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(Regn. No. 28 OF 2007)

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Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4^{th} edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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Strategies for Patenting in Biotechnology

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Abstract

Intellectual property rights (IP) is one of the major component of research in many organizations (both profit and non-profit), institutes and academics. Since 1970's, during which a Canadian non-governmental organization (ETC group) filed two patent applications for the first time on "the world's first-ever human-made life form", many companies including academic institutes or universities are encouraging their researchers to protect their findings through IP's. It is obvious for the researcher to surprise if he looks at the number of patents that were issued since 1970 on various entities over the advancement of science. Despite intense database on inventions and/or discoveries of various scientific organizations, the increasing interests of the scientists to protect their inventions/technology/discovery thorough IP is significantly reducing the accessibility of their findings and there by slowing advances in science. In this review, we are discussing on various components of patenting tools, protection and methodologies as an introductory material for scientists and students for the better understanding of intellectual property (IP) rights. We would like to promote the use of IP's to protect the technology being theft out for biological terrorism rather than a commercial motif to "Business" the science.

Key Words: Intellectual Property Rights, Patenting DNA, Bayh-Dole Act, Stevenson-Wydler Technology Innovation Act.

Abbreviations

cDNA- Complementary Deoxyribonucleic acid, DNA- Deoxyribonucleic acid,
ESTs- Expressed sequence tags, HGP- Human genome project.
mRNA- Messenger ribonucleic acid, NIH- National institutes of health.
PCR- Polymerase chain reaction, rDNA- Recombinant deoxyribonucleic acid.
RNA- Ribonucleic acid, UCSF- University of California, Sanfransisco.
USPTO- United states patent and trademark office.

Introduction

Intellectual property rights have been a recurring source of controversy in molecular biotechnology in recent years. A variety of developments have contributed to the increasing salience of intellectual property in biomedical research, including strong and growing commercial interest in the field, legal decisions that have clarified the availability of patent protection for a wide range of discoveries related to life forms, and changes in federal policy to encourage patenting of the results of government-

sponsored research. Protection of intellectual property rights has helped researchers and institutions to attract research funding and has helped firms to raise investment capital and pursue product development. But it has also periodically generated complaints and concerns about its effect on the progress of science and on the dissemination and use of new knowledge (1). The concerns have been particularly pressing for scientists when intellectual property rights have threatened to restrict access to materials and techniques that are critical for future research. Controversy over intellectual property rights in biomedical research has waxed and waned over the years. The current wave of concern was triggered in 1991 when NIH filed its first patent application on partial cDNA sequences, or expressed sequence tags (ESTs). Despite the later withdrawal of the patent applications, the concern over access to DNA sequence information continued to generate debate, both in the US and internationally. Another focal point of concern has been the patenting and licensing of polymerase chain reaction (PCR) technology. In 1992, the pharmaceutical giant Hoffman-La Roche, who holds the patent on the enzyme used in PCR (*Taq* polymerase), sued the biotechnology company Promega for breach of contract over the distribution of enzyme. During the course of the litigation, many research scientists received a letter from Promega suggesting them they had been named as infringers against the Roche patent. Although Roche stated they had no intention of naming any scientists in the suit, the letter sent a chill throughout the research community and raised fears that patents might be blocking access to research tools. In addition to those controversies, less notorious controversies have surrounded other research tools in molecular biology.

Increasing alliances among academe, industry, and government, driven by a combination of economic and legal changes, have challenged institutions in the public and private sectors to balance their sometimes competing interests in the protection of intellectual property. Over the last two decades, public investment in research has been rewarded by a dazzling series of advances in molecular biology. At the same time, scientists have had to adapt to declines in the growth of public funding to explore these research frontiers. The commercial potential of the advances has motivated the private sector to provide additional resources, and a series of laws, beginning in 1980 with the Bayh-Dole Act (2), have encouraged the pooling of public and private research funds. This environment has been favorable for the development of small, research-intensive biotechnology companies with close links to universities. Indeed, most of the early biotechnology companies were founded by university professors, and many universities now offer "incubator space" for start-up biotechnology companies working in collaboration with university researchers. Pharmaceutical companies are also increasingly eager to establish collaborations with university researchers (3).

