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Mini Review

Thermotolerant micro-organisms in Consolidated Bioprocessing for ethanol production: A review

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Microorganisms that withstand high temperatures (thermotolerant) will continue to gain global significant prominence in consolidated bioproduction of ethanol. Consolidated bioprocessing (CBP) technology, is an approach that merged together enzyme production, saccharification and fermentation in a single vessel. It offers considerable advantages for the production of bioethanol. It seems promising because of its reduction in utilities, substrate and raw material simplification during operation, making it cost effective. High temperature CBP is preferred, because cellulolytic optimum enzyme performances occur at elevated temperature of around 50ºC. While on the other hand, optimum microbial fermentation occur best at temperature between 28ºC - 37ºC. This necessitates the search for more thermo tolerant micro-organisms as suitable candidates for CBP. Despite the advances seen in this technology, it uses only highly engineered microbial strains, which poses public health risks and environmental concern. As promising as the approach might be, there are, as yet, no wild types microbial strain has been isolated that perform CBP at the required elevated temperatures. This review will focus on the recent features of CBP technology, its advantages and drawbacks towards the production of bioethanol, and provide selected characteristics features of some thermotolerant micro-organisms for the process compatibility. Furthermore, perspectives, challenges and emerging new directions were also briefly highlighted.

Key words: Consolidated bioprocessing, Thermotolerant Microbes and Bioethanol

Fuels reserves has been diminishing in recent years necessitating the need for alternative energy sources that are renewable, sustainable, efficient, cost effective and safe (Yu, XuZhang et al. 2008). Microbial ethanol production via consolidated bioprocessing in recent years has been focused and considered as an alternative fuel in the near future.

Consolidated bioprocessing, is an approach integrating enzymes production, saccharification and fermentation in to a single process, a strategy that is effective for ethanol production from lignocellulosic materials (Hasunuma and Kondo 2012). In addition, CBP requires microbial strains capable of hydrolyzing with enzyme produce on its own and producing high titre ethanol. To date, only engineered microbial strains have been known to perform this.

Furthermore, different researches were conducted to explore the engineering of different microbial strains to suit the intended process, where heterologous expression of cellulolytic enzymes has been pursued with yeast host and cell surface engineering has been successful (Hasunuma, Okazaki et al. 2013). Thermophilic yeast such as *Kluveromyces*

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marxianus has been engineered to explore its ability to perform CBP at elevated temperature for bioethanol production (Limayem and Ricke 2012) table 2. Extreme anaerobic thermophilic bacteria such as *Thermoanaerobacter saccharalyticum*, *Thermo-anaerobacter ethanolicus* and *Clostridium thermocellum* have been genetically modified to suit perform for CBP (Kumar, Singh et al. 2009; Limayem and Ricke 2012).

Few examples of thermotolerant micro-organisms suitable for CBP exist, and effort to optimize their performance at elevated temperature is hampered by little genetic knowledge and/ or tools for engineering and adaptations to high temperatures. It is our intents in this article to (1) Present recent significant features of CBP, including its advantages and disadvantages table 1. (2) Highlight the characteristics features of some selected microorganism involved in bioethanol fermentations table 2. (3) Briefly pinpoints the perspectives and challenges in this emerging area of Biotechnology.

Consolidated bioprocessing (CBP)

Consolidated bioprocessing (CBP), which combines enzyme production, saccharification and fermentation into a single vessel, is a promising strategy for effective ethanol production from lingo-cellulosic materials, this is because utilities are reduced, and substrate and other raw materials are simplified during operation (Hasunuma and Kondo, 2012). The use of this approach as an emerging technology in bioethanol production from lignocellulosic materials is of utmost importance because the cost of capital investment and enzyme production can be avoided. The recent advances seen in microbial strains with the capability of efficient cellulose hydrolysis and ethanol production represent significant achievement towards CBP (Hasunuma and Kondo, 2012). One of the peculiar features of CBP is that it requires highly engineered microbial strains that would be compatible with process parameters such as high temperature and simultaneously hydrolyzing biomass with enzymes on its own with high ethanol titre.

Further more, one of the major bottlenecks in CBP is the optimum temperature required for saccharification and fermentation stages. Best performance of cellulolytic enzymes is achieved around 50^{o}C, while on the other hand the optimum performance of most fermenting microbes occurred between 28^{o}C and 37^{o}C (Jørgensen, Kristensen et al. 2007). Some authors are of the opinion that it would be difficult in practice, to lower the optimum temperature of cellulases via protein engineering (Hasunuma and Kondo 2012). Accordingly, the demand for high temperature fermentation is increasing in recent years, because in CBP ethanol production at elevated temperature is cost effective. In this approach, sacharification and fermen-tation concurrently occur in single vessel at high temperature. This attracts the need for screening microorganisms for CBP based on temperature requirements. The adventages of CBP, high temperature process and its disadvantages are highlighted in table 1.

