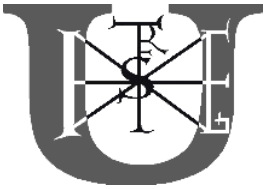

SZENT ISTVÁN UNIVERSITY

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The thesis of the dissertation

Attila Kónya

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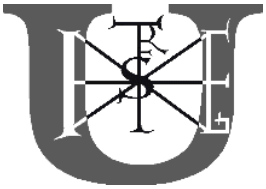

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BACKGROUNDS, AIMS

The intensive research of the antibiotics of microbial origin began after the discovery of the penicillin. Since then it was proved that microorganisms are a rich source of valuable, biologically active compounds. The isolation of mycophenolic acid from the fermentation broth of a *Penicillium brevicompactum* strain was an early result of the investigation of the antibiotics. Mycophenolic acid has a wide range of biological activity. Its antibacterial, antifungal, antiviral, antitumour and immunosuppressive effect has been described. The origin of the biological effects is the inhibition of the enzyme inosine monophosphate dehydrogenase by mycophenolic acid. Inhibition of the enzyme decreases the biosynthesis of guanosine and, therefore, reduces the rate of cell proliferation. Mycophenolic acid is used in human therapy as an immunosuppressive drug to prevent the rejection of different organs after transplantation.

The first successful kidney transplantation was carried out in 1954. By now the kidney, liver, heart, pancreas and lung transplantations became the accepted part of the therapy. The discovery of the immunosuppressive drugs improved the efficiency of the transplantation procedures. Because the number of transplantations is continuously increasing, it is important to investigate such compounds to increase their positive effects and decrease the side effects.

In our Institute a screening program has been carried out to isolate microorganisms able to biosynthesize compounds with different biological effects. In this program a fungal strain was isolated from a soil sample collected in India. The culture of this strain contained mycophenolic acid. We aimed to develop a process for the industrial preparation of mycophenolic acid using the isolated fungal strain. The aims of the research program were as follows:

1. Development of a microbial process for the preparation of mycophenolic acid
    - Improvement of the mycophenolic acid biosynthetic capacity of the isolated fungal strain with mutation selection methods
    - Optimisation of the composition of the fermentation medium
    - Optimisation of the fermentation parameters under shaken flask circumstances
    - Optimisation of the fermentation parameters in laboratory fermentors

2. Preparation of microbiologically produced mycophenolic acid derivatives
    - Performance of a screening program to isolate microorganisms with mycophenolic acid bioconversion ability
    - Isolation of the mycophenolic acid derivatives from the fermentation broth and determination their structure
    - Investigation of the biological activity of the isolated mycophenolic acid derivatives

3. Investigation of the electrophoretic karyotype of the strains
    - Determination of the electrophoretic karyotype of the mycophenolic acid producing fungal strain using pulsed field gel electrophoresis.
    - Comparison of the electrophoretic karyotypes of the wild type strain isolated from soil, and different mutant strains isolated in the strain improvement process
    - Comparison of the electrophoretic karyotype of our strain with that of the mycophenolic acid producer *Penicillium brevicompactum ATCC 9056* strain

MATERIALS AND METHODS

Microbiological methods

Maintenance, inoculation and cultivation of the microorganism strains were carried out with conventional techniques.

Optimisation of the fermentation parameters

The optimisation of the fermentation parameters was carried out under shaken flask conditions in 100 ml volume and in 5-litres volume laboratory fermentors. We used regression analysis for the optimisation of the fermentation medium.

Karyotype analysis

Protoplasts were formed from young mycelia of the strains and used for the preparation of chromosome samples. Chromosomes were separated by pulsed field gel electrophoresis.

RESULTS

Isolation of a fungal strain with mycophenolic acid producing ability

A screening program has been carried out to isolate microorganisms able to biosynthesize compounds with different biological effects. Mycophenolic acid was isolated from a fermentation broth with an antifungal effect. It was produced by a fungal strain isolated from a soil sample collected in India. The strain was identified as an isolate of *Penicillium waksmani* belonging to the *Monoverticillata* section. All species described in the literature as mycophenolic acid producers belong to the *Asymmetrica* section. The species, isolated by us, is the first species of the *Monoverticillata* section with mycophenolic acid biosynthesising capacity.

