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Review Article

Recent Advances in Microbial Production of Butanol as a Biofuel

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Abstract

In lieu of rising crude oil prices, exhaustion of petroleum feed stocks and environmental challenges, only renewable fuels have the potential to match the energy requirements of the future. Among the various renewable fuels, butanol has recently gained a lot of attention because of its advantages over other biofuels. Its microbial production by clostridia through ABE fermentation is being explored for improved yield and cost effectiveness. Using lignocellulosic wastes successfully for butanol production through ABE fermentation is a major breakthrough to deal with the future energy crisis. Genetic engineering of microbes to increase the carbon and redox balance, cell recycling, media optimization, mathematical modelling and tolerance improvement strategies are being attempted to overcome the hurdles of high production cost, by products formation leading to low yield and product toxicity. Along with genetic engineering major research is cantered on heterologous host engineering for improved butanol production and tolerance. This review highlights the recent advances in improving yield and tolerance to butanol in both *Clostridial* and heterologous hosts from genetic engineering and fermentation methodology aspects.

Keywords: Biofuels; Genetic Engineering; *Clostridia*; Butanol

Introduction

Increasing crude oil prices and awareness about the finite life span of fossil fuels have resulted in increased demand of renewable fuels that can be derived from sustainable resources. Further global warming and environmental pollution arising from these fossil fuels is also a major concern. "Biofuels" are emerging as the most promising alternative due to their renewable features and lesser emission of greenhouse gases. Biofuels include ethanol, methane, hydrogen, alkanes, diesel and butanol. Ethanol is a major biofuel which is already being produced at industrial scale and used as fuel in automobile engines after mixing in certain proportions with gasoline (Xue *et al*., 2013). It is produced mainly from two sources ie. corn and molasses with United states and brazil being currently the largest producers of ethanol in the world.

Hydrogen and methane (Biogas) are generally considered as ideal biofuels as the former can be directly converted into electrical energy and is produced in almost every bacterial anaerobic fermentation while the latter is also a sustainable fuel because it can be produced using household as well as industrial wastes. But both hydrogen and methane being gaseous in nature, require either liquefaction or storage conditions before they can be commercialized (Antoni *et al*.,2007). Biological production of alkanes is also gaining consideration with the main focus being their toxicity to the cell (Chen *et al*., 2013). While biodiesel produced from vegetable oils by trans-esterification can be used as a blending agent in diesel engines. Among the various biofuels butanol also known as next generation biofuel, is emerging as an ideal fuel for the transportation sector because of certain advantages over ethanol the most

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extensively used biofuel these days. Butanol offers higher energy content than ethanol and has lesser corrosive properties so can be easily transported through existing pipelines. Lower vapour pressure than ethanol allows blending into gasoline up to a higher concentration than ethanol and therefore it can be used into the existing automobile engines without any modifications either as a sole fuel or in combination with gasoline. Also, butanol has higher flash point, therefore it is safer to use (Lee *et al*.,2008 and Schwarz *et al*., 2006). Apart from being considered the next generation transportation biofuel it also has numerous important industrial applications such as paints, thinners, rubbers, resins, elastomers, perfumes, textiles, leather and pesticides (Mahapatra *et al*., 2017).

Though butanol can be produced chemically using fossil fuels but to discourage the use of fossil fuels for avoiding their exhaustion, biological production of butanol through fermentation is the main focus. Biological production of butanol using microbes was first reported by Louis Pasteur in 1861 but was industrialized by ChaimWiezmann in 1916. During world war I and II (early 20th century) butanol production through anaerobic ABE fermentation (acetone: butanol: ethanol :: 3:6:1) using molasses as substrate was exploited in *Clostridium* species. Infact at the time of world war II Japan used butanol as aviation fuel when the fossil fuel supply diminished (Schwarz *et al*., 2006, Mahapatra *et al*., 2017; Tashiro *et al*., 2010). Subsequently interest in butanol production started diminishing because of increasing substrate (ie. molasses) cost and competition with low cost fossil fuels. However again in the late 90's *ie*. 1973 butanol production regained interest because of increasing crude oil crisis and its price (Tashiro *et al*., 2010; Zheng *et al*., 2015).

ABE anaerobic fermentation consists of two phases: first the acid fermentation phase where exponentially growing clostridia produce acetic and butyric acids, carbon dioxide and hydrogen from sugars, followed by the solvent fermentation phase where acids are converted into acetone, butanol and ethanol, typically in the ratio of 3:6:1 by the stationary cells. More amount of butyrate is produced than acetate because butyrate favours redox equilibrium more favourably (NADH formed during glycolysis in consumed in butyrate pathway). Butyrate and acetate are converted into butanol and acetone respectively, illustrating almost double yield of butanol in ABE fermentation than ethanol (Jones and Wood 1986). The reducing equivalents such as NADH or NADPH formed by ABE-producing clostridia through glycolysis are oxidized during solvent fermentation phase, to produce butanol or ethanol with 4 mol of NADH being required to produce 1 mol of butanol. Thus carbon and electron flow, control the metabolism of ABE fermentation. In the butanol production pathway, the conversion of acetyl-CoA to butanol by Clostridium spp. involves a series of enzymes: *acetyl-CoA acetyltransferase* (thiolase; THL), *ß-hydroxybutyryl- CoA dehydrogenase* (HBD), 3-*hydroxybutyryl-CoA dehydratase* (crotonase; CRT), *butyryl-CoA dehydrogenase* (BCD), *butyraldehyde dehydrogenase* (BYDH) and *butanol dehydrogenase*. (Tashiro *et al*., 2010) as illustrated in Figure1.

The traditional ABE fermentation suffers from certain limitations described below (Jones and Wood 1986; Schwarz and Gapes 2006; Zheng *et al*., 2009; Niemisto *et al*., 2013; Lutke-Eversloh *et al*., 2011).

a) Strict anaerobic nature of clostridia makes their handling very difficult as there is need of stringent anaerobic conditions.
b) Low yield of butanol because of its toxic nature to microbes. The typical ABE fermentation cannot surpass the butanol production beyond 13g/L in the fermentation broth.
c) Low cell density due to loss of cells during solvent extraction leading to lower productivity during fermentation.
d) Formation of byproducts as acetone and ethanol, leading to costly downstream processing thus making the process economically less preferable.
e) Increasing cost of traditional substrate *ie.* Molasses

All these limitations have led to renewed interest of the researchers in improving the yield of butanol by cost cutting of the fermentation process (either by improving the efficiency of fermentation process, manipulations in the native *Clostridium* sp., exploration of renewable and economical substrate and engineering a new potential microbial host for butanol production). Though underestimated or misinterpreted as "Next generation biofuel" butanol has been produced since decades both as a by-product along with acetone as well as major fermentation product and is being used as very important industrial solvent. But today it is coming out as more potential fuel and solvent over the existing ones (Schwarz *et al*., 2006).

This paper essentially reports on advancement in fermentation process using C*lostridium sp*., engineering of *Clostridium sp*., search of natural butanol tolerant microbe and improvement in new engineered hosts for greater tolerance to butanol for higher production of butanol.

Advancement in Fermentation Process Using Clostridial sp.

The major work areas in *Clostridial* fermentation are the exploration of newer renewable substrates, increase in the cell density (cell immobilization, cell recycling), various methods to improve the yield of the process (in situ removal of solvent, optimization of media or process, pH maintenance), use of mixed culture and sugars (Zheng *et al*., 2015).

Exploration of Renewable Substrates

***Lignocellulosic substrates*:** Traditional food based substrates used in fermentation were whey, molasses, corn, cassava etc. However due to increase in demand of food crops hence rising price and competition for land there is an urgent need to rely on sustainable feed stock biomass for biofuel production. Lignocellulosic biomass composed of 3 constituents, 30–55% of cellulose, 25–50% of hemicellulose and 10–35% of lignin is the most promising feed stock to solve this problem. As lignocellulosic biomass comprises of complex between lignin, cellulose and hemicellulose so there is need of pre-treatment to convert these into simpler easily fermentable sugars. Most successful pre-treatment methods employed are acid treatment, alkali treatment and enzymatic treatment. But these treatment lead to secretion of unwanted inhibitory chemicals such as formic acid, acetic acid, levulinic acid etc. which are inhibitory to ABE fermentation. Removal of these inhibitors by evaporation, lime treatment, XAD resin treatment and charcoal adsorption etc. have been successfully employed (Lutke-Eversloh *et al*., 2011; Bharathiraja *et al*., 2017; Silva *et al*., 2013). (Table 1)

Table 1: List of various microorganism, substrates, treatment technology used, solvent production in ABE fermentation

