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A N I N T E R N A T I O N A L J O U R N A L

Genetic Engineering In BioButanol Production And Tolerance

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ABSTRACT

The growing need to address current energy and environmental problems has sparked an interest in developing improved biological methods to produce liquid fuels from renewable sources. Higher-chain alcohols possess chemical properties that are more similar to gasoline. Ethanol and butanol are two products which are used as biofuel. Butanol production was more concerned than ethanol because of its high octane number. Unfortunately, these alcohols are not produced efficiently in natural microorganisms, and thus economical production in industrial volumes remains a challenge. The synthetic biology, however, offers additional tools to engineer synthetic pathways in user-friendly hosts to help increase titers and productivity of bio-butanol. Knock out and over-expression of genes is the major approaches towards genetic manipulation and metabolic engineering of microbes. Yet there are TargeTron Technology, Antisense RNA and CRISPR technology has a vital role in genome manipulation of C.acetobutylicum. This review concentrates on the recent developments for efficient production of butanol and butanol tolerance by various genetically engineered microbes.

Key words: Butanol, CRISPR, biofuel, *Clostridium acetobutylicum*

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INTRODUCTION

One of the greatest challenges for mankind in the 21st century is to meet the growing demand for energy which is utilized in transportation, heating furnaces and other industrial processes in a suitable way [1]. Biofuel production is classified into four generation (based on raw material). First generation biofuel synthesized from edible plant material and second-generation biofuel derived from non-food vegetable like feed stocks (e.g. Lignocelluloses material). Third generation biofuel is derived from the oleaginous materials through heterotrophic mode (microbes like microalgae, yeast, bacteria). Fourth generation biofuel is based on direct use of CO_2 through phototrophic mode [1,2].

Ethanol is largely incorporated as biofuel in Brazil, USA and some European countries. Ethanol can be blended with petrol or used as neat alcohol in dedicated engines.Taking advantage of high octane number and heat of vaporization; it is an excellent fuel for future's advanced Flex-fuel hybrid vehicles [2]. In spite of all these qualities of ethanol, currently butanol, higher alcohols, alkanes, alkenes and biodiesel are preferred due to higher octane number and other physiochemical properties (as higher alcohol don't form azeotrope with water)[3-5]. Traditionally, bioalcohols are produced by fermentation process from naturally occurring microbes like yeast *Saccharomyces cerevisiae*, bacteria like *Zygomonas mobilis* and *Clostridium acetobutylicum* [6-8]. According to Grand View Research Inc. there is a steep rise in demand for biobutanol production in the next few years due to efficient fermentation technologies and cellulosic extraction technologies. There is a growing interest in butanol production from chemical based synthesis to biobased [9]. According to literature there are 6,600 articles titled with butanol out of which 746 articles has title of butanol production and 55 articles shows enhanced butanol production by engineered microbe (i.e genetic/metabolic/other type of engineering). The comparative analysis of articles entitled with butanol production and tolerance are explained graphically in figure 1.

The microbes producing butanol are of genus *Clostridia*, but are also reported in traces in various fungi (eg. *Penicillium*, *Aspergillus* species) and bacteria growing on the cereals[10]. The strain most commonly used in genetic engineering are *Clostridium acetobutylicum* and *Clostridium beijerinckii*. Other microbes which produce butanol are *E.coli*, *Pseudomonas* species and *S.cerevisiae*. The pathway followed by *Clostridium* species for acetone, butanol and ethanol production is depicted in figure 2. Table 1 shows a summary of all substrates utilized for biobutanol production, fermentation and purification process which was done in the year 2015. Table 2 depicts agricultural waste and industrial waste used for the production of butanol. There are various other microbes available for butanol production apart from *Clostridium acetobutylicum*. *Lactobacillus* and *Pseudomonas* were found to have butanol tolerance of 3% and 6% respectively [11-14].