Fungus for CBP

Fungal genera *Aspergillus, Rhizopus, Monilia, Neurospora, Fusarium, Trichoderma* and *Mucor*, which are considered predominantly filamentous fungi, have been explored for the improved production of ethanol from biomass (Hasunuma, Okazaki et al. 2013). It was recently reported by Okamoto, Nitta et al. (2011) that the white rot fungus *Trametes hirsute* was shown to be capable of fermenting rice straw, starch and wheat bran directly to ethanol without prior enzymatic and/or acid hydrolysis. The advantageous characteristics of *Rhizopus oryzae,* such as ability to utlize pentoses, low growth requirements, tolerance to some certain inhibitors present in acid hydrolysate of lignocellulosic biomass and its abilty to directly utilized non pretreated cellulose and hemicelluloses (Zhang and Yang 2012), make it a potential candidate for CBP. In addition, the methophilic fungus *Fusarium*

oxysporum is among the few microbial species that possess the enzymatic system to break down cellulose and hemicellulose while simultaneously fermenting the generated hexoses and pentoses to ethanol(Hasunuma, Okazaki et al.). This capability allows for single-step ethanol production from agricultural and forestry residues. (Xiros, Moukouli et al. 2009) Reported a yield of 109g ethanol per kg of dry brewer's spent grain (BG) obtained from alkali pre-treated BG using *F. oxysporum* cultivated under microaerobic conditions, which ultimately tally with 60% of the theoretical yield based on the total glucose and xylose content of BG(Xiros, Moukouli et al. 2009)

Bacteria for CBP

Many examples of thermophilic microorganisms have been documented with ability to perform optimally at high temperature operation. This could serve as alternatives for use as the major fermentatives and cellulolytic agents in bioprocessing for ethanol production (Limayem and Ricke 2012). Bacteria such as *Clostridium cellulolyticum* and *Thermo-anaerobacterium saccharalyticum* have been recently reported to serve as alternatives for this process, some with ability to perform fermentation at high temperature of approximately 50^{o}C (Joe Shaw, Jenney Jr et al. 2008).

Clostridium thermocellum, a thermophile that is predominantly anaerobic, has been known among the few bacteria that can ferment sugars, polymers and in turn cellulose to ethanol (Limayem and Ricke 2012). It posses additional physiological features that makes it a promising a candidate. These features includes, growth temperature selectivity of around 50^{o}C during fermentation process, couple with its ability to yield 0.3g/g ethanol by directly converting cellulose polymers at temperature of approximately 60^{o}C(Limayem and Ricke 2012), and hence could be considered suitable for CBP(Lynd, Zyl et al. 2005).

Table 1. Advantages and drawbacks of CBP (Hasunuma and Kondo, 2012)

Advantages of CBP	Advantages of High Temperature Process	Disadvantages of CBP
• Risk of contamination is reduced drastically by reducing glucose and producing ethanol • Total operational simplification • Raw materials and/or substances are reduced for reactions • Fermentation and saccharification vessels are reduced • Enzymes production utilities are totally eliminated • Capital investment is highly reduced to the lowest minimum	• Cooling cost reduction • Improvement of hydrolysis efficiency • Compatibility with high temperature of tropical countries • Elimination of chiller unit • Reduction in risk for contamination • Evaporation of ethanol continuously from broth under reduced pressure	• To date, only highly engineered microbial strains are known to perform optimally in CBP. • The use of recombinant strains is highly restricted in some countries, and there is growing public health concern and environmental risk associated with this. • No wild type bacterial / fungal species to date that are known to perform saccharification and fermentation at high temperature.

Table 2: Thermotolerant micro-organisms used for Bioethanol production

Species	Characteristics	Advantages	Draw back(s)	References
Zymomonas mobilis	Ethanologenic G-ve bacteria	Ethanol yield surpasses *S. Cerevisiae* (97% of the theoretical) High ethanol tolerance (up to 14%v/v). High ethanol productivity (five fold more than *S. cerevisiae* volumetric productivity). Amenability to genetic modification Does not require additional O_2.	Not able to ferment xylose sugar Law tolerance to inhibitors Neutral pH range.	(Limayem and Ricke 2012)
Kluveromyces marxianus	Thermophilic yeast	Able to grow at elevated temperature of above 52^{0}C. Suitable for SSF/CBP process. Reduces cooling cost Reduces contamination Ferments a broad spectrum of sugars Amenability to genetic modification	Excess of sugars affects its alcohol yield Law ethanol tolerance Fermentation of xylose is poor and lead only to the formation of xylitol	(Limayem and Ricke 2012)
Themophilic Bacteria: Thermoanaerobacter saccharalyticum *Thermoanaerobacter ethanolicus* *Clostridium thermocellum*	Extreme anaerobic bacteria	Resistance to an extremely high temperature of 70^{0}C. Suitable for CBP processing Ferments a variety of sugars Display cellulolytic activity Amenability of genetic modification	Low tolerance to ethanol.	(Kumar, Singh et al. 2009) (Limayem and Ricke 2012)

