

PRODUCTION, CHARACTERIZATION AND MOLECULAR DOCKING OF MYCOPHENOLIC ACID BY *BYSSOCHLAMYS NIVEA* STRAIN JSR2

RITIKA CHAUHAN, JERUSHA EMMANUEL AND JAYANTHI ABRAHAM*

Microbial Biotechnology Laboratory, School of Biosciences and Technology, VIT University, Vellore-632014, Tamil Nadu, India.
Email: jayanthi.abraham@gmail.com*

Received: 15 Feb 2014, Revised and Accepted: 02 Mar 2014

ABSTRACT

Objective: The present study was aimed to estimate the production of Mycophenolic acid by fungus *Byssoschlamys nivea* strain JSR2 in various substrates and to conduct *in silico* studies to determine the interaction of MPA with the NS5B receptor of Hepatitis C Virus.

Methods: The isolate was characterized as *Byssoschlamys nivea* strain JSR2 by 18S rRNA gene sequencing. The production of mycophenolic acid by strain JSR2 was determined on various substrates such as pineapple and apple extract and was estimated using chromatographic technique. The morphological and spore arrangement of strain JSR2 was determined by high resolution scanning electron microscope. *In silico* studies were also conducted to determine the interaction of MPA with the NS5B receptor of hepatitis C virus with ribavirin as the reference ligand.

Results: The mycophenolic acid production was found to be highest with pineapple juice substrate and was further identified by high performance liquid chromatography. The binding energy of MPA and ribavirin was found to be similar through Autodock Vina which further represents the same mechanism of action.

Conclusion: The strain JSR2 produced good amount mycophenolic acid in the presence of pineapple juice as substrate and it represents the similar mode of action against Hepatitis C virus when compare to ribavaran with reference to *in silico* studies.

Keywords: Mycophenolic acid, Biogas sludge, Molecular modeling, Ribavaran, Mycotoxins.

INTRODUCTION

Mycophenolic acid [(6-(4-hydroxy-6-methoxy-7-methyl-3-oxophthalanyl)-4-methyl-4-hexenic acid] (MPA) is a secondary metabolite isolated in 1896 by Gosio [1]. It is generated from the pro-drug Mycophenolate mofetil (MMF) on metabolism in the liver and known to have several biological properties. MPA has been well studied for its anti-neoplastic, immunosuppressive, anti-inflammatory, antiviral, anti-psoriasis and antifungal activities. In the past, it was used extensively for its antimicrobial properties [2]. Antifungal properties have also been reported with strains of *Candida albicans* displaying susceptibility to MPA in cell culture studies [3]. Several studies have indicated the antiviral activities of MPA. The replication of dengue virus in monkey kidney cells was dramatically reduced in the presence of MPA [4]. Western blot analysis revealed that MPA can reduce the gp120 levels in HIV [5].

MPA is a significant therapeutic in that it is not nephrotoxic. Graft rejection is also a much lower 17% as compared to the previous 40% seen in triple regime of cyclosporine, azathioprine and a glucocorticoid [6].

Presently, commercially available MPA is produced by chemical synthesis by an aromatic annulations strategy described by [7] Cyclobutanone and alkyl intermediates are heated with benzene to yield a penta substituted phenol compound. A nine step reaction yields MPA. However, there has been renewed interest in the biological methods of production of the same. A crystalline form of this mycotoxin has been isolated from the fermentation broths of *Penicillium brevicompactum*, *Penicillium carneum*, *Penicillium raciborskii* and *Penicillium roqueforti*. In an RFB, the mycelia is immobilized and a nearly cell free fermentation broth is obtained. Thus the operating parameters can be controlled effectively. Product recovery and purification is also facilitated by this method. A fed batch process can be used to optimize the production of MPA [8]. Thus there is a need for newer better yielding sources of MPA. *Byssoschlamys nivea* belongs to the phylum Ascomycota a member of the Trichocomaceae family. This large family of fungi is primarily saprobic in nature. Seventy five percent of all described fungi are grouped under this phylum. They are catabolically and anabolically