Substrate	**Microorganism**	**Technology**	ABE Production (g/L)	Butanol Production (g/L)	**Highlight of the process**
Palm waste					
POME	*Clostridium saccharoperbutylacetonicum* N1-4	Acid treatment	2.2		Acid conc. beyond 2% resulted in decrease in ABE production
		XAD-4 treatement after enzymatic hydrolysis of POME	4.29		
PFEB	*Clostridium acetobutylicum*	Enzymatic hydrolysis	1.15	1.47	
	Clostridium acetobutylicum ATCC 824	Alkali treatment followed by enzymatic treatment	2.75		Simultaneous sachharification and fermentation was carried out.
Corn Waste					
Fiber	*Clostridium beijerinckii*	XAD-4treatment after acid treatment		9.3	XAD-4 treatment increased production approx. by 9 times
		Enzymatic hydrolysis		9.6	No inhibitors produced
Stover	*Clostridium saccharobutylicum DSM13864*	Acid and enzyme treatment followed by dilution with water	16.0	10.4	Treatment led to no inhibitors Production
		Lime treatment	26.7	14.5	
Corbs	*Clostridium saccharobutylicum DSM13864*	NaOH pretreatment and enzymatic hydrolysis	12.27		Washing the corncorbs enhanced the yield
	Clostridium beijerinckii NCIM 8052	Enzymatic treatment +Iime treatment	16.8	9.8	Treated corncorbs gave much better yield due to removal of inhibitors
Degermed corn	*Clostridium beijerinckii* BA101	Non-sachharified degermed Corn		5.89	
		Enzymatically Saccharified degermed corn		14.16	Sachharification of degermed corn to release excess nutrients
Barley straw hydrolysate (BSH)	*Clostridium beijerinckii* P260	Acid + enzyme treatment	7.09		BSH produced more ABE than glucose
		Lime treatment	26.04	18.0	
Switchgrass hydrolysate	*Clostridium beijerinckii* P260	Acid + enzyme treatment	1.48		
		Lime treatment	14.61		
Rice straw	Clostridium mixed sp.	Alkaline hydrolysis followed by enzymatic treatment		2.92	Clostridial sp. were isolated from hydrogen producing sewage
	Clostridium acetobutylicum NCIM2337	Acid hydrolysis with simultaneous shear stress	13.5		Stress helped to release the maximum amount of fermentable sugars

Substrate	Microorganism	Technology	ABE Production (g/L)	Butanol Production (g/L)	Highlight of the process
	Clostridium sporogenes BE01	Acid + enzymatic treatment		5.52	Non acetone producing strain decreased downstreaming cost
Sugar cane	Clostridium mixed sp.	Alkaline hydrolysis followed by enzymatic treatment	2.29		Clostridial sp. were isolated from hydrogen producing sewage
Sugar maple hemicellulosic hydrolysate	*Clostridium acetobutylicum ATCC 824*	Nano Filteration		0.8	Nano filtration did not remove the inhibitors completely
		Lime treatment		7.0	
Wheat bran	*Clostridium beijerinckii 55025*	Acid hydrolysis		8.89	Mixed sugars were used
Lactuca sativa leaves	*Clostridium acetobutylicum DSM792*	Alkali pretreatment + enzymatic hydrolysis	1.44	1.11	
Banana pseudostem	*Clostridium sporogenes*	Alkali treatment		10.12	
Switch Grass	*Clostridium Saccharoperbutylacetonicum N1-4.*	Acid + enzymatic hydrolysis		8.6	Acetic acid the byproduct of ABE fermentation used for pretreatment
Wood pulp hydrolysate	*Clostridium beijerinckii*	Non treated pulp		6.73	
		Resin treated pulp		11.35	Resin treatment and gas stripping increased the yield about three times
		Gas stripping coupling		17.73	

Acid/ Alkali Treatment

Acid treatment of various lignocellulosic substrates involves treatment with concentrated acids mainly H_2SO_4 in a range of 0.5-2% (w/w) at 121°C for 20 to 60min. followed by lime, XAD, resin treatment or evaporation to remove the inhibitors. Acid hydrolyzed corn fiber treated with XAD yielded 9.3g/L butanolby *Clostridium beijerinckii* (Qureshi *et al*., 2008) while Liu *et al*. (2011) reported the production of 8.8g/L butanol by *Clostridium beijernickii 55025* using acid hydrolysed wheat bran as substrate for fermentation. Another ABE fermentation by *Clostridium acetobutylicum* with Palm empty fruit bunches(PEFB) the palm-oil industrial wastes after acid hydrolysis yielded 1.15 g/L butanol (Noomtim and Cheirsilp 2011). While Al-Shrogani *et al.,* (2012) (c) reported 2.2g/L ABE production with palm oil industry waste *ie*. Palm oil mill effluent (POME) based fermentation by *Clostridium saccharoperbutylacetonicum* N1-4. Fermentation of acid hydrolysed and resin treated wood pulp hydrolysate by *Clostridium beijerinckii* produced 11.35g/L ABE and further coupling with gas stripping resulted in 17.73g/L ABE (Lu *et al*., 2013). Fermentation by *Clostridium acetobutylicum* NCIM2337 of rice straw treated with shear stressalong with acid hydrolysis yielded 13.5g/L butanol (Ranjan *et al*., 2013) while alkali treated rice straw fermentation produced only 2.92g/L of butanol (Cheng *et al*., 2012). Acid and alkali treated pine apple peel based fermentation by *Clostridium acetobutylicum* B527, produced 5.23g/L ABE (Khedkar *et al*., 2017). Al-Shorgani *et al.* (2012b) reported the production of 7.72 g/L of butanol using acid treated de-oiled rice bran. Alkali treatment (2% NaOH w/v) of banana pseudostem at 30°C in the presence of *Clostridium sporogenes* resulted in 10.12g/L butanol production (Sivanarutselvi *et al.,* 2019).

Enzymatic Treatment

Enzymatic hydrolysis has been reported to be more effective as it could release more amounts of sugars and results in lower amount of inhibitors production than acid or alkali treatment and therefore higher ABE production (Lutke-Eversloh *et al*., 2011, Bharathiraja *et al*., 2017, Silva *et al*., 2013) Different substrates were incubated with various enzymatic suspensions within a temperature range of 40-55°Cat optimum pH, accompanied by agitation for 24-72 hrs for pretreatment and then used for fermentation (Niemisto *et al*., 2013; Lutke-Eversloh *et al*., 2011; Bharathiraja *et al*., 2017,). Ezeji *et al.* (2007) reported early termination of ABE fermentation by *Clostridium beijerinckii* BA101 using degermed corn based medium yielding 5.89g/L of butanol. This early termination was attributed to retrogradation. Saccharification of degermed corn (to reduce retrogradation) using gluco-amylase (pH-4.5, 1ml/L of 400U/ml) for 48-72 hrs, resulted in production of 14.16g/L butanol. ABE fermentation of corn fiber

hydrolysate treated with cellulase and cellobiase (1ml/100gm substrate of 0.7FPU and 250U/g resp. at pH 4.5) by *Clostridium beijerinckii* produced 9.6g/L butanol (Qureshi *et al*., 2008). Noomtim and Cheirslip (2011) reported 1.47g/L of butanol with cellulase (45U/g of substrate for 48hrs at pH 5.0) treated palm empty fruit bunches (PEFB) which was slightly higher than acid treated PEFB (1.15g/L mentioned earlier section 2.1.1). ABE fermentation based on corn cob residues (CCR) treated with cellulose (48 FPU/g at pH 4.8) followed by Lime treatment resulted in production of 16.8g/L ABE with 8.2g/L butanol (Zhang *et al*., 2012). Production of 4.29g/L ABE using cellulose hydrolysed POME (Palm oil mill effluent) as compared to acid treated POME(2.2g/L) in a fermentation by *Clostridium acetobutylicum* was achieved (Al- Shorgani *et al*., 2012(c))

Acid/Alkali Pre-Treatment and Enzymatic Hydrolysis

Barley straw pre-treated with 1% H_2SO_4 (v/w) followed by enzymatic hydrolysis (cellulase, β-glucosidase and xylanase mixture, 6ml/L each at pH 5.0) based butanol fermentation by *Clostridium beijerinckii* P260 produced 7.09 g/L ABE while barley straw hydrolysate (BSH) treated with lime prior to fermentation led to 26.64g/L of ABE and 18.01g/L butanol (i) (Qureshi *et al*., 2010). Untreated corn stover hydrolysate resulted in no fermentation while dilution of corn stover with water (1:2) resulted in ABE yield of 16g/L and 10.4g/L butanol. Further lime treatment of corn stover increased the yield to 26.27g/L ABE and 14.50g/L butanol (ii) (Qureshi *et al*., 2010). Alkali pretreated and enzymatically hydrolysed (cellulase, mixture of endogluconase (0.56U/ml) and β-glucosidase (0.3U/ml) at pH- 5.0) corncobs produced 12.27g/L butanol (Gao and Rehmann 2014). Further Ibrahim *et al*., (2015) reported the production of 2.75g/L butanol in cellulose (5U/ml at pH 5.5) treated PFEB based fermentation by *Clostridium acetobutylicum* ATCC 824. Apart from above treatments another treatment method employed by Sun *et al.* (2012) was nano-filteration. Nano filtered Sugar maple followed by lime treatment resulted in the production of 7g/L butanol. In a fermentation by *Clostridium acetobutylicum* DSM792, the residues of fresh cut vegetables *ie.* Lactuca sativa leaves used after alkali hydrolysis (NaOH 200 kg m^{-3}) followed by enzymatic hydrolysis (Cellic CTec 2 Novozymes) led to production of 1.44g/L ABE and 1.1g/L butanol (Procentese *et al*., 2017). Acid pretreatment of rice straw followed by cellulase (30 FPUs/g, 50°C for 48 hrs) treatment led to production of 5.52g/L butanol in a fermentation by *Clostridium sporogenes BE01* (Gottumukkala *et al*., 2013). Acetic acid pretreatment of switch grass (3g/L, 170°C for 20 min) followed by enzymatic hydrolysis (Cellic CTec 2 Novozymes) led to production of 8.6g/L butanol by *Clostridium Saccharoperbutylacetonicum* N1-4. (Wang *et al.*, 2019)

Among the various treatment methods such as acid, alkali and enzymatic treatment of various lignocellulosic wastes as substrates including corn wastes, barley straw, rice bran, palm waste and wood pulp etc. the best yield was achieved with wood pulp hydrolysate obtained with acid treatment followed by enzymatic treatment.

