H. Dong, Y. Zhu, Y. Zhang, Y. Li and Y. Ma (2012). Introducing a single secondary alcohol dehydrogenase into butanol-tolerant *Clostridium acetobutylicum* Rh8 switches ABE fermentation to high level IBE fermentation. *Biotechnology for biofuels,* 5: 44.

Dürre, P. (2008). Fermentative butanol production: bulk chemical and biofuel. *Annals of the New York Academy of Sciences,* 1125: 353-362.

Ezeji, T., N. Qureshi and H. Blaschek (2003). Production of acetone, butanol and ethanol by *Clostridium beijerinckii* BA101 and in situ recovery by gas stripping. *World Journal of Microbiology and Biotechnology,* 19: 595-603.

Higashide, W., Y. Li, Y. Yang and J.C. Liao (2011). Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. *Appl. Environ. Microbiol.,* 77: 2727-2733.

Ishimoto, M., M. Umeyama and S. Chiba (1974). Alteration of fermentation products from butyrate to acetate by nitrate reduction in *Clostridium perfringens*. *Zeitschrift für allgemeine Mikrobiologie,* 14: 115-121.

Jang, Y.-S., J.Y. Lee, J. Lee, J. Park, J. A. Im, M.-H. Eom, J. Lee, S.-H. Lee, H. Song and J.-H. Cho (2012). Enhanced butanol production obtained by reinforcing the direct butanol-forming route in *Clostridium acetobutylicum*. *MBio,* 3: e00314-12.

Jang, Y. S., A. Malaviya and S.Y. Lee (2013). Acetone–butanol–ethanol production with high productivity using *Clostridium acetobutylicum* BKM19. *Biotechnology and bioengineering,* 110: 1646-1653.

Janoir, C., C. Denève, S. Bouttier, F. Barbut, S. Hoys, L. Caleechum, D. Chapetón-Montes, F.C. Pereira, A. O. Henriques and A. Collignon (2013). Adaptive strategies and pathogenesis of *Clostridium difficile* from *in vivo* transcriptomics. *Infection and immunity,* 81**,** 3757-3769.

Jin, M., V. Balan, C. Gunawan and B.E. Dale (2011). Consolidated bioprocessing (CBP) performance of *Clostridium phytofermentans* on AFEX-treated corn stover for ethanol production. *Biotechnology and Bioengineering,* 108: 1290-1297.

Karadag, D. and J.A. Puhakka (2010). Direction of glucose fermentation towards hydrogen or ethanol production through on-line pH control. *International journal of hydrogen energy,* 35: 10245-10251.

Kaur, G., A.K. Srivastava and S. Chand (2012). Simple strategy of repeated batch cultivation for enhanced production of 1, 3-propanediol using *Clostridium diolis*. *Applied Biochemistry and Biotechnology,* 167: 1061-1068.

Khan, A. W. M. and W.D. (2008). Influence of Clostridium saccharolyticum on cellulose degradation by Acetivibrio cellulolyticus*. *Journal of Applied Microbiology,* 53: 379-383.

Koepke, M., C. Mihalcea, J.C. Bromley and S.D. Simpson (2011). Fermentative production of ethanol from carbon monoxide. *Current opinion in Biotechnology,* 22: 320-325.

Kundiyana, D. K., M.R. Wilkins, P. Maddipati and R.L. Huhnke (2011). Effect of temperature, pH and buffer presence on ethanol production from synthesis gas by "*Clostridium ragsdalei*". *Bioresource technology,* 102: 5794-5799.

Lee, J., Y.-S. Jang, S. J. Choi, J. A. Im, H. Song, J.H. Cho, E. T. Papoutsakis, G. N. Bennett and S.Y. Lee (2012). Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol-butanol-ethanol fermentation. *Appl. Environ. Microbiol.,* 78: 1416-1423.

Lee, J. Y., Y.S. Jang, J. Lee, E.T. Papoutsakis and S.Y. Lee (2009). Metabolic engineering of *Clostridium acetobutylicum* M5 for highly selective butanol production. *Biotechnology Journal: Healthcare Nutrition Technology,* 4: 1432-1440.

Levinson, W. E. (2016). *Review of Medical Microbiology and Immunology 14E*, McGraw Hill Professional.

Lütke-Eversloh, T. (2014). Application of new metabolic engineering tools for *Clostridium acetobutylicum*. *Applied Microbiology and Biotechnology,* 98: 5823-5837.

Lütke-Eversloh, T. and H. Bahl (2011). Metabolic engineering of *Clostridium acetobutylicum*: recent advances to improve butanol production. *Current opinion in Biotechnology,* 22: 634-647.

Mann, M. S. and T. Lütke-Eversloh (2013). Thiolase engineering for enhanced butanol production in *Clostridium acetobutylicum*. *Biotechnology and Bioengineering,* 110: 887-897.

Mao, S., Y. Luo, T. Zhang, J. Li, G. Bao, Y. Zhu, Z. Chen, Y. Zhang, Y. Li and Y. Ma (2010). Proteome reference map and comparative proteomic analysis between a wild type *Clostridium acetobutylicum* DSM 1731 and

its mutant with enhanced butanol tolerance and butanol yield. *Journal of Proteome Research*, 9: 3046-3061.

