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Itaconic Acid Production by Microorganisms: A Review

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Abstract: Itaconic acid ($C_5H_6O_4$) is an organic acid with unique structure and characteristics. In order to promote the bio-based economy, the US-Department of Energy (DOE) assigned a "top-12" of platform chemicals, which include numerous of organic acids. In particular di-carboxylic acids, like itaconic acid, can be used as monomers for bio-polymers. Thus the need to produce itaconic acid attracts much attention. The favored production process is fermentation of carbohydrates by fungi and *Aspergillus terreus* is the mostly frequently employed commercial producer of itaconic acid. This review reports the current status of use of microorganisms in enhancing productivity.

Keywords: Aspergillus, fermentation, itaconic acid, production

INTRODUCTION

Itaconic acid (methylene succinic acid) is a promising organic acid. It is a white crystalline unsaturated dicarbonic acid in which one carboxyl group is conjugated to the methylene group. Different microorganisms have been used in industry for the production of secondary metabolites and enzymes. For every case, the culture medium was optimized for a target product and the carbon source mainly used (glucose or sucrose) is either expensive and/or competes with the food industry (Menon and Rao, 2012). *Aspergillus terreus* NRRL 1960 has been used as microorganism for optimizing the fermentation process. The mycelial growth is sensitive to phosphate limitation and to carbon and nitrogen availability (van der Werf *et al*., 2009). Glucose and glycerol were reported as suitable for itaconic acid production (Jarry and Seraudie, 1995; Jarry and Seraudie, 1997) however the productivities of the process were strictly depend on the selected strain, media formulation and ambient conditions (Meyer, 2008; Okabe *et al*., 2009). The maximum of itaconic acid concentration produced by *A. terreus* TN-484 was 82 g/L from 160 g/L of glucose in a shaking flask, which is 1.3-times higher than that of the parental strain (Yahiro *et al*., 1995).

Current global scenario of itaconic acid production: Global itaconic acid announces the release of a comprehensive global report on itaconic acid market. The global market for itaconic acid is forecast to reach over US$398.3 million by the year 2017, driven by rising concerns over diminishing fossil fuel resources and the need for manufacture of environment friendly 'green' chemicals. There is an increasing concentrate on production of itaconic acid from renewable resources, a drastic shift from the currently prevalent sourcing from petrochemical feedstock. Asia-Pacific, backed by tremendous impetus from China is poised to emerge as the fastest growing market for itaconic acid at a Compound Annual Growth rate (CAGR) of over 9.0% through 2017. China, previously a major importer of itaconic acid, is currently one of the leading producers in the world, trailing closely behind Russia, Japan and the US. **(**prweb.com/releases/itaconic_acid/renewable_chemicals/prweb8831422.htm)

The economic importance of microbial itaconic acid: Itaconic acid has been applied in a numerous range of industries. Throughout the 1950s, itaconic acid was used in industrial adhesives. Overall, in this period, itaconic acid was used at an industrial scale and plenty amounts of it were required. The sulfonated form or alkali salt of poly itaconic acid is employed as a detergent and in shampoos. The polymerized ethyl, methyl, or vinyl esters of itaconic acid are used as plastics, elastomers, coatings and adhesives, (Mitsuyasu *et al*., 2009). Characteristics of the coating and plastic, which is compounded by using 1-5% itaconic acid and styrene, include light color, easy separation, easy to paint, water-fast and antisepsis; it can be used in the manufacture of high-strength enhanced plastic fiberglass and in the coating of carpets and book covers (Jin *et al*., 2010). Itaconic acid may also be used as artificial gems and synthetic glasses with special nonlinear characteristics (Kin *et al*., 1998).

Considering that the 1990s, the applications of itaconic acid have been developed to biomedical fields, like the ophthalmic, dental and drug delivery fields. Another application of itaconic acid is in the

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preparation of Glass Ionomer Cement (GIC). GICs demonstrated to be useful adjuncts in restorative dentistry (Nagaraja and Kishore, 2005). Crisp and Wilson (1980) synthesized a copolymer of acrylic and itaconic acid that turned out to be indefinitely stable in aqueous solution. This copolymer was the initial commercial marketable cement. An N-vinylcaprolactam-containing copolymer of acrylic itaconic acid (Moshaverinia *et al*., 2009) and poly (acrylic acid-co-itaconic acid) (Culbertson, 2006) was developed to be used in functional and mechanical GICs. These materials have found increasing applications in clinical dentistry (Okabe *et al*., 2009).