Glycerol (a waste of biodiesel industry): Glycerol is produced as a waste of biodiesel industry and using it as a carbon source can make the process economical. Using mutant strain of Clostridium pasteurianumMBEL_GLY2 with glycerol as substrate 17.8g/L butanol was produced (Malaviya et al., 2012). Khanna et al., (2013) reported the production of 8.83g/L butanol using crude glycerol in fermentation by Clostridium pasteurianum. While using glycerol as substrate coupled with in situ butanol removal by vacuum membrane distillation yielded a maximum of 29.8g/L butanol by Clostridium pasteurianum CH4 (Lin et al., 2015). Further addition of glucose to glycerol (glycerol:glucose: 3:1) resulted in 13.3g/L butanol by Clostridium pasteurianum CH4 (Kao et al., 2013). ABE fermentation of Glycerol in combination with thin stillage (liquid fraction of waste generated in ethanol fermentation after distillation process) and with spruce biomass hydrolysate by Clostridium pasteurianum 525 yielded 7.2g/L and 17 g/L butanol respectively (Ahn et al., 2011; Sabra et al., 2014). A mutant strain of Clostridium pasteurinum achieved by chemical mutagenesis through EMS treatment produced maximum of 12.6g/l of butanol used crude glycerol as substrate (Jensen et al., 2012). (Table 2)

***Algae*:** Algae is also being exploited as a substrate for butanol fermentation as it is present in abundance and gives no competition to other food crops in terms of arable land. Pretreatment of algal biomass mainly involves thermal decomposition at 90- 110 °Cin the presence of acid or alkali leading to conversion of complex sugars into easily fermentable sugars thus increasing the surface area for bioconversion by enzymes more efficiently. Clostridium acetobutylicum B-1787 cells immobilized on PVA cryogel using Arthrospiraplatensis biomass as substrate gave 380mg/L of butanol (Efremenko et al., 2012). Jamaica bay macroalgae based ABE fermentation by Clostridium beijerinckii and Clostridium saccharoperbutylacetonicum yielded 4.0g/.L butanol (Potts et al., 2012). Using algae growing in waste water lagoons as substrate for ABE fermentation by Clostridium saccharoperbutylacetonicum N1–4 led to production of 7.79g/L butanol and 9.74 g/L ABE (Ellis et al., 2012). Ulvalactuchydrolysate as substrate yielded 3.0g/L butanol while supplementation with glucose, xylose and rhamanose led to production of 8.4g/L butanol (Van der wal et al., 2013). Fermentation of microalgae Chlorella sorokiniana CY1 residues by Clostridium acetobutylicum yielded 3.86g/L butanol (Cheng et al., 2015). (Table 3)

Table 2: list of microorganism used, technology used and solvent yield using glycerol as substrate.

Microorganism used	Technology used	Yield of butanol (g/L)
*Clostridium pasteurianum*MBEL_GLY2	Chemical mutagenesis	17.8
Clostridium pasteurinum	Immobilization	8.83
Clostridium pasteurianum CH4	In situ product removal by vacuum	29.8
Clostridium pasteurianum CH4	Glycerol:glucose::3:1	13.8
Clostridium pasteurianum525	Glycerol with thin stillage	7.2
Clostridium pasteurianum525	Glycerol with spruce biomass	17.0
Clostridium pasteurinum	Chemical mutagenesis	12.6

Table 3: List of Algae used in ABE fermentation and solvent yield.

Microorganism used	Algae	Production of butanol (g/L)
Clostridium acetobutylicum B-1787	Arthrospiraplatensis	0.382
Clostridium beijerinckii and Clostridium saccharoperbutylaceto nicum	Jamaica bay	4.0
Clostridium saccharoperbutylacetonicum N1–4	Waste water algae	7.79
Clostridium beijerinckii	Ulvalactuchydrolysate	8.4
Clostridium acetobutylicum CICC 8012	Chlorella sorokiniana CY1	3.86

Various Methods to Improve the Yield of the Process

***Increase in the cell density*:** Immobilization of cells leads to increased cell count, viability and decreased cell loss as compared to suspension cultures. This leads to increased cell density during the fermentation and increased production. *Clostridium acetobutylicum* DSM 792 immobilized on wood pulp fibers with glucose and sugar mixture (glucose, mannose, galactose, arabinose, and xylose) as substrate produced 14.32 g/L ABE with approx. 11.0 g/L butanol (Survase *et al*., 2012). *Clostridium pasteurianum* cells immobilized on amberlite using glycerol as substrate produced butanol concentration of 8.83 g/L (Khanna *et al*., 2013). Using immobilized cells of *Clostridium acetobutylicum* CGMCC 5234 on pre-treated cotton towels with xylose as substrate, 10.02 g/L butanol production was reported, while using glucose in combination with xylose yielded 11.2 g/L (Chen *et al*., 2013). (Table 4)

***In situ product removal*:** The most traditional method for recovery of butanol is distillation but this is too much energy consuming and economically unfavorable (Visioli *et al*., 2014). Therefore, nowadays various new *in situ* product removal techniques such as gas stripping, cell recycling by dilution, bleeding and solvent – solvent extraction has been used in many studies to remove the products from the fermentation broth resulting in decrease in product inhibition caused by toxicity of solvent accumulation. All these techniques have been used either individually or in combination with each other to make the process more effective.

Gas stripping is the most commonly used method as it does not require any expensive membrane or chemicals and it has led to better yields than any other process (Ezeji *et al*., 2013). Vacuum process (gas stripping) was used for *in situ* product removal in a fermentation carried out by *Clostridium beijerinckii* yielding 41g/L butanol (Mariano *et al*., 2011). It was also inferred that intermittent vacuum resulted in better yield than continuous vacuum. Mariano *et al.*, (2012) reported that ABE fermentation coupled to intermittent gas stripping led to 39% decrease in consumption of energy without affecting the yield of butanol. ABE fermentation with *Clostridium acetobutylicum* JB200 using cassava baggase and glucose as substrate coupled to gas stripping resulted in increase in butanol production from 20g/L to 76.4g/L butanol and 113g/L butanol respectively (Lu *et al*., 2012; Xue *et al*., 2012). Further Xue *et al.* (2012) reported the coupling of process to phase separation by liquid- liquid extraction which increased the butanol production up to 610g/L. Rochon *et al.*, (2017) reported the production of 18.6g/L butanol by *Clostridium acetobutylicum* DSM 792 using sugarcane sweet sorghum juices in a fermentation coupled to gas stripping.

Continuous fermentation with high-density *Clostridium saccharoperbutylacetonicum* N1-4 achieved through cell recycling using xylose as substrateresulted in butanol productivity of 3.32 g/L/h (Zheng *et al*., 2013).While Ezeji *et al*.,2013 reported the additional impact of bleeding after regular intervals on ABE fermentation by *Clostridium beijerinckii* BA101 with glucose resulting in production of 232.8g/L and 461.3g/L butanol for fed batch and continuous

fermentation respectively with less accumulation of toxic compounds.