Development of a microbial process for the preparation of mycophenolic acid

We developed a fermentation process for the industrial production of mycophenolic acid. The mycophenolic acid producing capability of the strain was improved by genetic modification and by the optimisation of the fermentation parameters. After mutagenic treatments the biosynthetic capacity of the mutant strains was studied under shaken flask conditions. We had developed a fast and cost-effective pre-screening method in microtiter plates to select the strains with improved mycophenolic acid production. In the strain improvement process more than 7000 strains were examined. In addition to isolating strains with better mycophenolic acid producing capabilities we also isolated strains with mutations blocking the genes responsible for mycophenolic acid biosynthesis. One of the latter strains No. 9/14 was a mutant at one of the steps prior to closing the aromatic ring of mycophenolic acid. No. SP1, another strain

of this type had mutation at a step after the incorporation of the farnesyl side chain in mycophenolic acid. Because of the mutation the intermediates resulting from the shortening of the farnesyl side chain could be isolated from the fermentation broth.

To improve mycophenolic acid production fermentation parameters were also optimized. After selection of the most advantageous carbon and nitrogen sources in the fermentation broth, their concentrations were also optimized using regression analysis and the method described by Box and Wilson. For the calculations we wrote a computer program in Pascal and Delphi programming language.

After the shaken flask experiments, investigations were continued in laboratory fermentors. Having determined the optimum fermentation parameters we developed a fermentation process for the 5 litre scale preparation of mycophenolic acid under agitated-aerated conditions.

After strain improvement and the optimisation of fermentation parameters our fermentation method was able to produce around 2000 mg/l concentrations of mycophenolic acid in 5 litre volume laboratory fermentors.

Preparation of mycophenolic acid derivatives

In some cases, the effectiveness of biologically active compounds can be increased and the side effects decreased by preparing derivatives from the original compound. These derivatives can be prepared by synthetic or microbiological methods. We used some strains of the *Streptomyces* genus to convert mycophenolic acid. We prepared from mycophenolic acid as substrate hydroxy-mycophenolamide, mycophenolamide, hydroxy-mycophenolic acid and the rhamnosyl derivative of the hydroxy-mycophenolic acid. In pharmacological tests, the effectiveness of the amide and the rhamnosyl derivatives was similar to that of mycophenolic acid. The hydroxymethyl derivatives lost their activity.

Electrophoretic karyotype of the strains

Using pulsed field gel electrophoresis, we investigated the electrophoretic karyotype of the mycophenolic acid producer *Penicillium waksmani* strain isolated from a soil sample. We separated 4 chromosomes of the strain. The sizes of the chromosomes were 3, 4.7, 5.8 and 10.5 Mb, the size of the genome was 24 Mb as calculated from the sizes of the chromosomes. The strain seems to have two chromosomes of 3 Mb size, since this band was more intense in the gel than the other bands, and therefore the size of the genome might be 27 Mb. We compared the electrophoretic karyotypes of the wild type strain isolated from soil, and different mutant strains isolated in the strain improvement process. We examined mutant strains of higher mycophenolic acid producing capacity and mutant strains unable to biosynthesize mycophenolic acid. No differences were found in the electrophoretic karyotypes of the different strains. The differences between the strains studied were not detectable on the level of chromosomes.

We compared the electrophoretic karyotype of our strain with that of the *Penicillium brevicompactum ATCC 9056* strain, on which data are available in the literature. We separated two chromosomes of the *P. brevicompactum ATCC 9056* strain in the range between 3 and 10 Mb. One of the chromosomes was of 4.1 Mb, the other one was of 5.7 Mb. The karyotypes of the two investigated strains differed in the number and in the size of the chromosomes. This finding confirmed the results of the taxonomic investigation according to which the strain isolated by us is different from the *P. brevicompactum ATCC 9056* strain.

New results

1. In a screening program we isolated a strain of *Penicillium waksmani*, which biosynthesized 100 mg/l mycophenolic acid. This is the first report on the occurrence of a strain, that belong to the *Monoverticillata* section and capable of synthesizing mycophenolic acid.
2. We performed a strain improvement and optimized the fermentation parameters. As a result of this process 2000 mg/l mycophenolic acid could be prepared in laboratory fermentors.

3. We wrote a computer program for the optimisation of the fermentation parameters using regression analysis
4. In addition to isolating strains with better mycophenolic acid producing capabilities we also isolated strains in which genes responsible for mycophenolic acid biosynthesis was blocked.
5. We prepared four microbiologically produced derivatives of mycophenolic acid used *Streptomyces* strains. In pharmacological tests, the effectiveness of the amide and the rhamnosyl amid derivatives was similar to that of mycophenolic acid. However the hydroxymethyl derivatives lost their activity. The pharmacological investigations confirmed that the methyl group on the aromatic moiety of the mycophenolic acid is essential to the activity, its hydroxylation lead to the loss of the activity.
6. Using pulsed field gel electrophoresis, we investigated the electrophoretic karyotype of the mycophenolic acid producer *Penicillium waksmani* strain. Four chromosomes of this strain could be separated in this experiment. The sizes of the chromosomes were 3, 4.7, 5.8 and 10.5 Mb, the size of the genome was 24-27 Mb.
7. We compared the electrophoretic karyotype of our strain with that of the mycophenolic acid producer *Penicillium brevicompactum ATCC 9056* strain. Two chromosomes of *P. brevicompactum* strain *ATCC 9056* could be resolved in the range between 3 and 10 Mb. One of the chromosomes was of 4.1 Mb, the other 5.7 Mb in size. This finding confirmed the results of the traditional taxonomic investigations concluding, that the strain isolated by us is different from the *P. brevicompactum ATCC 9056* strain.