diverse and secrete extrolites known as mycotoxins. Several species are xerotolerant, while others exhibit thermotolerant [9]. The ascospores are present in eight celled asci which are heat resistant. The common association of *Byssoschlamys nivea* is with spoilage of apples, peaches, pears, grain and their products. The soil is the source of *Byssoschlamys nivea* spores which contact the fruit when in the field and are activated by the warmer temperatures encountered during processing [10]. Unlike other fungi, *Byssoschlamys nivea* spores are highly resistant requiring pressure of over 600 MPa and temperature above 60°C for inactivation [11]. Thus they continue to grow beyond the pasteurization stages during storage. Due to the hypoxic conditions of the cans, growth is not extensive. However, it is sufficient to cause bulging of cans and reduction of the organoleptic properties of the product. This makes the product unacceptable to the consumer leading to economic loss. Additionally, a number of secondary metabolites are produced during the process such as patulin and mycophenolic acid. *Byssoschlamys nivea* is also a common contaminant of silage and is a risk to animal health by causing a rise in infection. Thus this organism has largely been viewed as a health hazard and one that causes economic losses. However, due to the therapeutic properties role of the mycotoxins produced, the potential use of *Byssoschlamys nivea* in the production of therapeutics has been recognized.

The ability of *Byssoschlamys nivea* to produce mycotoxins MPA, Patulin and other mycotoxins has been reported [12]. In this study, we explore the production of MPA by a *Byssoschlamys nivea* with different substrates.

MATERIALS AND METHODS

Organisms

Byssoschlamys nivea JSR2 strain was isolated from sample collected from biogas sludge VIT University, Vellore, Tamil Nadu, India. The strain was revived by streaking onto Potato Dextrose Agar (PDA) plates. Incubation was carried out at 30°C for seven days.

Morphological characterization

Byssoschlamys nivea JSR2 strain was maintained on Potato Dextrose Broth (PDB) at 4 °C. The preliminary spore identification was

examined by Lactophenol cotton blue staining. The spore morphology and spore arrangement was identified by High Resolution Scanning Electron Microscopy (HR-SEM).

Taxonomic Identification of Fungal Strain

The isolation and purification of genomic DNA was carried using Chromous Genomic DNA Isolation Kit (Chromous Biotech Pvt. Ltd., Bangalore, India). The primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify 18S rRNA gene. The polymerase chain reaction mixture contained 100 ng template DNA, 400 ng each of universal primers, 2.5 mM dNTPs, and 10×Taq DNA polymerase assay buffer and Taq DNA polymerase enzyme was run on a Thermal Cycler ABI2720. The amplification reaction was followed by initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 15 min for 35 cycles. After PCR amplification, the amplified product was visualized using agarose gel electrophoresis. The product was directly sequenced with the primer using ABI 3130 Genetic Analyzer (Chromous Biotech Pvt. Ltd., Bangalore, India). The sequencing result was submitted to the GenBank National Center for Biotechnology (NCBI) database and the accession number obtained is JQ746683.

Fermentation and Extraction

A loopful culture of *Byssoschlamys nivea* JSR2 was inoculated on Potato Dextrose Agar Medium at 30°C. After 5d of incubation, spore suspension was freshly prepared with 0.05% of Tween 20. The fermentation medium of different substrates such as apple juice and pineapple juice was introduced into czapek dox medium separately. 10% of the freshly prepared spores of *Byssoschlamys nivea* JSR2 was inoculated into various fermentation mediums and were incubated for 21d at 30°C [12].

After 21d of incubation, the cell biomass was removed through centrifugation at 4,000 rpm at 4°C. 100 ml of collected supernatant was adjusted to pH 2.0 with 2 M H₂SO₄ and was extracted with 70 ml of ethyl acetate. The organic phase was separated in separating funnel and was evaporated at atmospheric temperature. The residue was dissolved in 200 µl of methanol, filtered through a 0.45-µm-pore-size and 20 µl was injected into a chromatographic column [13].

HPLC analysis

The production of mycophenolic acid by *Byssoschlamys nivea* JSR2 was analyzed using HPLC with C18 column [14]. The identification of the acid was performed with a linear elution gradient by using 33 mM acetic acid (solvent A) and acetonitrile (solvent B) according to the method described by [12]. For the identification of mycophenolic acid, the gradient program was: at time zero, 100% solvent A; linear gradient to 10% solvent B within 20 min, to 20% solvent B in 10 min, and to 90% solvent B in 4 min; a plateau for 4 min; and, finally, a decrease within 7 min to 10% solvent B.