Liquid – Liquid extraction methods have also been used for *in situ* product removal in several studies. Oleol alcohol + decanol mixture have been used in fermentation which resulted in production of 25.32 g/L ABE and 16.9 g/L butanol (Bankar *et al*., 2012).Earlier these solvents used for extraction were found to have inhibitory effect on microbes so Tanaka *et al*.,(2012) coupled the fermentation using 1-dedecanol as an extractant with MAE (membrane-assisted extractive fermentation) using polytetrafluoroethylene (PTFE) membrane and reported an increase in production of butanol from 16.0g/L to 20.1 g/L. This led to decreased microbial toxicity as highly hydrophilic nature of membrane helped in avoiding the direct contact of microbial cells with 1- dodecanol. Later Yen *et al*.,(2013) used biodiesel (which did not have any toxic effect on cell growth), as extractant to overcome the cost barrier of membrane coupled extractants resulting in increased butanol production from 9.85 g/L to 31.44 g/L. Apart from these a hydrophobic polymer resin Dowex Optipore L-493 used in expanded bed adsorption for product removal in a fed batch fermentation by *Clostridium acetobutylicum* ATCC 824 resulted in production of 27.2 g/L butanol and 40.7 g/L ABE (Wiehn *et al*., 2014). (Table 5)

Table 4: List of microorganisms, method to improve cell density and solvent production in ABE fermentation

Microorganism	Substrate	Technology	Highlight of the process	Production
Clostridium pasteurianum	Glycerol	Immobilized column reactor	Amberlite used as a carrier	8.83g/L Butanol
Clostridium acetobutylicum DSM792	Pulp industry waste	Immobilized column reactor	Immobilization lead to increase in cell density and decrease in cell loss	14.32g/L ABE 11.0g/L Butanol
Clostridium acetobutylicum CGMCC 5234 on	xylose	Immobilized column reactor	Pre treated cotton towels used as carrier	11.2g/L Butanol
Clostridium saccharoperbutylacetonicum N1–4	Xylose	Cell recycling and dilution rate variation	Cell recycling increased the cell density and dilution increased ABE productivity	3.32 g/L/h Butanol

Table 5: List of microorganisms, methods for *in situ* product removal and solvent yield

Microorganism	Substrate	Technology	Highlight of the process	Production Butanol
Clostridium beijerinckii BA101	Glucose	Gas stripping method Bleeding of system	Continuous product removal and bleeding of system lead to decreased accumulation of toxic substances hence increased yield by 10%	461.3g/L
Clostridium beijerinckii P260		Vacuum process for in situ product removal	Complete utilization of substrate and higher productivity due to decreased product inhibition	41g/L
Clostridium acetobutylicum	Cassava bagasse hydrolysate	Gas stripping method	In situ product removal lead to increased production of butanol and low amount of acid production	76.4g/L
Clostridium acetobutylicum JB200	Glucose	Gas stripping Liquid liquid extraction	Product removal enhanced the yield of the process by overcoming product inhibition hence 15% increase in productivity	113g/L
Clostridium acetobutylicum DSM792	Sugarcane sweet sorghum juice	Gas stripping		18.6g/L
Clostridium acetobutylicum B 5313	Glucose	Two stage chemostat system and liquid liquid extraction	Using oleol alcohol and decanol as extractants product inhibition was reduced	16.90g/L
Clostridium saccharoperbutylacetonicum N1–4		1-dodecanol used as extract	MAE increased the butanol production by avoiding the direct contact of cells with dedecanol	20.1g/L
Clostridium acetobutylicum		Liquid liquid extraction	Biodiesel used as extractant had no toxic effects	31.44g/L
145Clostridium acetobutylicum	Glucose	Expanded bed adsorption	Dowex Optipore L-493 used as adsorber	27.2 g/L

Table 6: List of microorganisms and methods for improved solvent yield and solvent yield

Microorganism	Substrate	Technology	Highlight of the process	Production
Clostridium beijerinckii	Maize stalk juice	RSM	Optimum conditions as pH, substrate conc. etc. were determined by RSM	0.27g/g sugar
Clostridium acetobutylicum	Corn straw	RSM	Optimum conditions as pH, substrate conc. etc. were determined by RSM	6.57g/L
Clostridium beijerinckii ATCC 10132	Beef extract+glucose	Media optimization	No product removal was done still the butanol production increased 6 times	20 g/L
Clostridium saccharobutylicum DSM 13864	Corn Stover	Media Optimization	-	12.3g/L
Clostridium beijerinckii TISTR 1461	Sugar cane molasses	Media optimization	Gas stripping increased the yield further to media optimization	14.13g/L
Clostridium acetobutylicum T64		Artificial simulation of bio-evolution (ASBE)	Butanol tolerance of the microbe was increased two times	15.3 g/L

***RSM (response surface methodology/Various mathematical models Evolution /selection to improve the yield of fermentation and optimization of various parameters*:** RSM (response surface methodology) was used for optimizing the parameters for fermentation by Clostridium beijerinckii NCIMB 8052 with maize stalk juice as substrate at pH 6.7, sugar concentration 42.2 g/L and agitation rate 48 rpm. Maximum butanol yield of 0.27 g/g-sugar was obtained under these optimum conditions. Further increase in the agitation rate and sugar concentration led to decreased production of butanol (Wang et al., 2011). Lin et al., (2011) optimized the process (CaCO3 concentration of 5.04g/L, temperature of 35°C with reaction time of 70 hrs) by Plackett-Burman (P-B) design and Central Composite Design (CCD) and obtained a yield of 6.57g/L butanol by Clostridium acetobutylicum CICC 8008.Optimized parameters for a fermentation by Clostridium beijerinckii ATCC 10132 nitrogen source (beef extract 50g/L), Carbon source (glucose 20g/L + Malt extract 50g/L), temperature of 37°C and pH-6.5) resulted in yield of 20 g/L butanol in a single chemostat culture without employing any method of product removal. This was attributed to increased tolerance of the strain owing to enhanced expression of chaperon, groESL and change lipid profile (Isar et al., 2012).Dong et al., (2013) reported a yield of 12.3g/L of butanol in an ABE fermentation by Clostridium saccharobutylicum DSM 13864 from corn stover with optimum conditions of 37°C temperature, 5% inoculums size and 7% biomass. Wechgama et al., (2017) reported that a molasses based fermentation by Clostridium beijerinckii TISTR 1461 at pH 6.5, sugar conc. of 40 g/L and a urea conc. of 0.81 g/L produced 12.55g/L butanol. Further coupling of the process to gas stripping increased the butanol titer to 14.13g/L. Through artificial simulation of bio-evolution (ASBE) by repetitive evolutionary domestication in a fermentation by Clostridium acetobutylicum D64an increase in butanol yield from 12.2 g/L to 15.3 g/L was obtained (Liu et al., 2013). (Table 6)

***Maintenance of pH*:** ABE fermentation is remarkably regulated by pH with an optimum pH in the range of 4-6 (Zheng et al., 2015; Bowles and Ellefson 1985). Immobilized Clostridium acetobutylicum cells in a continuous packed bed reactor with pH maintained in the range of 4-5, resulted in butanol productivity of 4.4g/Lh (Napoli et al., 2010). Further it was shown that maintaining a two stage pH control in a range of 5.5-4.9 resulted in 12% increase in butanol production ie.20.3g/L compared to process without pH control by Clostridium acetobutylicum XY16 (Guo et al., 2012). Li et al. (2011) reported butanol yield of 11g/L (which was 90% of total solvents produced) in a batch fermentation by Clostridium acetobutylicum by controlling the pH at 4.5. The study supported the fact that pH controlled batch system resulted in increased butanol ratio in the total solvent as compared to typical 3:6:1:: A:B:E ratio (Li et al., 2011).In a fermentation by Clostridium beijerinckii IB4 an increase in butanol and ABE production from 11.0 g/L and 14.1 g/L to 15.7 g/L and 24.6 g/L resp. by maintaining the pH of the process at 5.5 was reported by Jiang etal.,(2014). In a fermentation by Clostridium saccharoperbutylacetonicum N1-4 using glucose and acetate as substrate, maintaining the pH at 5.5 resulted in increase inbutanol production from 14.0g/L to 15.0g/L butanol (Gao et al., 2016). A non acetone producing novel Clostridium sp. A1424 was able to produce 9.86g/L butanol at pH 5.5 versus <8g/L at pH 6.0, 5.7, 5.2 and 5.0 (Youn et al., 2016). In a multi phase pH controlled ABE fermentationby Clostridium acetobutylicum SE25 25% higher titer of butanol ie. 16.24g/L was achieved as compared to without pH controlled process (Li et al., 2016). (Table 7)

Table 7: List of microorganisms, methods for pH maintenance and improved yield

Microorganism	Substrate	Technology	Highlight of the process	Production (Butanol)
Clostridium acetobutylicum	Lactose and yeast extract	pH maintenance	Keeping initial pH higher than require overcame the limitation of automatic decrease of ph during the process.	4.4g/Lh
Clostridium saccharoperbutylacetonicum N1-4	Glucose and acetate	pH maintenance	Using acetate as substrate led to increase in butanol production	15.13g/L
Clostridium sp. A1424	Glucose and Glycerol	pH maintenance	Using novel non acetone producing strain gave max. butanol yield	9.86g/L
Clostridium beijerinckii IB4	Glucose	pH maintenance		15.13g/L
Clostridiumacetobutylicum SE25	Cassava	Multi stage pH maintenance	$CaCO_3$ addition helped in pH maintenance and improved butanol yield	16.24g/L
Clostridium acetobutylicum XY16	Glucose	pH maintenance by continuous addition of HCL and NaOH	Initial pH set higher than required to counter the automatic gradual decrease to pH than optimum during the fermentation	20.3g/L
Clostridium acetobutylicum	Glucose	pH maintenance	pH maintenance resulted in increase in ratio of butanol in ABE	11.0 g/L
Clostridium saccharoperbutylacetonicum N1–4	Glucose +lactic acid	Batch and fed batch culture + pH control	Lactic acid consumption was verified during butanol production	15.5g/L
Clostridium saccharoperbutylacetonicum N1–4	Arabinose + lactic acid	Batch Fed batch Lactic acid effect	Non edible substrate used	7.11g/L 15.6g/L
Clostridium saccharoperbutylacetonicum N1–4	Glucose + butyric acid	Effect of butyric acid was studied	Butyric acid alone also produced very low amount of butanol	13g/L