Murray, W., K. Wemyss and A. Khan (1983). Increased ethanol production and tolerance by a pyruvate-negative mutant of *Clostridium saccharolyticum*. *European journal of Applied Microbiology and Biotechnology*, 18: 71-74.

Ng, T. K., A. Ben-Bassat and J. Zeikus (1981). Ethanol production by thermophilic bacteria: fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. *Appl. Environ. Microbiol.*, 41: 1337-1343.

Ni, Y. and Z. Sun (2009). Recent progress on industrial fermentative production of acetone–butanol–ethanol by *Clostridium acetobutylicum* in China. *Applied microbiology and biotechnology*, 83: 415.

Papanek, B., R. Biswas, T. Rydzak and A.M. Guss (2015). Elimination of metabolic pathways to all traditional fermentation products increases ethanol yields in *Clostridium thermocellum*. *Metabolic engineering*, 32**:** 49-54.

Papanikolaou, S., P. Ruiz-Sanchez, B. Pariset, F. Blanchard and M. Fick (2000). High production of 1, 3-propanediol from industrial glycerol by a newly isolated *Clostridium butyricum* strain. *Journal of biotechnology*, 77: 191-208.

Papoutsakis, E. T. (2008). Engineering solventogenic clostridia. *Current opinion in Biotechnology*, 19: 420-429.

Patakova, P., M. Linhova, M. Rychtera, L. Paulova and K. Melzoch (2013). Novel and neglected issues of acetone–butanol–ethanol (ABE) fermentation by clostridia: *Clostridium* metabolic diversity, tools for process mapping and continuous fermentation systems. *Biotechnology Advances*, 31: 58-67.

Rydzak, T. and A.M. Guss (2018). Gene modification in *Clostridium* for increased alcohol production. Google Patents.

Seedorf, H., W.F. Fricke, B. Veith, H. Brüggemann, H. Liesegang, A. Strittmatter, M. Miethke, W. Buckel, J. Hinderberger and F. Li (2008). The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features. *Proceedings of the National Academy of Sciences*, 105**,** 2128-2133.

Sillers, R., M. A. Al-Hinai and E.T. Papoutsakis (2009). Aldehyde–alcohol dehydrogenase and/or thiolase overexpression coupled with CoA transferase downregulation lead to higher alcohol titers and selectivity in *Clostridium acetobutylicum* fermentations. *Biotechnology and Bioengineering*, 102: 38-49.

Sillers, R., A. Chow, B Tracy and E.T. Papoutsakis (2008). Metabolic engineering of the non-sporulating, non-solventogenic *Clostridium acetobutylicum* strain M5 to produce butanol without acetone demonstrate the robustness of the acid-formation pathways and the importance of the electron balance. *Metabolic Engineering*, 10: 321-332.

Stanbury, P. F., A. Whitaker and S.J. Hall (2013). *Principles of fermentation technology*, Elsevier.

Steer, T., M.D. Collins, G. R. Gibson, H. Hippe and P.A. Lawson (2001). *Clostridium hathewayi* sp. nov., from human faeces. *Systematic and applied microbiology*, 24**,** 353-357.

Tummala, S. B., S.G. Junne and E.T. Papoutsakis (2003). Antisense RNA downregulation of coenzyme A transferase combined with alcohol-aldehyde dehydrogenase overexpression leads to predominantly alcohologenic *Clostridium acetobutylicum* fermentations. *Journal of Bacteriology*, 185: 3644-3653.

Turton, L., D. Drucker and L. Ganguli (1983). Effect of glucose concentration in the growth medium upon neutral and acidic fermentation end-products of Clostridium bifermentans, Clostridium sporogenes and Peptostreptococcus anaerobius. *Journal of Medical Microbiology*, 16: 61-67.

Ueki, A., K. Ueki, K. Tanaka, R. Takahashi and T. Takano (1991). End products and molar growth yield of *Clostridium fallax* isolated from an anaerobic digester. *Journal of Fermentation and Bioengineering*, 72: 274-279.

Wen, Z., M. Wu, Y. Lin, L. Yang, J. Lin and P. Cen (2014). Artificial symbiosis for acetone-butanol-ethanol (ABE) fermentation from alkali extracted deshelled corn cobs by co-culture of *Clostridium beijerinckii* and Clostridium cellulovorans. *Microbial cell factories*, 13: 92.

Wilde, E., M. Collins and H. Hippe (1997). *Clostridium pascui* sp. nov., a new glutamate-fermenting sporeformer from a pasture in Pakistan. *International Journal of Systematic and Evolutionary Microbiology*, 47: 164-170.

Younesi, H., G. Najafpour and A.R. Mohamed (2005). Ethanol and acetate production from synthesis gas via fermentation processes using anaerobic bacterium, *Clostridium ljungdahlii*. *Biochemical Engineering Journal*, 27: 110-119.

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