In silico studies

The molecular docking studies were carried out using Autodock Vina. According to previous reports mycophenolic acid exhibited antiviral activity via IMPDH blockade on West Nile Virus, Dengue virus, yellow fever virus, a similar mechanism was expected in Hepatitis C Virus. However in a study conducted by [16] IMPDH was found to be independent. Thus it was contended that MPA may also follow the similar mechanism of action as Ribavirin by competing with nucleotides during RNA replication in a transient manner. It has also been reported by that a mutant strain of Hepatitis C Virus (HCV) possesses a TYR415 residue which plays a key role in the active site by narrowing it. In the present study, the 3D structure of HCV and NS5B receptor (PDB ID 1QUV) that has been determined by X-ray crystallography was used for molecular docking studies. The 415th residue was replaced by phenylalanine and modeled prior to docking. The molecular docking was performed by designing 3D structure of ligand (ISIS Draw, ChemSketch, ArgusLab), modelling of Receptor (I-TASSER), model validation by Ramachandran Plot (PDBsum), docking simulations were carried out with Ribavirin as

reference ligand (AutoDock Vina) and visualization of results (PyMol).

RESULTS AND DISCUSSION

The fungal strain *Byssoschlamys nivea* JSR2 was isolated from agricultural waste and was characterized through 18S rRNA gene sequencing. The phylogenetic tree and related accession numbers by neighbor joining method is depicted in Figure 1. According to previous reports there has been very less study conducted on production of mycophenolic acid from *Byssoschlamys nivea*. Mycophenolic acid is a well established therapeutic compound with varied applications and is presently being used as an immunosuppressive agent in the management of chronic post transplant graft rejection. In large scale production it has been manufactured by chemical process however there has been a renewed interest in carrying out the process biologically.

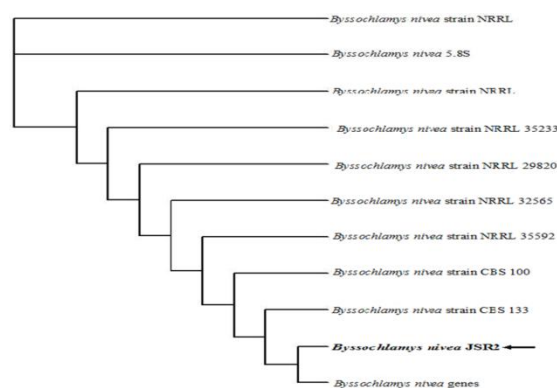


Fig.1: Phylogenetic tree representing *Byssoschlamys nivea* JSR2 strain by neighbour joining method.



Fig. 2: Microscopic view of *Byssoschlamys nivea* strain JSR2 using lactophenol cotton blue stain.

The need is thus created for better biological sources and substrates with improved yield. The strain JSR2 showed abundant growth on apple juice, pineapple juice and as well as czapex medium. The lactophenol staining of isolated strain JSR2 showed spores produced by *Byssoschlamys nivea* JSR2 as shown in Figure 2. The spore arrangement and morphology was determined by scanning electron microscope which depicts hyphae observed at a magnification of 5000X as shown in Figure 3. An ellipsoidal smooth walled spore was observed at higher magnification of 140000X. The spore structure morphology of the isolated strain corresponds to the previous reported studies done by [15] identified strains of *Byssoschlamys nivea*. The production of mycophenolic acid was analyzed using high performance liquid chromatography and was compared with mycophenolic mofetil (>98% pure, sigma Aldrich). Among three

substrates, apple juice showed commendable growth of *Byssoschlamys nivea* and production of mycophenolic acid. The peak at retention time 17.792 indicates the production of mycophenolic acid after 21d by *Byssoschlamys nivea* strain JSR using apple juice as substrate as shown in Figure 4. This correlates with the previous study conducted by [12].