***Use of organic acids*:** A novel high butanol production fed batch system was established by using pentose sugar (arabinose) as substrate in combination with lactic acid in fermentation by Clostridium saccharoperbutylacetonicum N1-4 yielding 15.60g/L butanol (Yoshida et al., 2014). ABE fermentation by Clostridium saccharoperbutylacetonicum N1-4 with lactic acid and glucose as substrate resulted in a maximum concentration of 15.5 g/l butanol in a fed-batch culture with a pH stat (Oshiro et al., 2010). ABE fermentation by Clostridium saccharoperbutylacetonicum N1-4 with glucose (10g/L) and butyric acid (20g/L) as substrates, 13g/L of butanol was produced. Using only butyric acid without glucose resulted in no acetone and ethanol production with only 0.7g/L butanol (Al-Shorgani et al., 2012a).

***Using mixed culture or mixed sugars*:** Co-culturing of different microbes with clostridial sp. was assumed to enhance the effectiveness of ABE fermentation. Co-culturing of Clostridium butylicum TISTR 1032 with an aerobic Bacillus subtilis WD 161 havig high amylolytic activity resulted in a yield of 8.9g/L ABE with 0.65 ratio of butanol. This was attributed to maintenance of anaerobic conditions without adding any reducing agent and enhanced utilization of starch by Bacillus subtilis WD 161 (Tran et al., 2010). Co-culturing of Clostridium acetobutylicumATCC 824 and Bacillus subtilis DSM 4451 in ABE fermentation using Spoilage date palm (Phoenix dactylifera L.) fruits as substrate resulted in maximum ABE production of 21.56 g/L and 15.0g/L of butanol (Abd-Alla et al., 2012). Clostridium thermocellum having high cellulolytic activity was co-cultured with Clostridium saccharoperbutylacetonicumN1-4in ABE fermentation using crystalline cellulose(avicel) as substrate. The resulting process led to production of 7.9g/L butanol (Nakayama et al., 2011) while use of mixed sugars ie. xylose and cellobiose instead of glucose, to overcome catabolite repression in ABE fermentation with Clostridium saccharoperbutylacetonicum N1-4 led to production of 16g/L butanol without catabolite repression (Noguchi et al., 2013). Co- culturing of Clostridium acetobutylicum ATCC 824 with Saccharomyces cerevisiae (secreting favorable amino acids) aided in production of 14.0g/L butanol due to favourable redox balance (Luo et al., 2016). Co–culturing of engineered Clostridium cellulovorans and Clostridium beijerinckii in fermentation using corn cobs as substrate resulted in production of 11.5g/L butanol (Wen et al., 2017).Co-culturing of Clostridium beijerinckii F6 and Sachharomyces cerevisiae resulted in production of 12.75g/L butanol (Wu et al., 2019) (Table 8)

Table 8: List of microorganisms used in mixed culture fermentation and solvent yield

Microorganism	Substrate	Technology	Highlight of the process	Production Butanol
Clostridium butylicum TISTR1032 + Bacillus subtilis WD161	Soluble starch Cassava starch	Coculturing of aerobe with clostridium	High amylolytic activity of bacillus increased the yield 5-6 times	8.9g/L ABE
Clostridium acetobutylicum ATCC824 + Bacillus subtilis DSM4451	Spoilage date palm	Coculturing of aerobe with clostridium	Addition of yeast extract and ammonium sulpahte increased the ABE yield	15g/L
Clostridium thermocellum + *Clostridium saccharoperbutylacetonicum N1–4*	Avicel	Coculturing of cellulolytic and butalogenic strains together	*Clostridium thermocellum* havin g high cellulolytic activity lead to increased cellulose degradation thus saving the cost of process	7.9g/L
Clostridium saccharoperbutylacetonicum N1–4	Mixed sugars used as substrate	CCR was overcome by use of mixed sugars		16g/L
Clostridium acetobutylicum ATCC824	Glucose	Coculturing with S.cerevisiae	*S.cerevisiae* led to secretion of amino acids for Butanol synthesis and NADH pool	14.0g/L
Clostridium sp.	Corn cobs	Co-culturing of *Clostridium cellulovorans* and *Clostridi um beijerinckii*	Enginnering of strains to delete competing pathway genes ack and ldh , overexpression of buk to increase carbon flux	11.5g/L
Clostridium beijerinckii F6		Co-culturing of *Clostridium beijerinckii* F6 and *Sachharomyces cerevisiae*	*S.cerevisiae* led to secretion of amino acids for Butanol synthesis and NADH pool	12.75g/L

Genetic engineering in Clostridial sp. for increased butanol production and tolerance

Clostridial sp. has been genetically modified either to increase the butanol yield or tolerance to butanol. These manipulations involved deletions of competing pathway genes, regulation of sporulating genes or over expression of certain butanol producing genes by random or targeted mutagenesis. Further studies were done to understand the genetic response of *Clostridial* cells in response to butanol stress.

Chemical and physical mutagenesis of *Clostridium acetobutylicum* CICC 8012 was used to improve its tolerance to butanol. The mutant F2-GA achieved after NTG (Nitrosoguanidine) or UV treatment followed by genome shuffling by protoplast fusion produced 22.21 g/L ABE with 14.15g/L butanol v/s 16.5g/L ABE with 10.46g/L butanol by wild type strain (Gao *et al*., 2012). Random mutagenesis of *Clostridium acetobutylicum* PJC4BK by NTG treatment yielded a mutant BKM19 which produced 32.5g/L ABE with 17.6g/L butanol which was 31% higher than parent strain producing 13.9g/L ABE with 7.6g/L butanol (Jang *et al*., 2013). Genome sequence analysis of *Clostridium acetobutylicum* EA 2018 mutant developed after repeated cycles of chemical mutagenesis by NTG treatment of *Clostridium acetobutylicum*ATCC824 revealed insertion of 46 genes and deletion of 26 genes in

addition to lower level of expression of acid forming genes and enhanced expression of *adhe* gene. Mutant *Clostridium acetobutylicum* EA 2018 produced 14g/L of butanol as compared to 9g/L by wild type *Clostridium acetobutylicum* ATCC 824 (Hu *et al*., 2011). NTG treatment followed by genome shuffling created a *Clostridium acetobutylicum* mutant strain GS4-3 able to produce 32.6 g/L of ABE and 20.1 g/L of butanol (Li *et al*., 2016).

Targeted mutagenesis was also done in some *Clostridial* species which was either aimed at deletion of *spoOA* (sporulationg transcription factor), few novel genes or competing pathways which lead to flux deficiency towards butanol synthesis or over expression of certain butanol producing genes. The sporulating transcription factor *SpoOA* being the master regulator of sporulation has always been assumed to be aiding in solventogenesis. It has also been reported that the strains lacking *SpoOA*, were deficient in butanol production (Woolley *et al*., 1990) whereas it has also been reported by Xu *etal.,* (2015) that the strains lacking *SpoOA* were able to produce higher level of butanol. Xu *et al.,* (2015) generated a mutant of *Clostridium acetobutylicum* ATCC 55025 by single base deletion in gene *cac3319* leading to knockout of histidine kinase gene involved in the activation of *SpoOA. This mutant* JB200, produced 45 % more butanol 19g/L vs. 12.6g/L.Subsequently it was demonstrated that knockout of *SpoOA* gene by NTG treatment of *Clostridium pasteurianum* ATCC 6013 resulted in the production of butanol (11.7 g/L)by the mutant (M150B) which was 80% higher than the wild strain(Sandoval *et al*., 2015).