The pervasive intertwining of public and private interests makes molecular biotechnology a particularly useful focal point for considering the effect of intellectual property rights on the dissemination and use of research tools. The potential implications of advances in molecular biology for human health raise the stakes of getting the balance between public and private right, particularly when public attention is riveted on the rising costs of health care. In this review we will be discussing about the research methodology, patenting tools and patent protection and patenting of research tools for molecular biology and biotechnology applications.

Research methodology

The research methodology adopted is of a descriptive-analytical method. The data has

been collected from primary and secondary sources which include internet sources (www.nap.edu/reading room/ books/ property /-), books, online transcripts of proceedings of meetings, conferences etc. The Bayh-Dole act and the Stevenson-Wydler technology innovation acts (4) which allow government contractors, small businesses and nonprofit organizations to retain certain patent rights in government sponsored research and permitted the funded entity to transfer the technology to third parties have been detailed. Cases that involved an important research tool in molecular biotechnology were chosen to illustrate a form of protection of intellectual property and a pattern of development involving both the public and the private sector. The strategies for patenting DNA sequences and multicellular organisms and the controversies surrounding them have been covered.

Bayh-Dole Act and the Stevenson-Wydler Technology Innovation Act

In 1980, Congress passed both the Bayh-Dole Act (5) and the Stevenson-Wydler Technology Innovation Act (6). Together these Acts allowed government contractors, small businesses, and nonprofit organizations to retain certain patent rights in government-sponsored research and permitted the funded entity to transfer the technology to third parties. The stated intent of Bayh-Dole was to ensure that the patented results of federally-funded research would be broadly and rapidly available for all scientific investigation. Bayh-Dole effectively shifted federal policy from a position of putting the results of government-sponsored research directly into the public domain for use by all, to a pro-patent position that stressed the need for exclusive rights as an incentive for industry to undertake the costly investment necessary to bring new products to market. The policy was based on a belief that private entities, given the incentives of the patent system, would do a better job of commercializing inventions than federal agencies. The Act for the first time established a largely uniform government-wide policy on the treatment of inventions made during federally supported R&D. Stevenson-Wydler is the basic federal technology law. A principal policy established by that Act is that agencies should ensure the full use of the results of the nation's federal investment in R&D. Another is that the law requires federal laboratories to take an active role in the transfer of federally-owned or originated technology to both state and local governments and to the private sector. Stevenson-Wydler required agencies to establish Offices of Research and Technology Applications at their federal laboratories, and to devote a percentage of their R&D budgets to technology transfer.

Patenting research tools

Over the past 15 years, a number of legal and commercial developments have converged to make intellectual property issues particularly salient in molecular biotechnology research. The figure 1 shows the number of patents issued in USA since 2001. A series of judicial and administrative decisions has expanded the categories of patentable subject matter in the life sciences. For many years it appeared that patents on living subject matter would violate the longstanding principle that one may not patent products or phenomena of nature (7). But in 1980 the US Supreme Court held in the case of *Diamond v. Chakrabarty* that a living, genetically altered organism may qualify for patent protection as a new manufacture or composition of matter under Section 101 of the US Patent Code (8). The US Supreme Court relied on this principle in *Funk Brothers Seed Co. v. Kalo Inoculant Co.,* holding invalid a patent on a mixed culture of different strains of bacteria used to inoculate the roots of different species of plants[9]. The court reasoned that, "The qualities of these