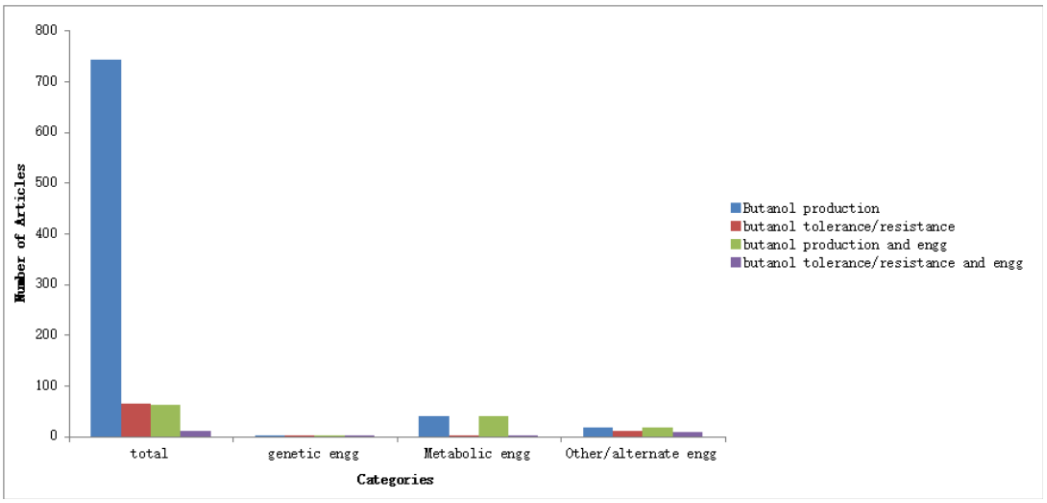

Figure 1. Graphical representation of butanol related (all key words in title) research article based on google scholar. (engg = engineering)

Table 1: List of microbes showing butanol production from 2015 publications

Microbe	Substrate	Mode of fermentation	Purification method	Butanol Yield	Ref.
S.cerevisiae ASA2BR Adh1+5g	Glucose	Batch	-	300mg/L	15
C.tyrobutyricum Δack–adhE2	Glucose	Fed batch	Gas stripping	55g/L	16
E.coli Bw2V	Glucose	Batch	-	2.8g/L	17
C.acetobutylicum ATCC 824	Glucose	continuous	Ex-situ recovery fermentation	146.9g/L	18

Table 2: List of microbe using waste (agricultural/domestic/industrial) as substrate for butanol production

Microbe	Waste used	Pretreatment	Butanol Yield (g/L)	Ref.
C.saccharoperbutylacetonicum N1-4	Palm oil waste	Enzymatic hydrolysis	4.37	19
C.acetobutylicum ATCC 824	Domestic organic waste	Enzyme hydrolysis	3	20
C.acetobutylicum	Industrial dairy waste	Enzymatic hydrolysis	7.5	21
C.beijerinckii			5.8	
Immobilized *C.acetobutylicum*	Waste starch	-	15.3	22

GENETIC ENGINEERING IN BUTANOL PRODUCTION

Clostridium acetobutylicum

Clostridium acetobutylicum a spore producing anaerobic solventogenic microbe was first isolated by Weisman in the early 1900. The fermentation carried out by the biofuel producer *C.acetobutylicum* is characterized by two phases, acidogenesis phase and solventogenesis phase. Acidogenesis occurs in exponential phase characterized by production of acid (acetate and butyrate). Solventogenesis occurs during transition of exponential and stationary phase characterized by production of solvents (acetone, butanol and ethanol)[23]. Amador-Noguez and his group reveal that in the kinetics of acetone, butanol and ethanol production there is a pH change in transition between acidogenesis and solventognesis. Metabolic remodeling reveals significant changes in an ordered series of metabolite concentration, involving all the metabolites synthesized during phase change from acidogenesis to solventogenesis [24]. Incorporation of induced gene with mathematical modeling of fermentation process provides a mechanical representation of pH induced switch between the two phases [25]. *Clostridium* is metabolically engineered for butanol production. Various mutation strains are formed, the genetic accessibility problem has been resolved by the in vivo methylation protocol using host strains lacking the very active restriction endonuclease Cac824 [26].