The deletion of novel protein SMB_G1518 (having conserved region of zinc finger which can modulate butanol tolerance) in *Clostridium acetobutylicum* resulted in increase in butanol tolerance showing 70% increased cell growth at 1%(v/v) butanol than wild type strain, thus suggesting that these proteins are the negative regulator of tolerance (Jia *et al*., 2012). Deletion of competing pathways *ie.* the knockout of *acetate kinase (ack* aiding in coversion of acetyl co-A to acetate) and *phosphotransbutyrylase (ptb* aiding in conversion of butyryl co-A to butyrate instead of butanol) and the over- expression of *alcohol dehydrogenase (adhe2)* gene from *Clostridium acetobutylicum* ATCC824 in non-solventogenic *Clostridium tyrobutyricum* ATCC 25755 strain resulted in higher butyryl Co-A production leading to 16g/L butanol and no acetone production by the mutant (Yu *et al*., 2011). Later on cloning of xylose utilization genes (*xylT*, *xylA*, and *xylB*) encoding a xylose proton-symporter, a xylose isomerase and a xylulokinase, respectively, into this strain led to the production of 15.7 g/L butanol using soyabean hull as substrate (Yu *et al*., 2015). *Zhu et al.* (2011) reported the expression of a glutathione producing gene in *Clostridium acetobutylicum*. Glutathione plays a significant role in various stress tolerance and metabolism in certain living organisms. Assuming it to protect *Clostridium acetobutylicum's* central metabolic pathway and enzymes under stress, glutathione biosynthetic genes (*gshAB* gene) were cloned into *Clostridium acetobutylicum* DSM1731 resulting in increased butanol yield from 11g/L to 15g/L.

Alsaker *et al.,* (2010), compared the cell physiology of *Clostridium acetobutylicum* by studying its transcriptional stress responses to fermentation products (acetate, butyrate and butanol). Up regulation of certain post translational modification genes and down regulation of translation machinery genes in response to stress caused by these metabolites was observed. Glycerol metabolism genes *glpA and glpF* were up regulated in response to butanol stress. A comprehensive proteome analysis of wild type *Clostridium acetobutylicum* DSM 1731 strain and its butanol tolerant mutant Rh8 revealed differential expression of around 73 proteins in butanol tolerant mutant which contributed to increased membrane stability (Mao *et al*., 2011). (Table 9)

Natural High Butanol Tolerant Microbe

Along with attempts to increase the tolerance to butanol though genetic engineering of *Clostridial* sp., another strategy was to isolate natural indigenous microbes tolerant to high concentration of butanol and then transfer the butanol producing gene in the butanol tolerant isolate.

Ruhl *et al.,* (2009) with four different strains of Pseudomonas sp. showed maximum tolerance to (3%v/v) butanol by *Pseudomonas* VLB120. Decrease in glucose consumption hence lower TCA cycle flux in butanol tolerant cells as compared to butanol sensitive strains indicated that cell membrane in *Pseudomonas* VLB120 is adapted to be maintained at lower energy level. Li *et al.,* (2010) reported that several strains which were reported to be tolerant against ethanol, did not show tolerance beyond 1.5% (v/v) to butanol. Screening of soil samples near butanol storage tank for butanol tolerant microorganism resulted in isolation of two isolates as *Enterococcus faecenium* and *Lactobacillus plantarum*, which could tolerate up to 2.5% (v/v) butanol. Li *et al.,* (2010) also tested a Lactic acid bacteria (LAB) culture collection of 49 cultures belonging to *Lactobacillus*, *Enterococcus* and *Pediococcus* genus for their tolerance to butanol. About 60% and 20% strains could grow in presence of 2.5 and 3% v/v butanol respectively. Later Katoka *et al.* (2011) isolated *Bacillus subtilis* GRSW2-B1 from marine samples which could tolerate up to 2.25%v/v butanol.The relation of hydrophobicity and butanol tolerance has been studied in LAB by Petrova *et al.*,(2019). They observed that the strains having tolerance to butanol had higher tolerance to butanol. (Table 10)

Table 9: List of microorganisms, technology used and solvent yield.

Microorganism used	Technology used	Yield of butanol (g/L)	
		ABE	Butanol
Clostridium acetobutylicum CICC 8012	NTG treatment for mutagenesis followed by genome shuffling	22.21	14.15
Clostridium acetobutylicum PJC4BK	NTG treatment for mutagenesis	32.5	17.6
Clostridium acetobutylicum EA2018	NTG treatment for mutagenesis	-	14.0
Clostridium acetobutylicum	Genome shuffling	32.6	20.1
Clostridium acetobutylicum ATCC 55025	Histidine kinase knockout	-	19.0
Clostridium pasteurinum ATCC 6013	spoA gene deletion	-	11.7
Clostridium tyrobutyricum ATCC 25755	*Ack* and *buk* gene knockout and adhe2 overexpression	-	16.0
Clostridium tyrobutyricum ATCC 25755	*Ack* and *buk* gene knockout and adhe2 , *xylT* , *xylA* and *xylB* overexpression	-	15.7
Clostridium acetobutylicum DSM 1731	*gshAB* over expression	-	15.0

Table 10: List of microorganisms, technology used, yield and other aspects.

Microorganism used	Technology used	Yield of butanol	Highlight of process
E.coli	Synthetic pathway	13.9mg/L	
	Thil substituted with atoB, ΔadhE, ΔfrdBC, ΔldhA, Δpta, M9 medium replaced with glycerol	552mg/L	*atoB* cloning, Deletion of competing pathways and using glycerol enhanced the yield to final 552mg/L
E.coli	Synthetic pathway	320mg/L	
	Using Adhe1 Using Adhe	1200mg/L	Adhe1 showed higher substrate specificity. Novel finding of Bcd-etfA-B complex activity
E.coli	Synthetic pathway ΔadhE, ΔfrdBC, Δfnr, ΔldhA, Δpta, ΔptfB, Gas stripping	50g/L	Deletion of competing pathways and decrease of butanol toxicity by product removal.
E.coli	Synthetic pathway Hosts own mixed acid fermentation genes were used to control butanol biosynthesis pathway	10g/L	Self regulatory *E.coli* was able to produce higher yield of butanol
E.coli	Synthetic pathway Polycystronic expression	34mg/L	-
	Monocystronic expression	200 mg/L	
	Thl substituted with atoB	220 mg/L	atoB showed higher substrate specificity.
	Fdh1 clonning	520 mg/L	*fdh*1 increased NADH flux.
	gapA overexpression	580 mg/L	*gapA* increased glycolytic flux.
E.coli	Synthetic pathway Co-culturing of two *E.coli* strains	5.8g/L	Fdh over expression and redox balance
E.coli	Synthetic pathway NADH flux increased, Thil enzyme substituted with atoB, Bcd-etfA-B substituted with Ter enzyme	30g/L	*atoB* showed higher substrate specificity. Ter reaction was irreversible
E.coli	Synthetic pathway AcrB pump was controlled by native e.coli promoter PgntK	5mg/ml 40% higher yield of butanol than control	AcrB pump controlled by PgntK lead to lesser cellular toxicity and higher butanol tolerance
E.coli	Keto acid pathway was used	8.0g/L	NTG mutation was done

Microorganism used	Technology used	Yield of butanol	Highlight of process
E.coli	Instead of synthetic pathway host's own amino acid synthesis pathway was used for butanol and propanol production	2g/L(butanol:propanol)	Butanol :Propanol::1:1
S.cerevisiae	Synthetic pathway	1.62g/L	Lower pdh activity to increase carbon flux Increase NADH flux by overexpression of mitochondrial malic enzyme (Mae1p)
S.cerevisiae	Synthetic pathway Thil replaced with *PhA* from *ralstonia eutropha*	1mg/L	
	Using *clostridial Hbd*, host's ERG10 was used instead of *PhA*	2.5mg/L	Hosts native *ERG10* showed better activity with *clostridial* Hbd instead of *PhA* or *Thil*
S.cerevisiae	Amino acid degradation pathway	92mg/L	
S.cerevisiae	Natural valine synthesis pathway was used	1.36mg/ml	Over expression of xylulose degrading genes
B.subtilis	Synthetic pathway	24mg/L	Butanol production was achieved only in anaerobic conditions
P.putida	Synthetic pathway Glucose substrate	44mg/L	No butanol production was achieved in anaerobic conditions
	Glycerol substrate	122mg/L	
L.brewis	Synthetic pathway	300mg/L	Host's native *adhe* showed lower activity than *clostridial adhe*
Synechococcus elongatus PCC 7942	Synthetic pathway	14.5mg/L	*atoB* and *Ter* instead of Thl and Bcd resp
	Direct photosynthetic butanol production through artificial ATP consumption	29.9mg/L	
	bldh substitution with oxygen tolerant CoA-acylating aldehyde dehydrogenase	404mg/L	bldh instead of adhe2 increased the yield 4 times
Thermoanaerobacterium saccharolyticum JW/SL-YS485	Synthetic Pathway	1.05g/L	

New Engineered Hosts for Improved Butanol Production and Tolerance

E.coli has been genetically manipulated for butanol production because of its well characterized and flexible genetic systems. Many synthetic biology tools and new versatile pathways are being developed in this organism to be used as host for production of biofuels and other important pharmaceutical chemicals (Xu *et al*., 2012; Atsumi *et al*., 2008).