Biochemical pathways of itaconic acid synthesis: A pathway for the biosynthesis of itaconic acid propose by Bentley and Thiessen (1957) which starting from a sugar substrate such as glucose the carbon molecules are processed through glycolysis to pyruvate. After that the pathway is scatter and part of the carbon is metabolized to Acetyl-coA releasing a carbon dioxide molecule. The other part is converted to oxaloacetate so the previously released carbon dioxide molecule is again incorporated. In the first step of the citric acid cycle, citrate and *cis*-aconitate are formed. In the last step, the only itaconic acid pathway dedicated step, *cis*-aconitate decarboxylase (*cadA*) forms itaconic acid releasing carbon dioxide. This pathway was confirmed by tracer experiments with ^{14}C and ^{13}C labeled substrates (Bentley and Thiessen, 1957; Winskill, 1983; Bonnarme *et al*., 1995) and also the necessary enzymatic activities have been all determined (Jaklitsch *et al*., 1991). The formation of carboxylic acids, such as citric and itaconic acid requires the shuttling of intermediate metabolites between different intracellular compartments and utilizes different enzymatic capabilities of the respective compartment. In the case of itaconic acid compartmentalization of the pathway was analyzed by fractionized cell extracts distinguishing the enzymatic activity of a mitochondrial from a cytosolic enzyme. It was discovered that the key enzyme of the pathway, *cadA*, isn't located in the mitochondria but in the cytosol (Jaklitsch *et al*., 1991), whereas the enzymes preceding in the pathway, namely citrate synthase and aconitase, are found in the mitochondria. Although a residual level of aconitase and citrate synthase activity is found in the cytosolic fraction. The proposed mechanism is that *cis*-aconitate is transported via the malate-citrate antiporter to the cytosol (Jaklitsch *et al*., 1991). Recent evidence (Strelko *et al*., 2011; Voll *et al*., 2012) shows that *cis*-aconitate decarboxylase activity organizes the general pathway for itaconic acid formation in nature. Also, itaconic acid was detected in mammalian cells, where it was found in macrophage-derived cells (Strelko *et al*., 2011). Those cells also possess a *cis*-aconitate decarboxylase activity and have the ability to form itaconic acid *de novo*. But, so far no specific gene encoding this enzymatic activity was recognized in mammalian cells (Matthias *et al*., 2013).

Fermentation mode for itaconic acid production: Many researchers have attempted to improve the itaconic acid production rate using various bioreactors. There have been different reactors tested such as the bubble column (Yoshida, 1988), tubular reactor (Moser, 1991), packed bubble column (Abraham and Sawant, 1990) and Air-Lift Reactor (ALR) (Siegel *et al*., 1986). Compared to stirred tank reactor (STR), the ALR uses one third of the energy required (Träger *et al*., 1989). In one research *A. terreus* IFO-6365 was used in the ALR using a modified draft tube for the production of itaconic acid (Okabe *et al*., 2009). It was noted that the production rate (0.66 g/L/h) increased to double the value from the STR, which is resulted from the morphological changes of the fungus from the filamentous form to the pellet type. In 1994 Park *et al*. (1994) reported that repeated itaconic acid production in the ALR was possible in 21 days and an itaconic acid production rate of 0.37 g/L/h was accomplishied. Itaconic acid producers have also been evaluated on the shake flask scale. Even when the same strain of *A. terreus* was used, the itaconic acid concentration differed between the flask culture and ALR, with a slightly higher concentration of itaconic acid being produced in the flask culture than in the ALR (Okabe *et al*., 2009; Park *et al*., 1994; Yahiro *et al*., 1995). Which may be due to oxygen limitations in the ALR because mixing in the ALR is milder than that in the rotary shaker.

Other researchers tried to immobilize *A. terreus* in order to improve the performance of various fermentation systems by immobilizing the mycelia: Polyacrylamide (Horitsu *et al*., 1983), polyurethane foam (Kautola *et al*., 1990, 1991), celite R-626 (Kautola *et al*., 1985), calcium alginate (Kautola *et al*., 1985) and porous disks (Naihu and Wang, 1986). It was found that production rates were relatively higher in immobilized cell bioreactors with porous disks or celite R-626, although the itaconic acid concentrations were still lower than 20 g/L. In batch cultures however it was found that production rate was similar and ranged between 0.26 and 0.32 g/L/h. In the continuous cultures the rate was twice the batch cultures. A concentration of (18 or 26 g/L) seems low for industrial purposes. It was found that the itaconic acid production rate in repeated batch culture was 0.37 g/L/h, which was 40% higher than of the rate in batch cultures through the use of ALR. According to Okabe *et al*. reported batch culture without the loss of itaconic acid formation activity may be expected in the ALR (Okabe *et al*., 2009).

