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A Group Contribution Analogy as Selection Criteria for Nutrients Fermentation Media in the Production of Tacrolimus, Sirolimus and Ascomycin

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Abstract: Tacrolimus, sirolimus, and ascomycin are macrolides related to a decrease in the occurrence and severity of refractory rejection episodes and other diseases, such as the skin and eyes.The determination of optimum initial sources of carbon and nitrogen in the medium is an essential step in optimizing the fermentation process to obtain these drugs. The current research proposes an innovative technique as selection criteria to culture media carbon and nitrogen sources.The concept is an analogy to group contribution from thermodynamics to identify molecular fragments. Tacrolimus, sirolimus and ascomycin molecular structures and fermentation results of these macrolides were analyzed. This approach can enhance the productivity of these important immunosuppressants.

Keywords: Macrolides, Molecular fragments, Fermentation.

1. Introduction

In Brazil, since the promulgation of the 1988 Federal Constitution, the right to health is universal, including comprehensive therapeutic and pharmaceutical assistance. Brazil has the largest public transplantation system in the world, with the Unified Health System (SUS) responsible for financing 96 % of all procedures related to the transplantation process [1]. However, in view of the covid-19 pandemic, there is a totally new situation in people's lives and a profound impact on the current generation. Mankind has a historic moment of extreme exceptionality, whose greatest recommendation in isolation, understanding that this isolation is not a personal choice, but a social necessity. Ordinary situations and demands could be assessed, circumvented, digested in such a way as to proceed with daily activities. It is not the case, because one lives in a universe that approaches science fiction, apocalyptic, but deeply real. Prudence and common sense are needed, with science and technology as determining allies, especially for the population considered at risk, such as those who needs transplants and those who have already had the transplant. This reflection clearly points to important social issue to be resolved, which necessarily involves investments in research and development and innovation in the production of immunosuppressants tacrolimus, sirolimus and ascomycin.Immunosuppressants stop your immune system from damaging healthy cells and tissues. People with organ transplants and stem cell transplants take these medicines to prevent transplant rejections. The drugs also treat autoimmune disease symptoms. Immunosuppressants are powerful drugs that require careful monitoring to avoid problems [2].

Tacrolimus, known as FK506 and fujimycin, is a macrolide lactone with molar mass 804.018 g/mol and empirical formula $C_{44}H_{69}NO_{12}$, can be obtained via fermentation by several species of *Streptomyces* genus, usually *Streptomyces tsukubaensis* [3]. Tacrolimus was discovered in 1987; it was among the first macrolide immunosuppressants discovered, preceded by the discovery of sirolimus (rapamycin) on Rapa Nui (Easter Island) in 1975. Tacrolimus is produced by a soil bacterium, *Streptomyces tsukubensis*.Tacrolimus is recommended as immunosuppressive drug for therapy of kidney and liver transplantation treatment[4]. In addition, tacrolimus (Fig. 1), is recommended for the treatment of autoimmune diseases, rheumatoid arthritis, and lichen planus [5], as well as in bronchial asthma treatments, dermatological disorders as vitiligo, psoriasis, atopic dermatitis [6], eye diseases like uveitis [7], and retinoblastomas [8].

Sirolimus (Fig. 2), empirical formula $C_{51}H_{79}NO_{13,}$ also known as rapamycin with molar mass 914.17 g/mol and sold under the brand name Rapamune is a macrolide compound that is used to coat coronary stents, prevent organ transplant rejection, treat a rare lung disease called lymphangioleiomyomatosis [9], and treat perivascular epithelioid cell tumor (PEComa)[10]. It has immunosuppressant functions in humans and is especially useful in preventing the rejection of kidney transplants [11].Sirolimus was initially developed as an antifungal agent [12]. However, this use was abandoned when it was discovered to have potent immunosuppressive and antiproliferative properties due to its ability to inhibit mTOR [13]. Sirolimus also acts in the treatment of dermatologic disease [14], diabetes [15] and, H1N1 [16] and H5N6 virus [17 and limitations of mTOR inhibitors in the treatment of cancer [18].