The strategies used for butanol production are disruption of butyrate, acetone, lactate and acetate pathways. The disruption of the butyrate acetone lactate pathway is done by 1) knockout/knockdown gene associated with butyrate, acetone, lactate and acetate 2) Inserting genes or over expressing genes (*SpoA* gene, *groESL* gene) associated with solvent production. Integrational plasmid technology, including replicating and non replicating plasmid is used, but due to low screening efficiency TargeTron technology is more preferred. Both technologies were used for improvement in butanol titer in solvent fermentation, but the disruption of *adc* gene causes increase in butanol production ratio from 70% to 80-85%. However Antisense RNA Technology is a potent and flexible tool for microbial manipulation (silencing) of gene without changing the regulation of gene expression. It is used in silencing of the *ctfB* gene for enhancing butanol ratio in solvent fermentation. The butanol production ratio was also improved when *aad* gene

is inserted in the strain M5 which is lacking megaplasmid pSOL1 (containing *aad* gene and acetone producing gene). In fermentation process of butanol production by *C.acetobutylicum* using different lignocellulosic material by heterologous minicellulosome was confirmed in two studies (metabolic engineering of *C.acetobutylicum* using *C.thermocellum* and *C.cellulolyticum*). The deletion of CRE (catabolic responsive element) causes 7.5 fold increase in butanol production [11,23-25]. Deletion of the gene *spo0A* performed by CRISPR analysis in *Closrtidium beijerinckii* also proved for ABE production. CRISPR analysis is a three year old method based on natural CRISPR (Cluster Regulatory Interspaced Short Palindromic Repeats) and CRISPR/Cas system (provide immune system by cleaving foreign DNA in bacteria and archea group). CRISPR analysis is a markerless and highly efficient deletion method of genome engineering done by simple cloning method. In this technique CRISPR array of target sequence transcribed and processed to form CRISPR-RNA (crRNA) guide Cas nuclease and cleave the target site with the help of protospacer-adjacent motif (PAM). In type-II CRISPR analysis Cas9 is activated only with Trans Activating CRISPR RNA (TracrRNA) and CrRNA. It is a dual RNA complex guiding system. CRISPR technique has some limitations like the accuracy of CRISPR analysis depends on homologous recombinant efficiency of microbe, and the precise prediction of insertion site especially when target site is short [27-29].

Escherichia coli

E. coli is engineered for various biotechnological applications. There are 62 articles found in google scolar with titled butanol and *coli* as key word out of which 22 articles related to butanol production . In last half decades, there are 13 articles (total 42 articles titled with *coli* butanol key words) showing butanol production from *coli*. Thus, it shows various advances in *E.coli* for biobutanol production [6,30]. The acetone-butanol-ethanol (ABE) fermentation pathway of *C.acetobutylicum* used in production of butanol was first constructed in *E.coli* to establish a baseline for comparison to other hosts 31,32. Improved titers were seem to be achieved due to the co expression of S.cerevisiae formate dehydrogenase while over expression of E.coli glyceraldehyde3-phosphate dehydrogenase to elevate glycolytic flux improved titers to 580mg/L and butanol production to 200mg/L [31,33]. Currently the overall production of n-butanol and isobutanol is 0.001g/L to 30g/L and 4 to 50 g/L respectively [6,17,31]. It was observed that the mutation of transcription factor of camp receptor protein causes increased tolerance of isobutanol up to 1.2% (v/v) against 2% isobutanol and productivity was 9.8g/L [34]. However butanol production reached upto 2278±29g/(L*d) due to stereo selectivity of butanone as proR over proS for production of R-2-butanol over S-2-butanol [35]. Recently *E.coli* was tested for potentials for the native promoter of hydrogenase I cluster Phya Bw2Vcarries plasmid pCNA-PHC and pENA-TA in anaerobic fermentation with extra glucose, the butanol production was up to 2.8g/l in batch culture bioreactor [17].