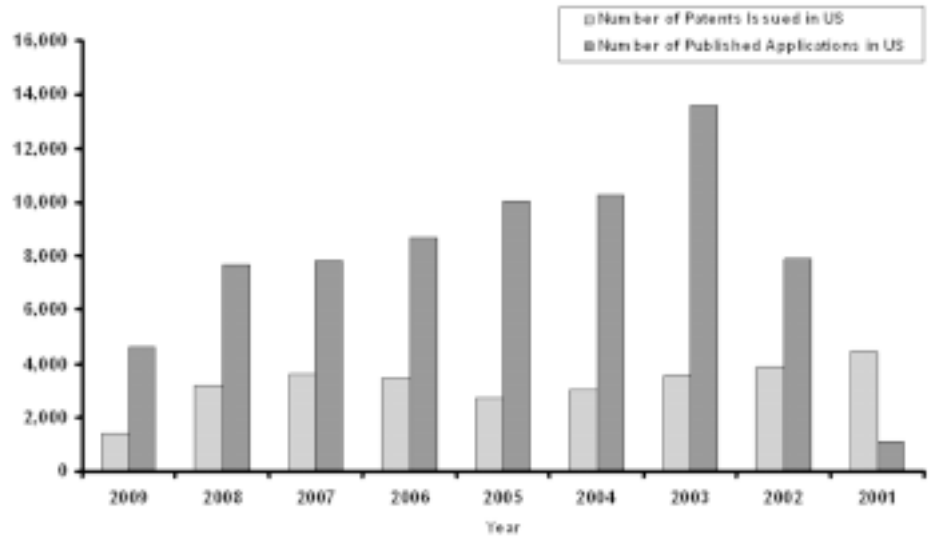

Fig.1: A comparative bar diagram of number of patents issued and number of patents published in USA since 2001.

bacteria, like the heat of the sun, electricity, or the qualities of metals, are part of the storehouse of knowledge of all men. They are manifestations of laws of nature, free to all men and reserved exclusively to none".

Characterizing Chakrabarty's invention as "a new bacterium with markedly different characteristics from any found in nature" and "not nature's handiwork, but his own," the Court indicated that Congress intended the patent laws to cover "anything under the sun that is made by man." With this broad directive from the Supreme Court, the US Patent and Trademark Office (PTO) expanded the categories of living subject matter that it considered eligible for patent protection to include plants (9) and animals (10). For example, in 1985, the PTO held that plants were eligible for standard utility patents, and not merely the more limited rights provided under special statutes for the protection of plant varieties[9] and the PTO held that oysters were patentable subject matter in *Ex parte Allen* (10). Shortly thereafter, the Commissioner of Patents issued a notice stating that the PTO would consider non-naturally occurring, non-human, multicellular living organisms—including animals—to be patentable subject matter (9). The notice hastened to add that PTO would not consider human beings to be patentable subject matter, citing restrictions on property rights in human beings. The first patent on a genetically altered animal was issued in April of 1988 to Harvard University for the development of a mouse bearing a human oncogene (11). The decision to extend patent protection to animals generated considerable public controversy and has been the focus of numerous hearings in the US Congress. Restrictive legislation has been proposed from time to time, including a moratorium on animal patenting, although no such legislation has been passed. During the same time period, the explosions of commercial interest in the field, and the concomitant emergence of commercial biotechnology companies, have amplified the importance of intellectual property in the biomedical sciences. Many biotechnology firms have found a market niche somewhere between the fundamental research that typifies the work of university and government laboratories and the end product development that occurs in more established commercial firms. To

survive financially in this niche, biotechnology firms need intellectual property rights in discoveries that arise considerably upstream from commercial product markets. This creates pressure to patent discoveries that are closer to the work of research scientists than to ultimate consumer products.

Another contemporaneous development that has contributed to the prevalence of intellectual property in biomedical research is the passage of the Bayh-Dole Act and the Stevenson-Wydler Act in 1980, and a series of subsequent acts that refine those statutes and expand their reach (12). These statutes encourage research institutions to patent discoveries made in the course of government-sponsored research.

For some institutions involved in health-related research, this represented a 180° shift in policy. A generation ago, the prevailing wisdom was that the best way to assure full utilization of publicly-sponsored research results for the public good was to make them freely available to the public. Today, federal policy reflects the opposite assumption. The current belief is that if research results are made widely available to anyone who wants them, they will languish in government and university archives, unable to generate commercial interest in picking up where the government leaves off and using the results to develop commercial products.