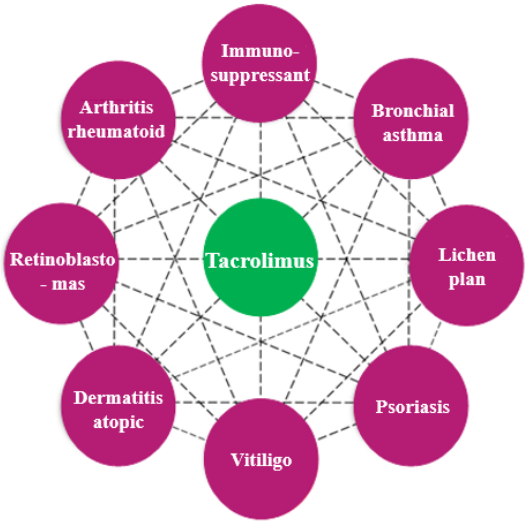


Figure.1. Tacrolimus ($C_{44}H_{69}NO_{12}$)applications.

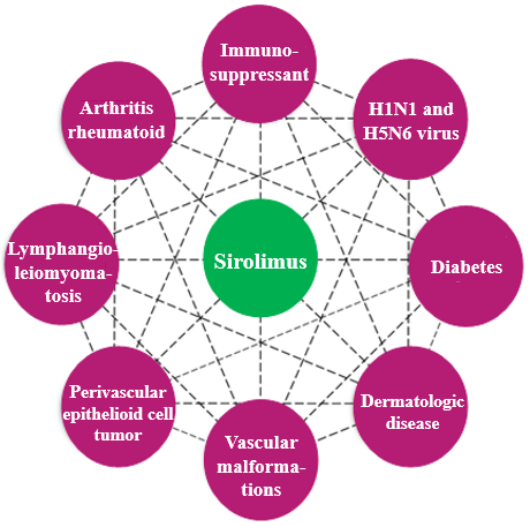


Figure. 2. Sirolimus ($C_{51}H_{79}NO_{13}$) applications.

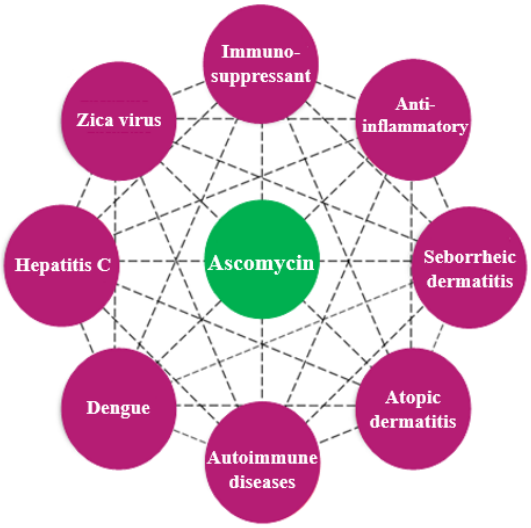


Figure. 3. Ascomycin($C_{43}H_{69}NO_{12}$) applications.

Ascomycin, empirical formula $C_{43}H_{69}NO_{12}$, also called Immunomycin, FR-900520, FK520, is an ethyl analog of tacrolimus (FK506) with strong immunosuppressant properties. Ascomycin (FK520),(Fig. 3),was initially referred to as FR-900520 and isolated from *S. hygroscopicus* KK317 in 1962 [19]. It was due to the high effectiveness of an orally used cyclosporin A that is a cyclic peptide accompanying 11 amino acids, which led to the discovery of this novel class of compounds. It has been researched for the treatment of autoimmune diseases and skin diseases, and to prevent rejection after an organ transplant [20].Additionally, ascomycin preferentially inhibits the activation of mast cells, an important cellular component of the atopic response. Ascomycin produces a more selective immunomodulatory effect in that it inhibits the elicitation phase of allergic contact atopic dermatitis and seborrheic [21] but does not impair the primary immune response when administered systemically. Ascomycin against Zika, hepatitis C and, dengue virus [22].