In the last decade butanol genes have been cloned into *E Coli* for enhanced butanol production. Atusmi *et al.,* (2008) engineered *E.coli* for production of butanol by cloning (*thl, hbd, crt, bcd-etfA-B, adhe*) genes coding for acetyl-CoA acetyltransferase, β-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, butyryl-CoA dehydrogenase, electron transfer flavoprotein A-B aldehyde dehydrogenase resp. from *Clostridium acetobutylicum*. The resulting engineered strain produced 13.9mg/L butanol. This low production was attributed to sensitivity of *Bcd/Etf*A-B complex towards oxygen (Atsumi *et al*., 2008), Since the expression of *Bcd/Etf*A-B complex was not detected in *E.coli* Later Inui *et al.,* (2008) was able to achieve successful expression of *Bcd/EtfA-B* complex in *E.coli* JM109 strain by cloning the complete butanol synthesis pathway genes, when the cells grown aerobically were incubated in anaerobic conditions. This study reported the successful expression of the genes *Thl , Hbd and Crt* having enzyme activities almost 30,20 and 500 times more than control JM109 strain leading to a yield of 1200gm/ml butanol (Inui *et al*., 2008).

It was also observed that only the expression of butanol pathway genes was not sufficient for an ideal heterologous host to increase the butanol production. The expression was regulated by the sufficient supply of redox balance and NADH pool. Regulation of the supply of redox balance and

NADH pool could be achieved by either deleting the native competing pathways which lead to reduced NADH consumption and therefore increase the availability of the NADH pool for butanol production or increase the NADH flux by incorporation of NADH producing pathways. In *E.coli* the formation of lactate (*ldhA),* formate (*frd),* acetate (*pta),* ethanol (*adhE)* and succinate (*frdBC)* as byproducts lead to NADH consumption. Deletions of these competing pathways resulted in the increase in butanol production up to 552gm/L in *E.coli* (Atsumi *et al*., 2008). Later Baez *et al.,* (2011) also engineered an *E.coli* JCL260 strain lacking these competing pathways to produce 50g/l iso- butanol. This high rate of butanol production was made possible by coupling to gas stripping to overcome the butanol toxicity. Later in an *E.coli* strain the endogenous mixed acid fermentation genes*lactate dehydrogenase (LdhA), fumarate reductase (FrdABCD), alcohol dehydrogenase (AdhE), and acetate kinase (AckA) lactate dehydrogenase (LdhA), fumarate reductase (FrdABCD), alcohol dehydrogenase (AdhE), and acetate kinase (AckA)* were used to self-regulate the butanol production on transcription and translation level resulting in production of 10g/L butanol (Wen *et al*., 2013).

Second approach was to increase the NADH flux by incorporating NADH producing pathway and its over expression i.e *fdh* (*formatedehydrogenase)* produces NADH while aiding the conversion of formate to carbon dioxide. Nielsen et al. (2009) cloned the *formatedehydrogenase (Fdh*) gene as well as over expressed the *gapA* (glyceraldehyde-3- phosphate dehydrogenase which aids in the conversion of glyceraldehydes-3 phospahte to 1-3 diphosphateglycerate) of *S.cerevisiae into E.coli.* The resulting clone yielded 580gm/L of butanol.*Fdh* expression and co culturing of two separate *E.coli* strains ie. *E.coli* BuT-8L-ato enabling production of butyrate from butyryl-CoA and acetate, and *E.coli* BuT-3E converting butyrate to *n* -butanol associated with acetate led to redox balanced state and yielded 5.8g/L butanol (Saini *et al*., 2013).

In addition to deletion of native competing pathways and *Fdh* over expression, the substitution of native butanol synthesis pathway genes with other genes coding for enzymes having either higher specificity or irreversible nature was also attempted. Hence *thl* substitution with *atoB* having higher specificity and substitution of *Bcd –etfA-etfB* complex catalyzed reaction with an irreversible reaction by *Ter*(trans enoyl coenzyme A) coupled with continuous removal of butanol led to maximum yield of 30g/l butanol (Shen *et al*., 2011). Meanwhile Smith*et al.,*(2011) reported a NTG created mutant *E. coli* NV3 strain able to produce 8.0g/L isobutanol using keto acid pathway.

Apart from manipulation of native metabolism to redirect the flux, another strategy was disorientation of central mechanism of cell. Carbon storage regulator (Csr) system of *E. coli*, the major controlling element for stringent response and other carbon metabolism uptake etc. was exploited to increase the production of butanol.Csr is controlled by the RNA-binding protein which regulates translation of specific mRNA targets. Its disorientation led to two folds improvement in the butanol production than control strain. A simultaneous decrease in the formation of byproducts as acetate and carbon dioxide was also observed (Mckee *et al*., 2012). Rather than using synthetic butanol production pathway from clostridium, *E.coli's* own native amino acid biosynthetic pathway was used for butanol production and it resulted in to co production of butanol and propanol in ratio of 1:1 with a yield of 2g/L (Shen *et al*., 2008)

Recently a new study was conducted by subjecting *E.coli* to error prone PCR based whole genome shuffling. The study revealed that the mutant *E.coli* strain BW1857 produced through genome shuffling showed approximately 15-18% improvement in growth as compared to control BW25113. Genomic analysis through resequencing revealed the mutations of *acrB* and *rob*gene and the deletion of *TqsA* genes in the mutant (He *et al.,* 2019)

One of the major aims to develop heterologous hosts for butanol production was to achieve better tolerance to butanol than the native *clostridial* strains(1.5%v/v). Though *E.coli* can stand as a potential host for butanol production but its use is limited due to its inability to tolerate butanol concentration beyond 1%(v/v). This low butanol tolerance problem can be overcome either by enhancing their tolerance ability or search for an alternate host having higher tolerance to butanol.

Various transcript analysis have indicated that cells develop various mechanisms in response to stress caused by organic solvents such as either accumulation various chaperons, heat shock proteins and Reactive oxygen species(ROS), expression of efflux pumps or modification of their membranes (Dunlop *et al*., 2011). To scavenge ROS, oxidative enzymes MTs (metallothionins) from various sources were isolated and introduced into *E.coli*. Out of all HMTs(human), MMTs(mouse) and TMTs(tilapia fish), later were able to show highest ROS scavenging abilities in 1.5%(v/v) butanol. Coupling of these MTs to Outer membrane protein C precursor (ompCs) was done as it was observed that ompC fused MTs were able to have higher detoxification abilities thus better butanol tolerance capability. In fact the strains expressing only ompC were also able to tolerate butanol up to a higher level than control *E.coli* strain proving that osmoregulation could enhance butanol tolerance by accumulating compatible solutes as well as increased cellular growth by up taking more glucose (Chin *et al*., 2011). Later on a maximum of 56 % increase in tolerance at 1%v/v butanol has been reported by over expression of *groESL* chaperon (facilitates protein folding)

from *Clostridium acetobutylicum* into *E.coli* (Abdelaal *et al*., 2015) .

As mentioned earlier by Dunlop *et al.* (2011) that microbes alter their membrane structure on exposure to butanol stress.In support of this fact a study was done in which out of a total of 16 butanol tolerant isolates, two isolates CM4A and GK12 identified as *Enterococcus faecalis* and *Eubacterium cylindroides* respectively, were studied with respect to their membrane structure. Both of these showed an increased amount of cyclic saturated and cyclo propane fatty acid (CFA) content in their cell membrane. Also the gene *cfa* (coding for CFA synthase) was cloned from CM4A into *E.coli* and there was increased fatty acid content in membrane and improvement in growth of *E.coli* harboring *cfa* gene than control in the presence of butanol (Kanno *et al*., 2013) .

To improve the tolerance in *E.coli*, efflux pump AcrB was engineered by directed evolution to secrete non native substances out of the cell to overcome their corresponding inhibitory effects. A single amino acid change in AcrB efflux pump resulted in up to 25% increase in tolerance of *E.coli* to butanol. In fact this approach increased the tolerance to other alcohols ie. n-heptanol and iso- butanol etc (Fisher *et al*., 2013). Later Boyarskiy *et al*., (2016) tested the efflux pump AcrB and its butanol secreting variant AcrBv2 under native stress promoter *i.e.* PgnktK of *E.coli*. The PgnktK controlled AcrBv2 conferred higher yield of butanol in*E.coli* ie. 5mg/ml vs 0.8mg/ml.