To make government-sponsored research discoveries attractive candidates for commercial development, institutions performing the research are encouraged to obtain patents and to offer licenses to the private sector. As a result, institutions that perform fundamental research have an incentive to patent the sorts of early stage discoveries that in an earlier era would have been dedicated to the public domain. A big part of the resulting increase in patenting activity among public sector research institutions has been in the life sciences. Taken together, these factors have created a research environment in which early stage discoveries are increasingly likely to be patented, and access to patented discoveries is increasingly likely to be significant to the ongoing work of research laboratories.

Requirements for patent protection

The basic requirements for patent protection are novelty, utility, and non-obviousness. *Novelty* means that the invention did not exist before. Determining whether an invention is new requires searching through certain categories of prior art to determine the state of knowledge in the field at the time that the invention was made. Sources of prior art include prior patents, publications, and inventions that were previously in public use. If an invention was already known or used before the time that the inventor claims to have made it, the public gains nothing by conferring a patent. The patent will take something away from the public that it was previously free to use without in any way enriching the public storehouse of knowledge.

The prior art is also relevant to the standard of *nonobviousness*. This standard asks whether the invention constitutes a significant enough advance over what was known previously to justify patent protection. Under US law, the requirement is satisfied if, at the time the invention was made, it would not have been obvious to a person of ordinary skill in the field and who was knowledgeable about the prior art. This determination looks to the level of inventive skill of others working in the field, as well as the state of the prior art. In principle, the requirement might be justified as a means of distinguishing between trivial inventions that require no special incentive to call forth, and more elusive (and, perhaps, more costly) inventions that might not be developed without the enhanced assurance of profitability that patent protection offers. But how the standard will apply in any given case is often

difficult to predict, and this uncertainty reduces the value of patents.

The *utility* requirement limits patent protection to inventions with practical applications, as opposed to basic knowledge. The meaning of this requirement has varied over the years from a minimal standard that the invention not to be positively harmful to people to a stricter requirement in recent years of safety and effectiveness that has sometimes approached what the FDA would require for approval of a new drug. Recent developments in the courts and in the PTO suggest that the utility requirement may be receding from its recent all-time high level as an obstacle to patent protection. The conceptual underpinnings of the utility requirement are not always clear, but in theory it can be justified as a means of distinguishing between basic research discoveries that are more likely to be effectively utilized if left in the public domain and more practical technological applications that may require a patent to ensure adequate incentives for commercial development. The Supreme Court has stated that discoveries whose only value is as an object of scientific inquiry do not satisfy the utility standard, suggesting that utility could be an important limitation on the use of the patent system to protect research tools.

Research tools in molecular biotechnology

Molecular biology provides a useful focal point for examining the effect of intellectual property on the dissemination of research tools. It is a dynamic and productive field of research that provides a wealth of new discoveries that are simultaneously inputs into further research and also candidates for commercial development. The obvious implications of discoveries in molecular biotechnology for human health raise the stakes of striking the right balance between public access and private property, particularly when public attention is riveted upon the rising costs of health care. And it profoundly affects the interests of two different types of commercial firms—young biotechnology firms and large, integrated pharmaceutical firms—both of which are sensitive to intellectual property but for different reasons.

This dichotomy between biotechnology firms and pharmaceutical firms oversimplifies the wide range of firms with interests in molecular biotechnology, but it is nonetheless a useful heuristic assumption to help sort through the interests of different sorts of firms. Young biotechnology firms typically need to raise funds to keep their research operations moving forward before they have products to sell to consumers. For these firms, an intellectual property portfolio might be critical at an early stage in their R&D to give them something to show investors as evidence of their potential for earning high returns in the future. With this purpose in mind, they are likely to seek patents on discoveries that are several stages removed from a final product that is ready to be sold to consumers.