The improving FK-506 production can be done by mutants of *S. tsukubaensis*[23] or from different nutrient media for ordinary *S. tsukubaensis* bacteria [24,25,26,27,28]. In thesecond case, these authors use distinct carbon and nitrogen sources. Analysingthe metabolic pathways of tacrolimus production it is possible to observe some precursor, such as methylmalonyl-CoA, malonyl-CoA, methoxymalonyl-CoA, pipecolate among others [29]. Some authors apply precursor in the media nutrients to improve the tacrolimus production [30,31]. It is important to point that the structures of these precursors are present in tacrolimus structure or, in other words, the tacrolimus structure presents fragments of this precursors or classical structures, such as amino acids (L-lysine, L-proline for example).

The sirolimus biosynthesis genes from *S. hygroscopicus* have been identified by hybridization with DNA from the PKS genes for erythromycin biosynthesis [32], whereas most of the nutritional control and regulatory mechanism for sirolimus biosynthesis remain unknown. Metabolic engineering for the improvement of sirolimus production has not been achieved, yet most efforts have mainly focused on the production of sirolimus analogues [33], and strain mutagenesis [34]. Although some different processes for producing sirolimus have been disclosed in various scale, the production and productivity of sirolimus on the industrial scale is still low. There still faced the challenge for developing an efficient process for sirolimus production. Macrocycle biosynthesis of sirolimus is completed by a series of condensations from acetate and propionate building blocks via a common polyketide pathway, and incorporation of pipecolic acid (a lysine derived amino acid) unit to form the 31-membered macrolide [35]. Based on this biosynthesis logic, precursor amino acids and nutrient regulation can play an important role for improving the biosynthesisof sirolimus. However, fewer attempts have been made so far to understand the important of amino acids and nutrient components to regulate the sirolimus biosynthesis.

A semisynthetic derivative of ascomycin called pimecrolimus has been used as the first-line treatment for mild-to-moderate atopic dermatitis and plays an important role in the market of immunosuppressive drugs [36]. Due to its complex macrolide structure, ascomycin is difficult to synthesize by chemical methods, and thus is mainly produced by microbiological fermentation [37]. However, the yield of ascomycin produced via microbiological fermentation is still low and the production costs are high. Recently, various efforts have been made to improve ascomycin yield through genetic manipulation. For example, the overexpression of some key genes involved in ascomycin biosynthesis, such as hcd, ccr, fkbR1, and fkbE, led to a marked increase in ascomycin yield [38]. In addition, an engineered *S. hygroscopicus* strain with increased chorismatase (FkbO) activity and inactivated pyruvate carboxylase (Pyc), named TD-ΔPyc-FkbO, showed the highest reported ascomycin yield to date, 610.0 mg/L [39]. Nonetheless, this yield is considered low, as it is not high enough to meet the demands, and the lack of complete genomic information for *S. hygroscopicus* limits further modifications of the strain by genetic manipulation [40]. It is necessary to optimize the composition of the culture medium to further enhance ascomycin production.

The tacrolimus, sirolimus and ascomycin structurespresents other fragments as fatty acids, that occur in classical carbon sources for its productions. As can be seen, it is possible to identify molecular fragments in macrolides in analogy of group contribution methods from thermodynamics, in which the molecular fragmentation is used to estimate

many physicochemical properties of pure compounds and mixtures. The crucial advantage of these methods is they need knowledge only of the chemical structure of the compounds without any other input information [41]. Then, these methods could be extended for identification of fragments of complex molecule, whose objective is to improve its production.