Increase in tolerance to 1.5% v/v butanol was achieved by using Artificial transcription Factor (ATF) and Cyclic AMP receptor Protein (CRP) in *E.coli* (Lee *et al*., 2011). To study the phenomenon behind tolerance to butanol, an *E.coli* strain SA481 was isolated after evolution from iso- butanol producing *E.coli* JCL260 strain. The whole genome of both the organisms was sequenced and it was identified that *acrA*(encoding *AcrB-Tol-C*), *gatY*(encoding *tagastose-1,6-bisphosphate aldolase*), *tnaA* (encoding *l-cysteine desulfhydrase/tryptophanase*), *yhbJ* (encoding *ATPase*) and *marCRAB*(encoding a transcriptional activator)were the main key mutations responsible for increased tolerance in*E.coli* strain SA481. Also the introduction of all these mutations into the host *E.coli* JCL260 strain successfully resulted in increased iso-butanol tolerance (Atsumi *et al*., 2010). In a similar study by experimental evolution followed by genome re-sequencing and a gene expression study in *E.coli*, set of gene loci were identified playing role in increased tolerance to isobutanol. After examining genotypic adaptations it was found that there is parallel evolution in marC (conserved protein for transporter), *hfq* (HF-I, host factor for RNA phage Q β replication), mdh (malate *dehydrogenase, NAD(P)-binding*), *acrAB* (multidrug *efflux system protein*), *gatYZABCD* (D-*tagatose 1,6-bisphosphate aldolase*) and *rph* (defective *ribonuclease PH*) genes encoding for conserved protein for transporter in response to isobutanol stress (Minty *et al*., 2011).

Microbes reported to have natural organic solvent tolerance as *Saccharomyces cerevisiae, Bacillus subtilis, Pseudomonas putida* and *Lactobacillus brewis* were also explored for butanol production (Knoshaug *et al*., 2009). *Saccharomyces cerevisiae* being an existing industrial strain for ethanol production, genetically well characterized and ability to tolerate two carbon alcohol (ethanol) grabbed the attention to be used as a host for butanol production. Various isozymes of butanol synthesis pathway from other microorganisms were used in native clostridial spp. by Steen *et al.*, (2008). Along with *clostridium beijerinckii* (thl) gene,its various isozymes such as thiolase from *Ralstonia eutropha(phaA*), and *E.coli(atoB)* were tested. The best activity was shown by the strain employing *PhaA* and it produced 1mg/L butanol. Then isozymes for *3-hydroxy butyrylco A dehdrogenase* were used.The best activity was shown by strain ESY7 harboring clostridial *hbd* gene in combination with host's native thiolase ie.*PhaA*. The resulting strain produced 2.5gm/L butanol. The natural valine synthesis pathway of *S. cerevisiae* was also exploited for iso- butanol production. The location of valine synthesis pathway from mitochondria to cytosol and over expression of *xylA* gene for xylose utilization resulted in the production of 1.36mg/ml iso- butanol (Brat and Bowles 2013 and Brat *et al*., 2009). Reducing the activity of pyruvate dehydrogenase (PDH) complex thus increasing the carbon flux towards iso butanol synthesis and over expression of transhydrogenase-like shunts *ie*. mitochondrial malic enzyme (Mae1p) which contributed to increased supply of NADPH resulted in production of 1.62g/L iso butanol in *S. cerevisiae* through keto acid pathway (Matsuda *et al*., 2013). Using the amino acid degradation pathway and glycine as substrate by *S. cerevisiae* resulted in the production of 92mg/L butanol (Branduardi *et al*., 2013).

Bacillus subtilis was also engineered to produce butanol. As *Bacillus* can prove to be a potential host because of its, easy genetic traceability, non-pathogenic nature and it has the capacity to export proteins into extracellular medium which is needed for heterologous gene expression.The engineered stain BK1.0 harboring synthetic butanol pathway from clostridium produced 24mg/L butanol an aerobically. No butanol production was achieved when the culture was grown in aerobic conditions (Nielsen *et al*., 2009)

Butanol synthesis was cloned in *Pseudomonas putida*as well for butanol production because of its reported high tolerance to organic solvents. Engineered strain produced 122mg/l butanol with glycerol as substrate in contrast to 44mg/l produced using glucose as substrate (Nielsen *et al*., 2009).

In the search of potent microbial host for butanol production lactic acid bacteria (LAB) were also explored because of it

is assumed that LAB possibly possess some hereditary butanol tolerance property. Even it was reported by Afschar *et al*., (1990) that most frequent contaminants found in ABE fermentation were found to be LAB. The native crucial enzyme activity *aldehyde dehydrogenase (bldh)* and *alcohol dehydrogenase(bdh)* activities were higher in *Lactobacillus* sp. supporting the fact that these native enzymes can contribute to butanol synthesis. But Berzenia *et al.,* (2010) reported that substituting the hosts aldehyde and alcohol dehdrogenase with clostridial genes led to higher yield of butanol. Infact despite the presence *Lactobacillus* own 3-hydroxybutyryl-co-A dehydrogenase gene(*Hbd*) its activity was not detected after introduction of the rest of butanol synthesis genes. The recombinant *Lactobacillus brevis* strain was able to synthesize only 300mg/L butanol.

Expression of butanol synthesis pathway genes into *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 resulted in the production of 1.05g/L butanol (Bhandiwad *et al*., 2014). Cyanobacteria being natural phototrophs, having fast cell growth and being capable of growth in even those areas which are not fit for cultivation, were exploited for biofuel production. Moreover, increasing carbon dioxide emission could be utilized in useful manner by converting into biofuel with the help of cyanobacteria (Machado *et al*., 2012). Lan and Liao (2011) reported the production of 14.5mg/L butanol by *Synechococcus elongatus* PCC 7942 harboring *crt*, *hbd*, *adhe2*, *atoB* instead of *Thl* and *Ter* instead of *Bcd* in anaerobic conditions. Later Lan and Liao (2012) achieved direct photosynthetic butanol production through artificial ATP consumption in *Synechococcus elongatus* PCC 7942. Through artificial ATP consumption the acetyl CoA condensation to produce acetoacetyl CoA was made thermodynamically favorable. The substitution of adhe2 (aldehyde/alcohol dehydrogenase) gene with butyraldehyde dehydrogenase (*Bldh*) resulted in approximately 4 times increase in yield from 6.5- 29.9mg/L butanol. Further Lan and Liao (2013) substituted the *bldh* with oxygen tolerant CoA-acylating aldehyde dehydrogenase as *bldh* was found to be oxygen sensitive and achieved the yield of 404mg/L butanol with the same organism (Lan *et al*., 2013). (Table11).

Industrial Aspect

In 1990 Austria introduced a continous fermentation based pilot scale plant, which employed new improved and economically favourable technologies for butanol production (Nimcevic *et al*., 2000). Companies such as DuPont, British Petroleum, Cobalt Technologies and Gevo Inc. are exploring biobutanol as a biofuel and its production. These companies are also targeting its industrial scale production. These companies have proposed a plan to produce 30,000 tons butanol per year. There many other companies as Butyl fuels, Cobalt Biofuels, Green Biologics, Metabolic Explorer etc. which are claiming to enhance the butanol production from pilot scale to industrial scale Currently, 11 fermentation plants for butanol production are in operation in China (plus an additional 2 under construction) and 1 in Brazil. (Ni *et al*., 2009; Durre *et al*., 2011)

Conclusion

According to current scenario butanol production seems to be rather fascinating than challenging. Numerous efforts are being made to increase butanol production from clostridia but saving the production cost is also very important. Therefore, exploration of lignocellulosic substrates has gained lots of interest but their pretreatment also adds burden to the cost of the process. So the genetic engineering of production hosts with the genes responsible for lignocellulosic waste degradation to avoid extra cost in treatment processes. Apart from this co-culturing of butanol producing microbe with microbes able to degrade Lignocellulosic substrates has also been done. Genetic engineering of clostridial hosts was also attempted to increase butanol production. In the process of achieving high yield of butanol, major hurdle was toxic nature of butanol to the microbes. To overcome this problem various in situ product removal methods were successfully employed.

Instead of achieving high yield through *Clostridial* sp., new heterologous hosts were also explored. Even the heterologous hosts faced the problem of butanol toxicity which resulted in low butanol yield therefore further studies were done to improve their tolerance against butanol. Though increased tolerance did not guarantee increased butanol production but increasing tolerance was mandatory to increase the yield of butanol. This will decrease the burden caused due to butanol toxicity. Though tolerance mechanisms were specific to different organisms and biofuels as ethanol tolerance did not ensure butanol tolerance in certain microbes.

Apart from developing tolerance in heterologous hosts, naturally tolerant hosts also came as promising candidates for butanol production. Further genetic studies to use them as production hosts is also very important. Analysis of butanol tolerant microbes in terms of their genetic constitution and membrane composition have opened new strategies to develop butanol tolerant microbe. Using *clostridial* sp. and heterologous hosts both is being explored at another level and equally important. To make biological production of butanol viable for industrialization *in situ* product removal, energy consumption and economics of the process need to be evaluated carefully

Table 11: list of microorganisms, technology used and improvement in butanol tolerance reported

Microorganism	Technology used	Improvement in tolerance
E.coli	Artificial transcription factor	Increased tolerance at 1.5% butanol
E.coli	groESL were expressed from clostrdia	56% increase in 1% butanol
E.coli	OMPcs fused TMT were expressed	2.04% increase in 1.5% butanol
E.col	Single amino acid change in AcrB pump by directed evolution	25% increase in tolerance
E.coli	AcrB pump under native promoter	Increase in yield from 0.8 to 5mg/ml butanol

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