Zymomonas mobilis is also known to be an attractive alternative candidate for CBP. It has characteristics of being ethanologenic, high ethanol yield and resistant to high temperatures in the range of 40^{o}C among others. Engineering efforts were made where different genes have been inserted in to *Z. mobilis*, to expand its effectiveness towards utilizing varieties of substrates, namely xylose and arabinose since originally the strain is only able to ferment glucose. The high ethanol yield of this strain and its amenability to genetic modification is an interesting attribute for use in CBP.

Perspectives and Challenges

Many examples from the discipline of bioprocess engineering have proven that the ability of microorganism particularly those capable of withstanding high temperature for the production and/or synthesis of biofuels are extensive and amenable to modification. The progression of notable example is the engineering of *K. marnianus* and *S. cerevisiae* to suit CBP for bioethanol production. The CBP as an emerging technology has been found to be cost effective at high temperature, as it allows selection of microorganisms by temperature and does not require cooling cost and cellulase addition (Limayem and Ricke 2012), but yet it suffer some draw backs. Recently, thermotolerant yeast strains *Kluyveromyces, Sacchromyces* and *Fabora genera* has been documented as an attractive candidates, because they can produce more than 5% (w/v) at elevated temperature (>40^{o}C) (Hasunuma and Kondo 2012). *K. marnianuus* has been reported to have ability to co-ferment both hexoses and pentoses sugars and survive high incubation temperature of 42-45^{o}C (Limayem and Ricke, 2012). In addition, some thermophilic bacteria such as *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacter ethanolicus* and *Clostridium thermotherum* have been also

reported to ferment both hexoses and pentoses sugars to ethanol (Limayem and Ricke, 2012). These organisms are strictly anaerobic, and some authors are of the opinion that it is difficult in practice to maintain a complete anaerobic condition in large fermentation facility (Hasunuma and Kondo 2012).

The current challenges are to obtain an ideal microbes for CBP with the characteristics of high target productivities, high ethanol titre, prolonged cell viability during the process of fermentation (Hasunuma and Kondo 2012). In the same vein, CBP has a unique drawback of the optimum temperature differences in saccharification (50^{o}C) and fermentation (28^{o}C and 37^{o}C) (Hari Krishna, Janardhan Reddy et al. 2001; Jørgensen, Kristensen et al. 2007; Hasunuma and Kondo, 2012). Microbial strain engineering to improve the strain process compatibility has been useful but it is beset with public health risks and environmental problems as genetically engineered microorganisms may escape into the environment (Limayem and Ricke, 2012)**.** Under the umbrella of bioprocess engineering, one can exploit enabling technologies such as DNA synthesis and the use of computational tools for the prediction, design and construction of robust cell factories for target compound production. Furthermore, system wide intracellular metabolic pathways modifications using advanced engineering tools such as host, vectors, genetic controllers and characterized enzymes (Keasling, 2012) are needed to make this a reality. This would improve not only the target productivities, but cell growth and viability during fermentation process. Although this will solely depends on headway progress seen in data systemization, synthetic microbiology and computational breakthroughs. It is deemed imperative to recognize that the ultimate goal of the entire process is to produce an ideal candidate chassis host that would be implemented into a large scale facility, which is a real factory. Accordingly, difficulties and challenges exists in computational approaches , but yet the tools/softwares breakthroughs currently seen with their sketchy history of success in this arena-will continue to have prominence and broad applicability (Prather and Martin 2008)

Conclusion

In this review, recent advances in CBP for the use of thermotolerant microbial stains for fermentative bioethanol production and cellulosic materials were briefly highlighted. The high temperature requirement for CBP in relation to microbial fermentation, its advantages and drawbacks were briefly emphasized. The need for integrating different biological disciplines, such as systems and Synthetic biology, Bioprocess engineering, Metabolic engineering, Computational biology and/or data systemization were briefly stated in the perspectives and challenges section of this review. In light of the obvious requirements for increasing yields and lowering production costs, this proof-of-concept could be significant, as it will allow the application of the aforementioned disciplines in microbial strains improvement to advancing CBP technology.

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