2. Methodology

The metabolic route suggests precursors that are used as strategies to assess productivity [30,31, 42]. In this context, the optimization of fermentation media using response surface methodology is common [30, 43]. The concept used in this work is based on the group contribution to identify molecular fragments to increase the studied macrolides productivity. It is an analogy to the classical group contribution used in the estimation of many physicochemical properties of pure compounds and mixtures, in which the fragmentation scheme is relevant to the calculation property. The macrolides molecules were fragmented, and the groups are the precursors in the fermentation media. In order to investigate this application, studies were evaluated to obtain tacrolimus, sirolimus and ascomycin from fermentation of *Streptomyces*. These studies are reported in Table 1, Table 2, and Table 3, respectively. The selected studies considered similar operational conditions about temperature, pH, time, and rotation, the values of these variables were, respectively, 27-30 °C, 6.7-7.0, 5-7 days, and 110-240 rpm. From these previous studies it is possible to relate the structure-dependence in media culture sources to macrolides productivity.

Table 1

Medium fermentation to produce tacrolimus.

Authors	Strain	Carbon source (including amino acids and proteins)	Nitrogen source	Tacrolimus production (mg/L)
[24]	*Streptomyces tsukubaensis*	Glycerol, corn starch, glucose, corn steep liquor, soluble starch, yeast extract	Corn steep liquor	13.6
[44]	*Streptomyces tsukubaensis*	Sacarose, glucose, cottonseed meal, corn steep liquor, glycerine, soluble starch	Dryed yeast	14-23
[31]	*Streptomyces tsukubaensis*	Glucose, maltose, malt extract, yeast extract, corn steep liquor, soy peptone, picolinic acid	Corn steep liquor, soy peptone	32.5
[45]	*Streptomyces tsukubaensis*	Malt extract corn steep liquor, soy peptone, picolinic acid, Brazil nut oil	Corn steep liquor, soy peptone	47.4
[27]	*Streptomyces tsukubaensis*	Glucose, maltose, malt extract, yeast extract, corn steep liquor, soy peptone, Brazil nut oil	-	41.7
[30]	*Streptomyces sp.*	Soy oil, soybean meal, L-lysine	$(NH_4)_2SO_4$	135.6
[46]	*Streptomyces sp. (MA 6858) ATCC n. 55098*	Glucose, dextrose, asparagine, soluble starch	Dried yeast, corn steep liquor, asparagine	10 - 37.8
[47]	*Streptomyces sp. (Strains PSCS) FERM B027; MA 6858, ATCC n. 55098; Mutant P5C*	East extract, malt extract, glucose, glycerin, cottonseed oil, ground oil, soy oil, sunflower oil	Cotton seed meal, corn steep liquor, dried yeast, feather meal, peanut powder, $(NH_4)_2SO_4$	150 - 250
[25]	*Streptomyces tsukubaensis* ZJU01	Glycerol, soybean meal, soluble starch, glucose, soybean oil, L-lysine	$(NH_4)_2SO_4$	46.9
[42]	*Streptomyces tsukubaensis* D852	Yeast extract, soybean meal, soy peptone	Soypeptone	177.8
[48]	*Streptomyces tsukubaensis*	Glucose, yeast extract, malt extract, maltose, glucose, coconut oil	Soypeptone	-

Table 2
Medium fermentation to produce sirolimus.