Established pharmaceutical firms are also very sensitive to intellectual property rights, but for different reasons and at a different stage in the R&D process. Pharmaceutical firms do not need to go to the capital markets to fund their research; they typically fund new research projects out of profits on existing products. For these firms, intellectual property is not a means of raising capital, but simply a means of ensuring an effective commercial monopoly for their products. A monopoly position in a new drug will help them recoup what might amount to hundreds of millions of dollars required for FDA-mandated clinical testing before they can bring that drug to market. For this purpose, they seek patent rights that cover the downstream products that they sell to consumers, not the upstream discoveries that they may use along the road to product development.

Since they have different reasons for requiring intellectual property rights, these different types of firms are likely to be affected differently by different legal rules. We need to keep the interests of both of these types of firms in mind, along with the interests of researchers and the institutions that fund research, as we think about how to manage intellectual property rights in research tools. Strategies that work for some players could be disastrous for others.

Patents on research tools

"Research tools" is not a term of art in patent law. No legal consequences flow from designating a particular discovery as a research tool. Research tools are not categorically excluded from patent protection (except insofar as they lack patentable utility), nor is the use of patented inventions in research categorically exempted from infringement liability.

Nonetheless, there are reasons to be wary of patents on research tools. Although the ultimate social value of research tools is often difficult to measure in advance, it is likely to be greatest when they are widely available to all researchers who can use them. For years, we have sustained a flourishing biomedical research enterprise in which investigators have drawn heavily upon discoveries that their predecessors left in the public domain. Yet the nature of patents is that they restrict access to inventions to increase profits to patent holders. An important research project might call for access to many research tools, and the costs and administrative burden could mount quickly if it were necessary for researchers to obtain separate licenses for each of these tools.

The effects of patenting research tools will vary. For example, patents are unlikely to interfere substantially with access to such research tools as chemical reagents that are readily available on the market at reasonable prices from patent holders or licensees. Many of the tools of contemporary molecular biology research are available through catalogs under conditions that approach an anonymous market. Some are patented, but the patents are unlikely to interfere with dissemination. Indeed, it might be cheaper and easier for researchers to obtain such a tool from the patent holder or from a licensed source than it is to infringe the patent by making it themselves. But not all research tools are of that character.

Some research tools can only be obtained by approaching the patent holder directly and negotiating for licenses; in this context, patents potentially pose a far greater threat to the work of later researchers. Negotiating for access to research tools might present particularly difficult problems for would-be licensees who do not want to disclose the directions of their research in its early stages by requesting licenses. Another risk is that the holders of patents on research tools will choose to license them on an exclusive basis rather than on a nonexclusive basis; this could choke off the R&D of other firms before it gets off the ground. Such a licensing strategy might make sense for a startup company that is short on current revenues, even if it does not maximize value in the long run from a broader social perspective.

Another risk is that patent holders will use a device employed by some biotechnology firms of offering licenses that impose "reach-through" royalties on sales of products that are developed in part through use of licensed research tools, even if the patented inventions are not themselves incorporated into the final products. So far, patent holders have had limited success with reach-through royalty licenses. Firms have been willing to accept a reach-through royalty obligation for licenses under the Cohen-Boyer patents on basic recombinant DNA techniques, perhaps because those patents include broad

claim language that covers products developed through the use of the patented technology. But reach-through royalties have met greater market resistance for other patents, including the patents on the Harvard onco-mouse and the polymerase chain reaction (PCR).

Licenses with reach-through royalty provisions might appear to solve the problem of placing a value on a research tool before the outcome of the research is known. One difficulty in licensing research tools is that the value of the license cannot be known in advance, so it is difficult to figure out mutually agreeable license terms. A reach-through royalty might seem like a solution to this problem, in that it imposes an obligation to share the fruits of successful research without adding to the costs of unsuccessful research. But it takes little imagination to foresee the disincentives to product development that could arise from a proliferation of reach-through royalties. Each reach-through royalty obligation becomes a prospective tax on sales of a new product, and the more research tools are used in developing a product, the higher the tax burden.