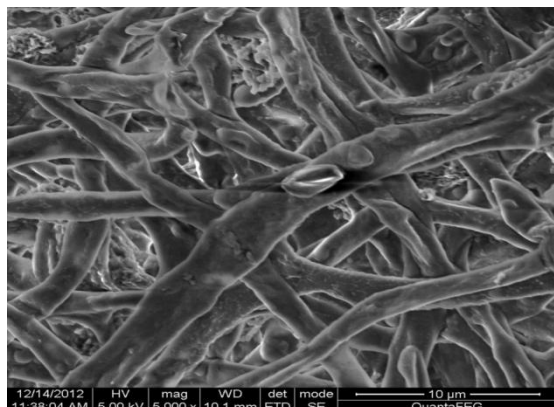


Fig. 3: Scanning electron micrograph of *Byssoschlamys nivea* strain JSR on Potato dextrose agar.

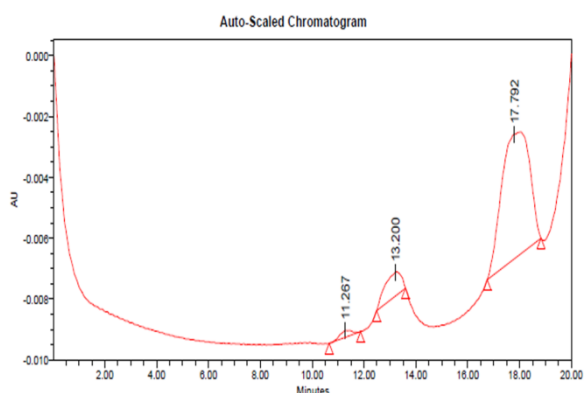


Fig. 4: HPLC chromatogram showing production of mycophenolic acid.

In silico analysis was carried out to determine the interaction of MPA with the NS5B receptor of Hepatitis C virus with Ribavirin as the reference ligand as shown in Figure 5 and 6 respectively. The sequence was modeled and validated using Ramachandran plot. Autodock Vina was used for docking simulations. The similarities in binding energies of both ligands indicate a similarity in the interactions. The key residues involved in interaction were determined to be PHE415, CYS386, TYR195,

ARG386, TYR428. I-TASSER was used to generate models of the NS5B receptor. Four models were generated, among these I-TASSER was chosen because it has a Tm score of 0.97 and the highest C score of 1.62.

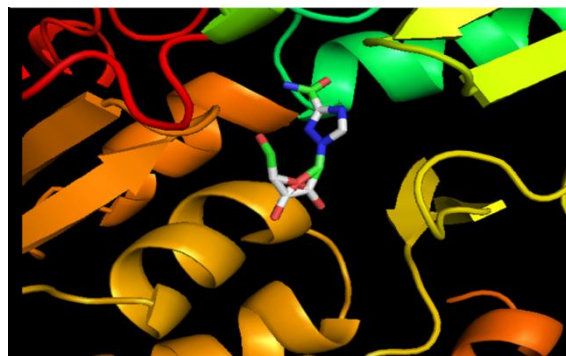


Fig. 5: Molecular binding of ribavirin by autodock vina at binding pocket.

The structure was further validated using Ramachandran Plot. Since 89.1% was in the allowed regions the model was accepted and used for docking simulations. For the molecular docking, Autodock Vina was used.

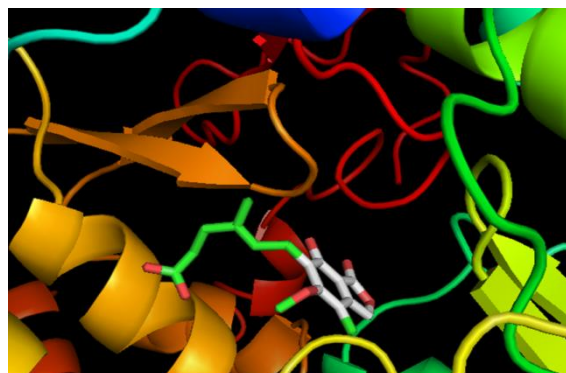


Fig. 6: Molecular binding of mycophenolic acid by autodock vina at binding pocket.

The binding energies and residues involved in the interactions were analyzed and compared to ribavirin ligand as shown in Table 1. The binding energies of both ligands were compared and were found to be similar. As one amino acid was involved in both the interactions, it can be considered that the mechanism of action is similar. It was also found that some of the residues were aromatic and thus the nature of the binding pocket was similar.