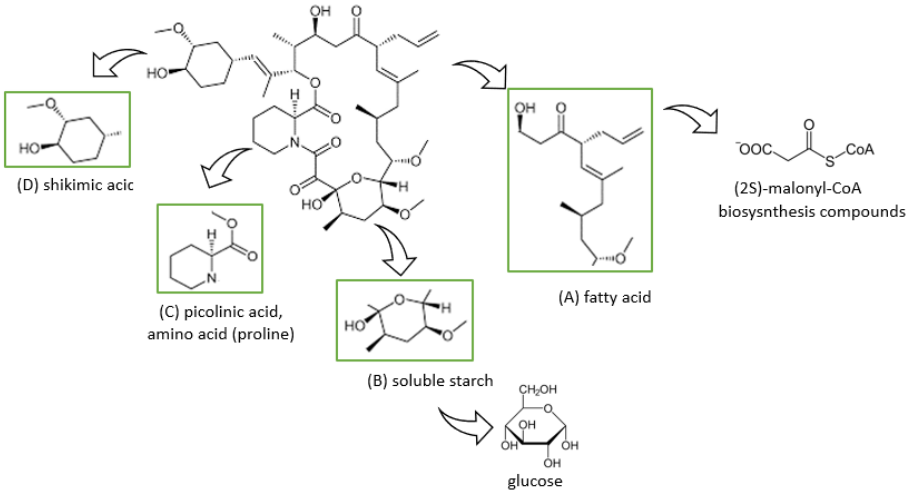
Authors	Strain	Carbon source	Nitrogen source	Sirolimus production (mg/L)
[49]	*Streptomyces hygroscopicus* strain C9	D-fructose, mannose	$(NH_4)_6MO_7O_{24}.4H_20$, L-arginine, L-histidine, L-aspartate	134
[50]	*Streptomyces hygroscopicus* ATCC 29253	Fructose, mannose	L-arginine, L-histidine, L-aspartate	357
[51]	*Streptomyces hygroscopicus* FC904	Glycerol, glucose, sucrose, glycine, soybean, oatmeal, dry yeast	Peptone, Polypeptone, dry yeast, L-lysine, peptone, polypeptone	139

Table 3
Medium fermentation to produce ascomycin.

Authors	Strain	Carbon source	Nitrogen source	Ascomycin production (mg/L)
[52]	*Streptomyces hygroscopicus* KK 317	Starch	Soybeanflour	100
[53]	*Streptomyces hygroscopicus*sp. ATCC 53771	Glucose, malt extract, soluble starch, yeast extract	Yeast extract, N-Z-amine type A	35% higher than previously published
[54]	*S. hygroscopicus* var. *ascomyceticus FS35*	Soluble starch, dextrin, corn steep liquor, soybean oil, yeast powder	$(NH_4)SO_4$, yeast powder, peptone, corn steep liquor, L-arginine	626.30

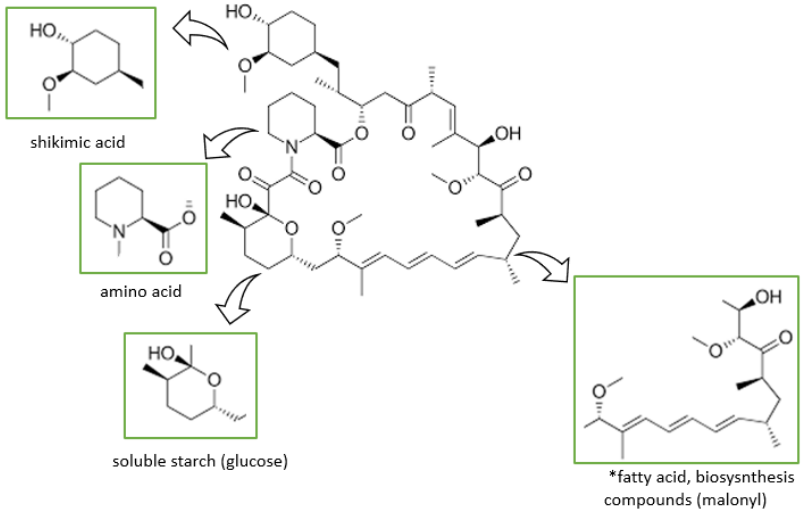
3. Results

Analyzing Figures 4, 5 and 6 it is possible to observe fragments analogous to different precursors, containing nitrogen and carbon sources, are there proteins and amino acids, fatty acids, picolinic acid, shikimic acid, soluble starch, and others. Additionally, can be seen fragments analogous to methylmalonyl-CoA, malonyl-CoA, methoxymalonyl-CoA from metabolic pathways of these macrolides production.