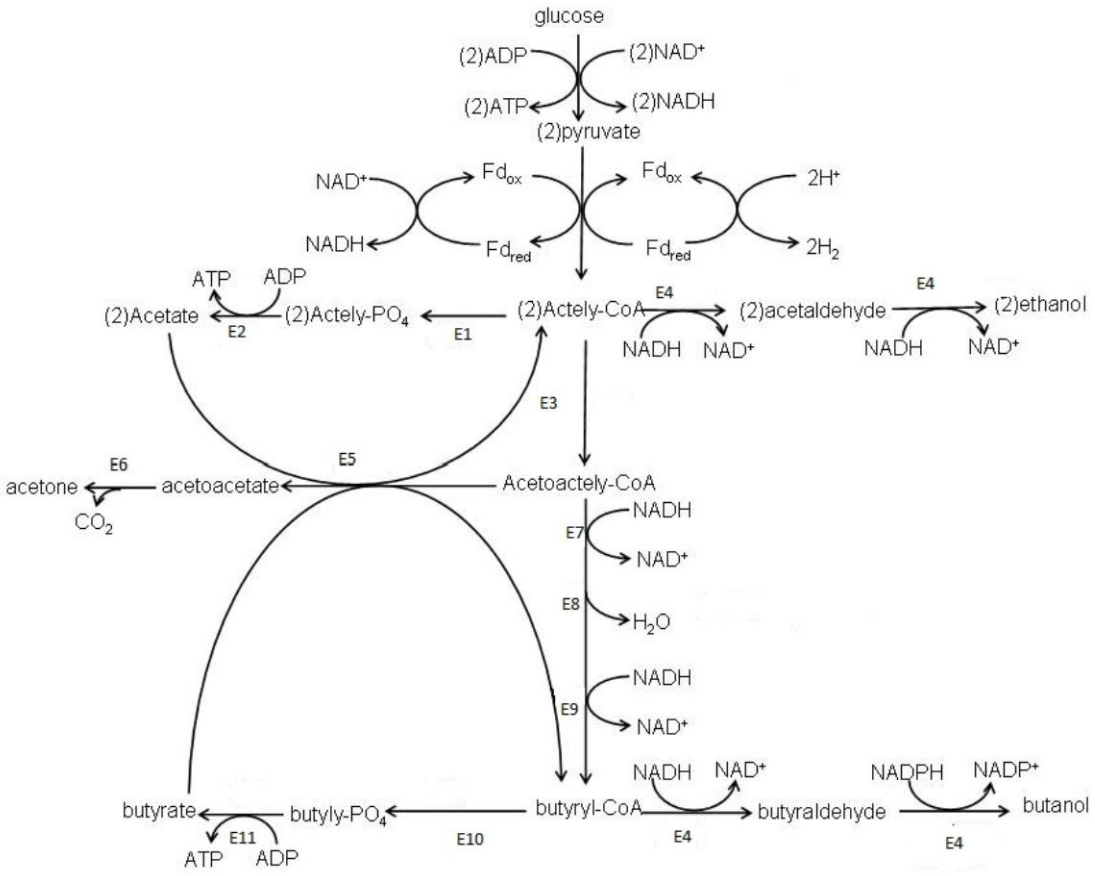

Figure 2. Pathway depicting butanol, acetone and ethanol production.E1 to E9 are enzymes involved in ABE pathway. E1-PTA-Phoshate acetyl transferase, E2-AK-Acetate kinase, E3-THL-ThiolaseA, E4-AAD-Alcohol aldehyde dehydrogenase, E5-CoAT- Co-A transferase, E6-AADC-Acetoacetate decarboxylase, E7-HBD-3 Hydroxybutryl CoA dehydrogenase, E8-CRO-Crontonase, E9-BCD-Butyryl-CoA dehydrogenase, E10-PTB-Phosphate butryl-transferase, E11-BK-Butyrate Kinase (modified 36)

Cyanobacteria

Carbon Dioxide as a sole source of carbon for all plants, which can also be used for chemicals as well as in biofuel production [37]. Synthetic pathway (Figure 3) was constructed in cyanobacteria *Synechococcus elongates* PCC7942 for the production of ethanol, n-butanol and 2,3-butandiol [38-41]. The n-butanol production was 14.5mg/L in strain EL14 containing plasmid NSI T.d- *ter* (his tag) and plasmid NSII *atoB, hbd, crt* and *adhE2* whereas NADH driven metabolism (NADP dependent *Adh* from *E.coli* and *Bldh* from *C.beijerinckii*) in *Synechococcus elongates* EL22 shows 29.9mg/L. The low productivity was due to toxicity [38-40]. Butandiol was targeted because of the less toxicity and matches with the pathway of cyanobacteria. Production of butandiol was 2.38g/L, which is significant in terms of exogenous pathway in cyanobacteria [41].