A further complication arises in the case of inventions that have substantial current value as research tools but might also be incorporated into commercial products in the future. It might be necessary to offer exclusive rights in the ultimate commercial products to innovating firms to give them adequate incentives to develop the products, but it might be impossible to preserve this option without limiting dissemination of the inventions for their present use as research tools.

For all of these reasons, exclusive rights risk inhibiting the optimal use of research tools and interfering with downstream incentives for product development. Much depends on whether the holders of exclusive rights can figure out how to disseminate research tools broadly without undermining their value as intellectual property.

These are difficult problems that defy facile solutions. One of the purposes of this article is to examine the solutions that different institutions have come up with and see how they have operated in practice. Which mechanisms have worked well, which have worked badly, and what can we learn from the experiences of others? We need to keep in mind that this issue implicates the interests of many different players who value intellectual property in different ways and for different purposes.

Patenting DNA sequences

On the surface, any device, process, or compound that meets the criteria of novelty, inventiveness and utility should be patentable. Since 1980, thousands of patent applications for whole genes have been approved by patent offices through out the world. The most valuable human gene patent is for the production of recombinant erythropoietin, which had sales of about $4 billion in 2001.Erythropoietin, stimulates the formation of red blood cells and is used to prevent anemia in patients with kidney failure who require dialysis. Many of the other patented gene sequences are used as biomarkers.

With the advent of HGP and, in particular, with the undertaking of the partial sequencing of thousands of human cDNA molecules from different tissues and organs, the patenting of these sequences became extremely contentious[13]. In 1991, the issue of patenting gene fragments was broached when the U.S. NIH filed for the patent rights for 315 partially sequenced human cDNAs. Two additional filings brought the total number of sequences to 6869. In 1994, in a preliminary ruling, the US PTO notified the NIH that it would reject the patent application on the grounds that the functions of the sequences were not known. In other words, partial sequences by themselves did not fulfill the requirement of utility and were not patentable. How ever, by 1997, over 350 patent applications for more than

500,000partial DNA sequences had been filed mostly by private companies, which purportedly met the standard for usefulness. One of these patent proposals sought protection for about 18,500 ESTs. Consequently; serious concerns were raised about granting patents for large numbers of sequenced genes and partially sequenced DNA fragments with broadly based applications.

Individuals who opposed the patenting of DNA fragments of unknown or loosely defined function contended that genes and partial DNA sequences are discoveries or, more likely, products of nature and definitely not inventions. Others conceded that, although some of these inventions might be useful, it was premature and speculative to award patents with out additional information about the functions of the sequences. In addition, it was argued that granting of such patents would not only give the patent holders too much control but would act as a constraint against the development of various diagnostic and therapeutic agents. In this context, thousands of ESTs are considered to be "means to ends" and not the actual end points (16). On the other hand, those who favored the patenting of ESTs maintained that these collections novel because they defined the normal mRNA complement of various tissues and organs and consequently had utility because each collection could be used as a diagnostic assay to determine the extent to which a disease alters the normal complement of mRNAs in various organs. In addition, these individuals asserted that, historically, the existence of patents had not deterred the development of new products and but, to the contrary, stimulated the process.

After developing some ad hoc rules, the U.S. PTO examined in more detail a full range of issues and concluded that genes and partial DNA sequences were patentable (16). On January 5, 2001 a set of guide lines for gene patenting was released. The key requirement for this type of application was that each DNA sequence must have "specific and substantial credible utility." Moreover, the written specifications and claims for each sequence must be thorough and demonstrate the actual use of each sequence and not merely a potential function. The guidelines have established the criteria for patenting incomplete DNA sequences in the United States, although there is still opposition against granting patents for any human DNA sequence.

Patenting multicellular organisms

The patenting of multicellular organisms continues to raise ethical and social concerns. However, there is nothing intrinsically new about the exclusive ownership of living material[14]. In the past, microorganisms were routinely patented and specific laws were promulgated to give plant breeders the right to own various plant varieties. The transgenic mouse that carries a gene that makes it susceptible to tumor formation has been the precedent-setting case in many jurisdictions to determine whether genetically modified animals are patentable. Currently, patenting of genetically modified animals is sanctioned in most developed countries, including the United States, members of the European union, Japan, Australia and New Zealand and others.