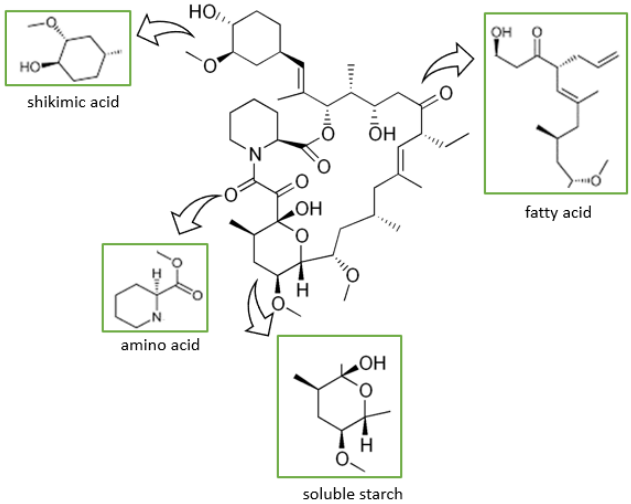
*This fragment also is analogous a biosynthesis compound from metabolic pathways.

Figure.4. Molecular structure of tacrolimus decomposed into fragments.



*This fragment also is analogous a biosynthesis compound from from metabolic pathways.

Figure. 5. Molecular structure of sirolimus decomposed into fragments.



*This fragment also is analogous a biosynthesis compound from from metabolic pathways.

Figure. 6 Molecular structure of ascomycin decomposed into fragments.

It is important to point that the identified precursors are present in tacrolimus, sirolimus and ascomycin structures. Some fragments show more similarities than others, as can be seen in Figure 5, in which the fatty acid fragment is analogous to linoleic acid in terms of unsaturated bonds.By inspection of Tables 1, 2 and 3, and similarly to the first level of contribution founds in the thermodynamic approach, the fragments were identified.

In tacrolimus molecular structure (Fig. 4):

Fragment (A) (Fig. 4) correspond to linoleic acid, the major fatty acid present in soybean oil, sunflower oil, cotton seed oil, and Brazil nut oil added in the composition of the media studied [25, 26, 27, 30, 45, 47].

Fragment (B) (Fig. 4) corresponds to starch added directly as glucose cited by [48], fructose, maltose, sucrose or other carbohydrates and cereal alcohols.

Fragment (C) (Fig. 4) corresponds to the structure of amino acids, such as L-Lysine, and picolinic acid. The soy peptone is also present in the composition of the most productive media [25, 26, 27, 31, 42, 45]. [44] also presents the soy peptone, however, unlike the other studies it does not insert vegetable oil in the composition of the medium. The picolinic acid is present in the media studied by [31] and [45].

Fragment (D) (Fig. 4) corresponds to the structure shikimic acid, although not identified neither mentioned directly in the composition of the medium, that may have contributed to the increase in productivity. This fragment appears in the molecular structures of the three macrolides studied.

Analyzing Table 1,it is verified that with more complex medium compositions, such as the addition of picolinic acid or Brazil nut oil, productivity becomes more expressive. In this respect, the medium studied by [31] and [45] are similar. However, the addition of linoleic acid through Brazil nut oil may explain the higher productivity in [27] and [45] studies, 47.4 mg/L and 41.7 mg/L, respectively. Mutant strain of *Streptomyces tsukubaensis* were used (Table 1). Higher yields are associated with compositions in which vegetable oils rich in linoleic acid, L-lysine and soy peptone have been added. The study by [42] showed higher productivity, 177.8 mg/L. It´s important to signalize that the interaction between the compounds L-lysine from the soybean meal combined with soy peptone can be associate to a second level of contribution.

Adding precursors to the culture medium is an option to increase yield. The media composition is essential to define the productive process for the three macrolides studied. Enrichment of the fermentation medium with possible tacrolimus precursors as picolinic acid and pipecolic acid, or growth promoters as nicotinic acid and nicotinamide, also increased the tacrolimus production in *Streptomyces tsukubaensis* by three to seven times [31]. Pipecolic and picolinic acids, which are direct tacrolimus precursors, were the most effective promoters of tacrolimus production described by [31].