Microorganisms producing itaconic acid other than *Aspergillus*: There are many studies on biosynthesis of organic acids in filamentous fungi (Mattey, 1992). Hence, fungi such as the genus *Aspergillus* are mostly

used for industrial production of organic acids like itaconic acid (Billington, 1969). *A. terreus* is frequently applied for itaconic acid production that's grown under phosphate-limited conditions (Lockwood, 1979) (Roehr and Kubicek, 1996; Willke and Vorlop, 2001), Also difficulties working with filamentous organisms in bioreactors and the sensitivity of *A. terreus* fermentations to metal concentrations (Lockwood, 1979) has led to the testing of yeasts for possible itaconic acid production. The patent literature in this subject, reviewed by Willke and Vorlop (2001), there are reports of itaconic acid production by the Candida mutant strain and *Rhodotorula* species. Tabuchi *et al*. (1981) isolated a strain, putatively recognized as a Candida that produced itaconic acid at the 35% yield when grown under phosphate-limited conditions. William *et al*. (2006) reported on the ability of *Pseudozyma antarctica* NRRL Y-7808 to produce itaconic acid from glucose and also other sugars under nitrogen-limited growth conditions. Species of *Pseudozyma* are basidiomycetes and are presumed to be closely relevant to *Ustilago* (Boekhout *et al*., 1998). However some species of the plant pathogenic fungal genus *Ustilago*, a basiodiomycete, are known to produce itaconic acid during fermentation (Willke and Vorlop, 2001). *Ustilago maydis* grows as single cells (yeast-like) in submerged cultivations and it is extremely robust in high osmotic media and real seawater. Moreover, *U. maydis* can grow on the hemicellulosic fraction of pretreated beech wood. Thereby, this fungus combines important advantages of yeasts and filamentous fungi (Tobias *et al*., 2012). *U. maydis* appears to be a candidate for an alternative producer of itaconic acid. It may produce high amounts of itaconic acid about 68.36 g/L under certain cultivation conditions. Under unlimited conditions cells from Glucose (Ramesh and Sastry, 2011).

Strain improvement of *A. terreus*: There have been some investigations on strain improvement for itaconic acid production and several types of microorganisms have been used for it. A strain that designated TN-484 was reported by Yahiro *et al*. (1995), selection using an itaconic acid concentration-gradient agar plate technique after NTG-treatment of *A. terrerus* IFO 6365 was successfully established for producing itaconic acid with a high yield. The itaconic acid concentration produced by *A. terreus* TN-484 was 82 g/L from 160 g/L of glucose in a shaking flask, which is 1.3-times more than that of the parental strain. Tsai *et al*. (2001) was reported a method to produce itaconic acid that it's solid-state fermentation method. Peeled sugarcane press mud or sugarcane press mud is the support used to adsorb liquid medium for the production of itaconic acid by an *A. terreus* mutant strain. The successive mutation was created this mutant strain from *A. terreus* ATCC 10020. The suitable amount of liquid medium that could be added to the support is 8 to 14 times its dry weight for the peeled sugarcane press mud and 4 to 6 times its dry weight for the sugarcane press mud. The optimal pH of the medium is between 2.0-3.0. The fermentation temperature is between 30-40 degrees centigrade.

Generally itaconic acid is produced by fungal cells of *A. terreus* in the branch of the TCA cycle via decarboxylation of cis-aconitate. It is known that highly branched filamentous morphology results in high viscosities of culture broth in fungal cell fermentations, leading to considerable decrease in mass and oxygen transfer capacity (Lin *et al*., 2004). To get more facilitated utilization of dissolved oxygen by the high-yielding mutants, an effective expression vector with Vitreoscilla Haemoglobin (VHb) gene plus gpdA promoter was constructed and after that introduced in to the high-yielding producers (Zhang *et al*., 2007). It was achieved through Southern blot analysis that 8 copies of VHb gene were integrated in to the transformants' chromosomes. Notably, a resulting transformant harboring the wild VHb gene showed approximately 40% higher productivity of itaconic acid compared to the parallel nontransformed strains in the shake flask fermentations performed under the similar fermentation conditions. In addition, production stability of the majority of the strains further screened from the transformants was enhanced, exhibiting sharp contrast to the results obtained from the corresponding nontransformants. According to these consequences, it absolutely was concluded that optimal supply of oxygen was prerequisite for the enhanced biosynthesis of itaconic acid as well as production stability of the high-yielding producers (Shin *et al*., 2009).