CONCLUSIONS AND SUGGESTIONS

A great number of drugs with different type of activity have been prepared microbiologically during the last few decades. The publications related to newly isolated compounds of microbiological origin prove that the importance of the drugs of

microbiological origin will not decrease. Although most drugs of microbiological origin are used as antibiotics, some natural compounds are also used in the therapy of other diseases. One of the latest therapeutic areas is the immunsuppression used after transplantation to prevent the rejection of the transplanted organs. Due to the increasing number of transplantations, it is important to investigate immunosuppressive compounds. One of the immunosuppressive drugs, applied more recently in the therapy is the morpholinoethyl ester derivative of mycophenolic acid, which is synthesized by microorganisms.

In a screening program we isolated a fungal strain, which is able to synthesize mycophenolic acid. One aim of our work was the development of a microbial process for the preparation of mycophenolic acid using our strain. According to taxonomical investigations the fungal strain was identified as an isolate of the *Penicillium waksmani* species, belonging to the *Monoverticillata* section. All species described in the literature as mycophenolic acid producer belong to the *Asymmetrica* section. Studies on our strain proved that the mycophenolic acid producing capability is not limited to the species belonging to the *Asymmetrica* section.

During the development of the industrial process the strain's biosynthetic ability to produce mycophenolic acid was improved by genetic modification and by the optimisation of the fermentation parameters. After strain improvement and the optimisation of fermentation parameters we could produce around 2000 mg/l concentrations of mycophenolic acid in 5 litre useful volume laboratory fermentors. The fermentation method is suitable for the scale up experiments. In addition to isolating strains with better mycophenolic acid producing capabilities in the strain improvement process we also isolated strains in which genes responsible for mycophenolic acid biosynthesis were blocked. One of them was a mutant at one of the steps prior to closing the aromatic ring of mycophenolic acid. Another strain of this type had mutation at a step after the incorporation of the farnesyl side chain in mycophenolic acid.

In some cases, the effectiveness of biologically active compound can be increased and the side effects decreased by preparing derivatives from the original compound. We investigated the microbial bioconversion of mycophenolic acid. Using species of the Streptomyces genus we prepared the amide, the hydroxymethyl, the

hydroxymethyl amide and the rhamnosyl amide derivative of mycophenolic acid. In pharmacological tests, the effectiveness of the amide and the rhamnosyl amide derivatives was similar to that of mycophenolic acid. However the hydroxymethyl derivatives lost their activity. These results confirmed earlier observations stating that the methyl group located on the aromatic moiety of the mycophenolic acid is essential for its activity.

Using pulsed field gel electrophoresis we determined the electrophoretic karyotype of the mycophenolic acid producer *Penicillium waksmani* strain, isolated by us. Four chromosomes of the strain were separated. The sizes of the chromosomes were 3, 4.7, 5.8 and 10.5 Mb. The strain may possibly have two co-migrating chromosomes of 3 Mb size, since this band was more intense in the gel than the other bands, and therefore the size of the genome might be 24-27 Mb. It is also possible that the strain isolated by us may have another, unresolved chromosome, because the size of the genome of the *Penicillium* species investigated, was shown, in the literature, to be larger than 30 Mb. We compared the electrophoretic karyotype of our strain with that of the *Penicillium brevicompactum ATCC 9056* strain, for which data are available in the literature. We separated two chromosomes of the *P. brevicompactum ATCC 9056* strain in the range between 3 and 10 Mb. One of the chromosomes was of 4.1 Mb, the other one was of 5.7 Mb. The karyotypes of these two strains differed greatly both in numbers and sizes of the chromosomes. This finding confirmed the results of the traditional taxonomic investigations stating that the strain isolated by us is different from the *ATCC 9056* strain *P. brevicompactum.*

PUBLICATIONS RELATED TO THE DISSERTATION

Articles

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Patents

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Jekkel, A., Barta, I., **Kónya, A.**, Sütő, J., Boros, S., Horváth, Gy. and Ambrus, G. Microbiological transformation of mycophenolic acid. 4th International Symposium on Biocatalysis and Biotransformation, Naxos, 1999.

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