Proline, leucine, threonine, and valine have been shown to be target amino acids for improving tacrolimus production. They significantly stimulate the yield of FK506 when added at 72 h of fermentation because they produce a significant increase in the FK506 precursors (acetyl-CoA and methylmalonyl-CoA) [55]. L-lysine is a precursor to L-pipecolic acid, which closes the macrolide ring of tacrolimus.

Similar procedure fragmentation was applied to sirolimus andascomycin structures, Figures 5 and 6, respectively. Tables 2 and 3 were used to support the fragmentation scheme. The higher productivity to sirolimuswas related to [50], in which the fermentation medium is enriched with amino acids and soluble starch. Ascomycin presented the higher productivity to [54], a complete fermentation medium enriched with soluble starch, soybean oil and amino acids.

In sirolimus molecular structure several competitive incorporation studies using precursors demonstrated that the heterocyclic ring originates from pipecolic acid, which is formed from lysine [56]. Rapamycin is a macrolide containing nitrogen, and the immediate precursor of the nitrogen-containing ring is pipecolic acid [56]. [57] in a preliminary study of nitrogen sources for the growth of *Streptomyces hygroscopicus*, found that the combination of the amino acids aspartate, arginine, and histidine are an effective mixture. The production of rapamycin is stimulated by L-lysine and decreased by L-phenylalanine and L-methionine.

Xu et al. (2011) increased the quantity of malonyl-CoA, a key precursor to the synthesis of rapamycin. Among the primary metabolic targets, ppC and accA are responsible for the synthesis of precursors of rapamycin methyl malonyl-CoA and malonyl-CoA, respectively.

Biosynthetic pathways may also support ascomicyn fragmentation. Ascomycin production, strain *Streptomyces hygroscopicus* var. *ascomyceticus*is studied well and previous scientific reports concluded that wild strain produced ascomycin in a very little amount; therefore, various yield improvement techniques are applied [19].

A study conducted by [59] where three pathways (aminoacyl-tRNA biosynthesis; phenylalanine, tyrosine, and tryptophan biosynthesis; and pentose phosphate pathways) were studied, from which aromatic amino acid and pentose phosphate biosynthesis pathway were seen to be responsible for the synthesis of precursor molecule involved in ascomycin biosynthesis.

The selection of shikimic acid resistant strain *Streptomyces hygroscopicus* var. *ascomyceticus* and the addition of 3 g/L shikimic acid at 24 h increased the production of FK520 to 450 mg/L, which was 53.3% higher than in the initial strain FS35 [60]. In *Streptomyces hygroscopicus* var. *ascomyceticus*, FK520 is assembled from 12 precursor molecules [61], with the majority being malonyl-CoA (2 molecules) and methylmalonylCoA (5 molecules). Thus, the biosynthesis of malonyl-CoA and methylmalonyl-CoA is crucial to produce FK520.

Based on these results, it is possible to affirm that the knowledge of the compound molecular structure and the decomposition into fragments can support the selection of more efficient carbon and nitrogen sources.

4. Conclusions

The year 2020 enters to history of humanity, exposing its fragility due to a virus, affecting people that remain invisible to public policies. In this case, it is essential to look for cientific and technological solutions to overcome the difficulty of those who depend on medicines, such as tacrolimus. Once this immunosuppressant is obtained by fermentation, it is essential to define the nutrient medium for the action of the microorganism, which, in the present study, refers to *Streptomyces* sp. The medium basic is defined by presence of carbon and nitrogen source. The strategy proposal in this paper is to analyze the tacrolimus, sirolimus and ascomycin molecular architecture and to find central fragments that contains carbon and nitrogen source, considering the analogy with thermodynamics group contribution approach. The knowledge of molecular architecture can be the auxiliar key to optimizing productivity by fragmentation of tacrolimus, sirolimus and ascomycin structure. This approach can enhance productivity of this important immunosuppressants and can be extended to other compounds produced from fermentative processes.

Acknowledgments

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