Thermoanaerobacterium saccharolyticum

Thermoanaerobacterium saccharolyticum strain JW/SL-YS485 closely related to thermophilic anaerobe, a gram positive bacteria. *Thermoanaerobacterium* were well characterized and engineered for the production of biohydrogen, ethanol and butanol[42,43]. The gene cluster used were *hbd*, *crt*, *bcd*, *eftA*, *eftB* from *Thermoanaerobacterium thermosaccharolyticum* DSM571 and *adhE2* from *C.acetobutylicum*. The pathway for butanol production from *C.acetobutylicum* which shows that from 10g/L of xylose produces 0.84g/L (21% of theoretical) however lactate deficient strains shows 1.05g/L (26% of theoretical)[44].

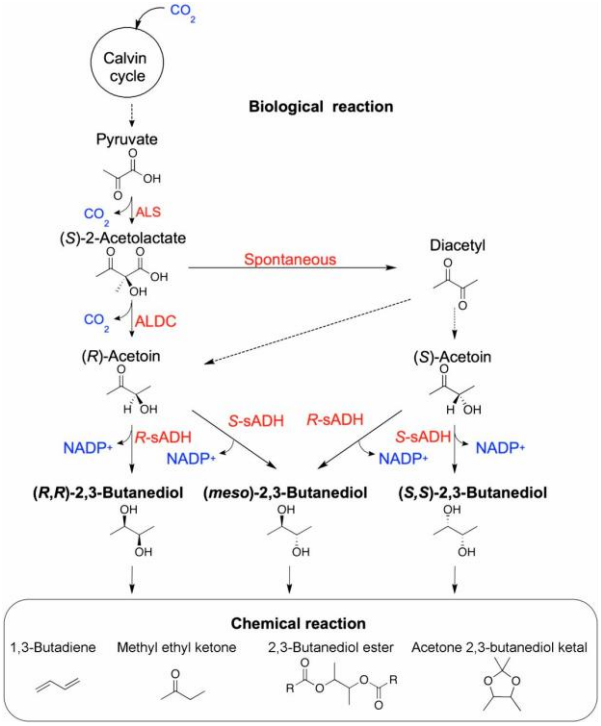

Figure 3:The pathway for acetoin and 2,3 butandiol production in *S.elongates* PCC7942. The acetoin/2,3-butandiol production pathway contains three enzymatic steps from pyruvate 41.

Klebsiella pneumoniae

Klebsiella a gram negative, rod shaped bacteria was genetically modified for 1-butanol, 2-butanol, butandiol, propanediol, ethanol and hydrogen [45-47]. *Klebsiella pneumoniae* was engineered to produce 2-butanol and 1-butanol from crude glycerol as a sole carbon source. 1-butanol production from the *Klebsiella* was done by modifying CoA-dependent pathway and 2-2-keto acid pathway was established by expressing the genes for *ter-bdhB-bdhA* and *kivd* respectively. The butanol titer and butanol production were found to be 15.03 mg/L and 27.79 mg butanol/g-cell and 28.7mg/L and 51.58mg butanol/g cell. The native products are suppressed by antisense RNA strategy [46]. 1-butanol was produced by engineering a-ketoisovalerate decarboxylase (*kivd*) and alcohol dehydrogenase (*adh*) from *Lactococcus lactis* into *Klebsiella pneumoniae* which bypassed the pathway for production of 2,3-butandiol. The yield was 320mg/L which shows increment by 2 folds [48-50].