Table 1: Binding energies of ligand and their interactions

Ligand	Binding energy- Ribavarin (kcal/mol)	Residues involved in interaction
Ribavarin	-6.3	PHE 415, SER556, GLN446, THR267
Mycophenolic Acid	-6.3	PHE415, CYS386, TYR195, ARG386, TYR428

CONCLUSION

The present study concludes that strain JSR2 isolated from biogas plant efficiently produces mycophenolic acid in the presence of pineapple substrate when compare to other substrates. The chromatographic analysis confirms the production of mycophenolic

acid produced by strain JSR2. Mycophenolic acid is well known therapeutic agent which is widely used for various treatments.

The molecular docking studies through autodock vina further represent the mechanism of action against hepatitis C virus compare to ribavirin as the ligand.

CONFLICT OF INTEREST

There is no conflicts of interest to declare.

REFERENCES

- Muth W L, Nash C H. Biosynthesis of mycophenolic acid: Purification and characterization of S-adenosyl-l-methionine:demethylmycophenolic acid O-methyltransferase. *Antimicrob Agents Chemother* 1975; 8: 321-327.
- Abraham E P. The effect of mycophenolic acid on the growth of *Staphylococcus aureus* in heart broth. *Biochem J* 1945; 39: 398-404.
- Kohler G A, Gong X, Bentink S, Theiss S, Pagani G M, Agabian N, Hedstrom L. The functional basis of mycophenolic acid resistance in *Candida albicans* IMP dehydrogenase. *J Biol Chem* 2005; 280: 11295-302.
- Takhampunya R, Padmanabhan R, Ubol S. Antiviral action of nitric oxide on dengue virus type 2 replication. *J Gen Virol* 2006; 87: 3003-3011.
- Hideaki U, Asanuma S, Chiba H, Takahashi A, Yamaguchi Y, Masuma R, Mura S, Tanaka H. Mycophenolic acid inhibits syncytium formation accompanied by reduction of gp120 expression. *J Antibiot* 2005; 58: 514-518.
- Allison A C, Eugui E M. Mycophenolate mofetil and its mechanisms of action. *Int Immunopharmacol* 2000; 47: 85-118.
- Danheiser R L, Gee S K, Perez J J. Total synthesis of mycophenolic acid. *J Am Chem Soc* 1986; 108: 806-810.
- Xu Z, Yang S. Production of mycophenolic and *Penicillium brevicompactum* Immobilized in a rotating fibrous-bed bioreactor. *Enzyme Microb Technol* 2007; 40: 623-628.
- Houbraken J, Samson R A. Phylogeny of *Penicillium* and the segregation of *Trichocomaceae* into three families. *Stud Mycol* 2011; 70: 1-51.
- Zimmermann M, Miorelli S, Massaguer P R, Aragao G M F. *Byssoschlamys nivea* growth in papaya juice as influenced by water activity and ascospore age. *Braz J Microbiol* 2011; 42: 203-210.
- Dombrink M A, Engberg A E. The isoeopoxydon dehydrogenase gene of the patulin metabolic pathway differs for *Penicillium griseofulvum* and *Penicillium expansum*. *Mycol Res* 110: 111-1118.
- Puel O, Galtier P, Oswald I P. Biosynthesis and toxicological effects of patulin. *Toxins* 2010; 2: 613-631.
- Rajendiran R K, Sekar V K, Namadevan B D, Anamalai J K, Devarajan S. UV-Spectrophotometric and RP-HPLC methods for the estimation of prasugrel hydrochloride in bulk and tablet formulation. *International journal of Pharma Tech Research. Pharmtech*, 2014; 6: 220-225.
- Rajkumar B., Bhavya T., KUMAR A A. Reverse phase HPLC method development and validation for the simultaneous quantitative estimation of alpha lipoic acid and allopurinol in tablets. *International journal of Pharma Tech Research Pharmtech*, 2014; 6: 307-312.
- Samson R A, Houbraken J, Varga J, Frisvad J C. Polyphasic taxonomy of the heat resistant ascomycete genus *Byssoschlamys* and its *Paecilomyces* anamorphs. *Persoonia* 2009; 22: 14-27.
- Henry S D, Metselaar H J, Lonsdale R C, Kok A, Haagmans B L, Tilanus H W, Van der Laan L J. Mycophenolic Acid Inhibits Hepatitis C Virus Replication and Acts in Synergy With Cyclosporin A and Interferon- α . 2006; 131: 1452-1462.