A. niger doesn't naturally produce itaconic acid since it lacks the essential enzyme cis-aconitate decarboxylase. The *cadA* gene encoding this enzyme in *A. terreus* has been recognized using different approaches, including an enzyme purification approach (Kanamasa *et al*., 2008) and a clone-based transcriptomics approach (Li *et al*., 2011). The expression of the *cadA* gene in *A. niger* leads to extremely low amounts of itaconic acid production (0.05 g/L), representing that the sole expression of the enzyme is not sufficient for effective production of itaconic acid. In the *A. terreus* genome, the *cadA* gene is located close to the lovastatin cluster (Tsao *et al*., 1999) and flanked by the putative mitochondrial transporter *mttA* and a putative plasma membrane transporter *mfsA*. The co-regulation of these transporters with *cadA*, reported by Li *et al*. (2011) proposed that the putative mitochondrial transporter might be involved in itaconic acid production in *A. terreus*. Recently, Li *et al*. (2013) reported that the effect of these putative transporters on itaconic acid production in *A. niger* led to a slight increase in itaconic acid production levels. Though, the highest titer of 1.5 g/L itaconic acid that was reached is far from the theoretical titer of above 135 g/L under conditions of

high citric acid production. Laura *et al*. (2014) reported that moreover to the *cadA* gene, the *mttA* gene from *A. terreus* is also crucial for efficient itaconic acid production in *A. niger*. Expression of the *mttA* gene, encoding a putative mitochondrial transporter, in the strain that expresses *cadA* resulted in an over twenty-fold increased secretion of itaconic acid. Expression of the *A. terreus* itaconic acid cluster, consisting of the *cadA*, *mttA* and *mfsA* genes, led to *A. niger* strains with over twenty five-fold higher levels of itaconic acid and a 20-fold increase in yield when compared to a strain that expressed only *cadA*.

Efforts to reduce the production cost of itaconic acid: Even though crystalline glucose may be costly, it is noticed that using glucose as substrate leads to higher itaconic acid. Corn starch is another useful source of carbon with the benefits of being cost effective, easily accessible and its purity (Reddy and Singh, 2002). Due to the difficulty in sterilizing corn starch, it may not be a good candidate. In order to solve this problem it was proposed to hydrolyze starch using acid or enzymes. Hydrolysis using glucoamylase (5,000 AUN/mL) led to itaconic acid yields of up to 0.36 g/g starch, whereas hydrolysis with nitric acid at pH 2.0 yielded 0.35 g/g starch. When raw corn starch was utilized for itaconic acid production, the production medium consisted of just corn starch which had been pre-treated by partial hydrolysis with either with glucoamylase or nitric acid at pH2 before autoclaving at 121°C for 20 min. Over 60 g/L of itaconic acid was obtained from *A. terreus* TN-484 in a 2.5-L air-lift bioreactor using 140 g/L of corn starch without any nitrogen source (Yahiro *et al*., 1995). Itaconic acid obtained from the corn starch consumed was over 50% and looked similar to the produce from crystalline glucose. According to Dwiarti *et al*. (2006), the medium containing nitric acid for both hydrolysis and itaconic acid production from sago starch was optimized and 48.2 g/L of itaconic acid was produced with a yield of 0.34 g/g sago starch. Market refused, banana and apple were also used as substrates for itaconic acid production and itaconic acid yields of 28.5 and 31.0 g/L were obtained using acid- and α-amylase-hydrolyzed corn starch (Okabe *et al*., 2009). In 2007 Jaheer Hussain *et al*. reported that Jatropha seed cake is one of the best carbon sources among various carbohydrates for itaconic acid production. The rapid spectroscopic method was used to measure itaconic acid concentration and Jatropha seed cake leads to maximum yeild of 24.45 g/L after 120 h. Also submerged substrate fermentation of Jatropha seed cake, a by-product of oil extraction from Jatropha curcas seed using *A. terreus* was done in order to produce itaconic acid (Amina *et al*., 2013). By dilute acid hydrolysis using 50% sulphuric acid, the Jatropha seed cake was initially converted into fermentable sugars. Maximum yield of itaconic acid was 48.70 g/L at 5 mL of inoculum size, 50% substrate concentration and pH 1.5. There was an increase in the residual glucose concentration for the first 48 h of fermentation, then it decreased while itaconic acid concentration increased.

Decreases in production cost can be preserved by using low-cost carbon sources. Lignocellulosic residues as a renewable carbohydrate source could be a raw material for biotechnological processes, considering their widespread distribution, low price and abundance. Production of industrially important products, xylanase and itaconic acid by *A. terreus* NRRL 1960 from agricultural residues was investigated by Aytac *et al*. (2014). Sunflower stalk, cotton stalk and corn cob were used as carbon sources as lignocellulosic material. Among them, maximum xylanase production was obtained on corn cob. About 70 IU/mL xylanase and 18 g/L itaconic acid production levels were achieved by application of two-step fermentation.

CONCLUSION

Commercially, itaconic acid is produced from *Aspergillus terreus* using glucose or molasses as carbon sources, which contribute to high production cost. Decreases in production cost could be achieved by using low-cost carbon sources such as Lignocellulosic raw material and production of another industrially important product. Integrated generation of multiple products and stepwise usage of microorganism has good potential. Strain improvement by genetic engineering and optimization technique resulted an increased itaconic acid concentration.

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