Geobacillus thermoglucosidasius

The *Geobacillus* is a facultative anaerobic, rod-shaped, gram-positive and endospore-forming bacterium. *Geobacillus* species are capable to grows between 40°C and 70°C [51]. The *Geobacillus* was engineered for the production ethanol and isobutanol [52,53]. The *Geobacillus thermoglucosidasius* was engineered with acetohydroxy acid synthase gene and 2-ketoisovalerate dehydrogenase gene from *B.subtilis* and *L.lactis* respectively and promoter region of lactate dehydrogenase gene from Geobacillus thermodenitrificans. The isobutanol produced was 3.3g/L from glucose as substrate. Lin et al., showed that isobutanol was produced at elevated temperature of 50°C [53].

Pyrococcus furiosus

Pyrococcus furiosus is a heterophilic archaebacteria. It is cocci shaped, flagellated bacterium whose metabolic products are CO_2 and H_2 [54,55]. The *Pyrococcus furiosus* was genetically engineered for butanol production at elevated temperature. Lactate dehydrogenase gene from *Caldicellulosiruptor bescii* was expressed in *Pyrococcus* for the production of 3-hydroxypropionate (further used as electrofuel) using hydrogen as a substrate [56-59]. 1-butanol and 2-butanol production pathway was established in *Pyrococcus furiosus*. Genes responsible for the enzyme involved in first three reactions acetylCoA to crontylCoA isolated from *Thermoanaerobacter tengcongensis* and trans-2-enoyl-CoA reductase (*ter*) was from *Spirochaete thermophila* and butyraldehyde dehydrogenase (*Bad*) and butanol dehydrogenase (*Bdh*) was obtained from *Thermoanaerobacter* sp. X514. The production of 1-butanol and 2 butanol was 70mg/L and 15mg/L after 48 hr from genetically engineered *Pyrococcus furiosus* at 60°C respectively [60].

Yeast

Saccharomyces is well known as yeast used in various fermentation processes, especially beverage industry and alcohol production [61]. *Saccharomyces cerevisiae* has been genetically modified, for the production of 1-butanol, isobutanol and 2-butanol. The optimal 1-butanol and isobutanol production was approximately matched with the theoretical production of butanol product. The maxima was 242.8mg/L from glucose by deleting gene Δ*adh1*, Δ*ilv2* of YSG52 strain and 92mg/L from glycine as a single protein source by using novel pathway by converting glycine into glyoxylate further β-ethylmalate then α-ketovalerate into butanol by following Ehlich pathway [62]. The maxima for optimum production of isobutanol was 1620mg/L in a YPH499 strain by using full cytoplasmic pathway with concomitant mitochondrial gene *ILv2,ILV2*,

ILV2Δ54, ILV3Δ41, ILV5Δ47,ADH6, MAE1
Lactococcus lactis *gene kivD* [63].

Table 3: List of genetically engineered microbes to produce butanol

Microorganism	Modification	Product	Yield	Ref.
E.coli	Deletion of *adh, ldh, frd, fnr* and *pta* and insertion of *bcd-etfAB* from *C.acetobutylicum*	n-Butanol	0.37g/L	31
	GapA from *E.coli* over-expression *Fdh1* from *Saccharomycesis cerevisiae adhE2, bcd, etfAB* from *C. acetobutylicum*		0.58g/L	33
	over-expression of *kivd (L.lactis), ADH2 (S.cerevisiae),* and the *E.coli ilvA, leuABCD, thrAfbrBC*		1.250g/L	79
S.cerevisiae	*ERG10 (E.coli) hbd, crt, adhE2 (C.beijerinckii) ccr* from *S.collinus*	n-Butanol	2.5mg/L	80
C.acetobutylicum	Synthetic isopropanol operon	n-Butanol	14.74g/L*	81
	Knock out of Plasmid SOL1, and *aad* (*ptb* promoter)		11.396g/L#	82
	Ribosomal engineering		12.48g/L(butanol tolerance of 1.2 to 1.4%)	83
	Coprodution of riboflavin		14.09*#	84
	Adc promoter insertion		8.9g/L	85
	Integrated DNA tech approach		23.4g/L	86
C.tyrobutyricum	Knock out of *ack*	n-Butanol	16g/L	87
S.elongatusEL14	Plasmid NSI T.d- *ter*(his tag) Plasmid NSII *atoB, hbd, crt* and *adhE2*	n-butanol	14.5mg/L	39
S.elongatusEL22	NADP dependent *Adh* from *E.coli* and *Bldh* from *C.beijerinckii*		29.9mg/L	40
S.elongatus PCC7942	*Adh* from *C.beijerinckii*	butandiol	2.38g/L	41
T.saccharolyticum	*C.acetobutylicum*	n-butanol	1.05g/L	44
K.pneumoniae	Modifying CoA-dependent and 2-2-ketoacid pathway *Ter-bdhB-bdhA* and *kivd*	1-butanol	27.79mg 1-butanol/g cell 51.58mg 2-butanol/g cell	50
	α-ketoisovalerate decarboxylase and alcohol dehydrogenase from *L.lactis*	2-butanol	320mg/L	48
G.thermoglucosidasius	Aceto-hydroxy-acid sythase (*B.subtilis*) and 2-keto-isovalerate dehydrogenase (*L.lacti)*	Isobutanol	3.3g/L	53

P.furiosus	Enzymes from acetylCoA to crontylCoA (*T.tengcogensis), ter (S.thermophila)Bad* and *Bdh (Thermoacetobacter sp.X514*)	1-butanol 2-butanol	70mg/L 15mg/L	60

Keys # = calculated; * used for n-butanol however isopropanol operon is not produced that much amount at optimized condition; *ldh* (lactate dehydrogenase); *pta* (phosphate acetyltransferase), *Kivd* (2-ketoacid decarboxylase); *ter* (NADH dependent crotonyl-CoA specific trans-enoyl-CoA reductase); T.d-*ter* histidine tag (NADH dependent crotonyl-CoA especific to trans-enoyl-CoA reductase from *Treponema denticola*); *ccr* (Butyryl-CoA dehydrogenase); *ERG10* (acetoacetyl-CoA thiolase); *fnr* (DNA-binding transcriptional dual regulator, a global regulator for anaerobic growth), *gapA* (glyceraldehyde-3-phosphate dehydrogenase A); *ack* (acetate kinase); *hbd* (beta-hydroxybutyryl-CoA dehydrogenase); *crt* (crotonase); *bcd* (butyryl-CoA dehydrogenase); *ptb* (phosphor-trans-butyrylase); *adh* (alcohol dehydrogenase); *bdh* (butanol dehydrogenase); *adhE2* (aldehyde-alcohol dehydrogenase); *frd* (fumarate reductase); *atoB* (acetyl-CoA acetyltransferase); *Bad* (butyraldehyde dehydrogenase); *aad* (alcohol aldehyde dehydrogenase); *etfAB* (electron transfer flavoprotein); *bldh* (butyraldehyde dehydrogenase); *fdh1* (NAD(+)-dependent formate dehydrogenase); *ilvA; leuABCD* (2-isopropylmalate synthase (LeuA), Isopropylmalate isomerase (consisting of two subunits LeuC and LeuD), metal-dependent 3-isopropylmalate dehydrogenase (LeuB)) ; *thrAfbrBC* (thiolase Afeedback resistant with A and B thiolase)

GENETIC ENGINEERING FOR BUTANOL TOLERANCE

Solvent toxicity, is a one of the major limiting factors which hampers the cost-effective bio-production of butanol and ethanol. Butanol as like other alcohol is toxic to cells in slightly higher concentrations. In *Clostridium acetobutylicum*, a functionally unknown protein encoded by SMB G1518 showing the alcohol interesting site was identified. Disruption of SMB G1518 and/or its down regulating gene SMB G1519 resulting increase in butanol tolerance, while decrements was observed when overexpressed. These genes also influence the production of pyruvate:ferredoxin oxidoreductase (PFOR) and flagellar protein hag, which maintain cell motility [64]. The mutants of *C.acetobutylicum* ATCC824 shows tolerance to 1.8% butanol [65-67]. Membrane composition shows similarity with a strain of *Staphylococcus haemolyticus* which has shown tolerance to increased solvent concentration [6]. However limited growth in butanol was found in *S.cerevisiae* upto 2% but some microbe shows tolerance to 3% butanol while simulation results showed maximum tolerance of 4% by *C.acetobutylicum* [73,91,95,96,98,99]. Shuttle vector pCAC1839 due gene have similarity with the xenobiotic responsive element and it shows an increase in tolerance of 13 to 81% on introduction to *C.acetobutylicum* ATCC 824 [6,68]. The over expression of genes *entC* (isochorismate synthase) and *FeoA* (small iron tansport protein) shows an increase in butanol tolerance by 32.8% and 49.1% respectively, and by *astE* gene deletion butanol tolerance was enhanced by 48.7%. By knock out of *Cac*-3319 gene (histidine kinase production) by cis tron group II intron based inactivation system it enhances the biobutanol tolerance by 44.4% [69]. Isobutyrlaldehyde (an intermediate metabolite) toxic to cyanobacteria due to its high concentration. Therefore isobutyrlaldehyde production was eluded by use of different pathway for the production of 2.3-butandiol [70].

Table 4: List of microbes used for butanol tolerance

Microorganism	Method	Tolerance	Ref.
E.coli	Over-expressing *rob*	2.1%	88
	Proton irradiation	1.2%	89
	Protoplast fusion	2%	90
	Deletion of *astE* over-expression of *entC*, **FeoA,** factor cyclic AMP and *OmpT*	3%	91
L.brevis	-	3%	73
C.beijerinckii	Antisense RNA down regulating *gldA*	0.6%	92

C.acetobutylicum	Over-expression of *groESL*	0.75%	93
	Auxotrophic method	1.6%	67
	Ribosomal mutation	1.9%	94
	Nitrogen Ion beam implantation	3%	95
	Artificial simulation of bioevolution	4%	96
S.cerevisiae	Mutation	1.9% (2% very less strains)	97
Enterococcus faecalis	Natural	3.5%	98
Eubacterium cylindroides	Natural	3%	
Synechocystis	Over-expression of *SigB*	2.5-3%	99

Keys*: rob*(Right oriC binding); *astE*(Succinylglutamate desuccinylase); *entC* (enterobactin C); *FeoA*(ferrous iron transport protein A); *ompT*(outer membrane protein); *gldA*(glycerol dehydrogenase); *groESL*(Heat shock proteins); *SigB* (RNA polymerase sigma B factor)

Integration of heterologous (*HSPs*) has been used to improve the tolerance of solvent in *E.coli* [70,71]. Overexpression of autonomous *HSPs* genes mainly *GroES, GroEL, ClpB, GrpE* and *Lpl* promoter increases E.coli tolerance to ethanol and biobutanol [67,72-74]. In addition to *HSPs* gene, mar-sol regulon genes which are responsible for solvent tolerance, mmsB, zwf a member of mar-sol was used for the ethanol tolerance. The researchers indicate that this regulon changes the membrane pumps for exportation of solvents [75-77].

CONCLUSION

Butanol or isomer of butanol was not up to the mark for commercial use as biofuel. There are various microbes, including cyanobacteria, thermophilic bacteria, archeobacteria used for the production of butanol. The thermophilic bacteria is used as a key microbe for increasing the yield of butanol production and it also reduces the steps involved in downstream processing. Yet productivity was not satisfactory. *Geobacillus thermodenitrificans* and cyanobacteria are promising microbes for butanol yield and in case of eukaryotes isobutanol production of yeast was less than 1g/L. Sterioselectivity also shows promising results. Heat shock proteins plays important role in enhancing cell tolerances towards solvent toxicity. In addition to it there is a regulon which increases the cell permeability towards butanol extraction by changing the membrane composition and increasing the number of solvent extraction pumps. Cyanobacteria and themophilic bacteria seem to be the best option in the future for the production of butanol as biofuel. The butanol tolerance and butanol ratio were most concerned factors for enhanced production of biobutanol in industrial scale.

CRISPR approach is a new technique and can be used as efficient technology for improving butanol tolerance, production and downstream processing. A wide range of thermophilic fungi and bacteria are identified which can be genetically manipulated for cost effective butanol production.

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