Vigorous challenges to patenting transgenic animals have been put forward on moral grounds (15). In other words, the issue is whether society considers this form of patenting acceptable. From a historical perspective, it is unlikely that a position based on ethical considerations will be completely successful in preventing the patenting of transgenic animals. For example, if an invention purports to facilitate a new treatment for human disease, the currently prevalent view is that human rights and needs supersede those of animals .However, patenting is not an absolute right, and governments, by

passing specific laws, can what can or cannot be patented. If an invention is considered by various special interest groups to have a potentially negative impact on an existing agricultural practice, for example, then it is quite possible that a law preventing the implementation of the new technology could be passed

Experimental use exemption

In some cases, the courts have recognized what has come to be known as an experimental use exemption, or research exemption, from infringement liability. On its face, the patent statute does not appear to permit any unlicensed use of a patented invention, in research or otherwise, but language in some judicial opinions nonetheless suggests that use of a patented invention solely for research or experimentation is, in principle, exempt from infringement liability. The experimental-use doctrine was first expounded in 1813 by Justice Story in *dictum* in the case of *Whittemore v. Cutter*16. Here the legal term *dictum* refers to something said in a judicial opinion that was not necessary to resolve the case before the court, and therefore does not create binding precedent in subsequent cases.

He observed "that it could never have been the intention of the legislature to punish a man who constructed [a patented] machine merely for philosophical experiments or for the purpose of ascertaining the sufficiency of the machine to produce its described effects". It is difficult to discern the scope of this exception with any precision, inasmuch as experimental use becomes an issue only in patent infringement actions, and patent holders are unlikely to file a lawsuit against an academic researcher whose use of the invention is commercially insignificant. Judicial pronouncements on the scope of the experimental use exemption address situations in which a patent holder has found a defendant's activities sufficiently annoying to be worth the trouble of pursuing a lawsuit; this factor has undoubtedly skewed the distribution of cases in which the defense arises toward cases with high commercial stakes. Within this universe, the experimental use defense has been frequently raised, but almost never sustained. Nonetheless, courts have consistently recognized the existence of an experimental use defense in theory, although the defense has almost never succeeded in practice.

Recent case law suggests that the experimental use defense may be available only for pure research with no commercial implications, if such a thing exists. In *Roche Products v. Bolar Pharmaceutical Company* (17), 1984 decision of the US Court of Appeals for the Federal Circuit,the court rejected the arguments of a generic drug manufacturer that the experimental use defense should apply to its use of a patented drug to conduct clinical trials during the patent term. The purpose of the trials was to gather data necessary to obtain FDA approval to market a generic version of the drug as soon as the patent expired. The court characterized the defense as "truly narrow", noting that the defendant's use of the drug was "no dilettante affair such as Justice Story envisioned".

"The court held that the defense does not permit unlicensed experiments conducted with a view to the adoption of a patented invention for use in an experimenter's business, as opposed to experiments conducted for amusement, to satisfy idle curiosity, or for strictly philosophical inquiry. Although it is not entirely clear what sort of research the court would exclude from infringement liability as a mere "dilettante affair", the language of the decision offers little hope of an exemption for research scientists who use patented inventions with an aim to discover something of potential usefulness. It certainly suggests that the defense would be unavailable whenever the defendant's research is motivated

by a commercial purpose. As a practical matter, this parsimonious approach could seriously limit the availability of the defense in fields of research with commercial significance, in which even academic researchers are often motivated, at least in part, by commercial interests. For example, the Bayh-Dole Act in effect directs academic institutions to be alert to potential commercial implications of their research so that they can obtain patents as appropriate.

Congress has partially abrogated the decision of the Federal Circuit in *Roche v. Bolar* in the specific context of clinical trials of patented drugs by an amendment to the patent statute (18). As amended, the statute explicitly permits the use of patented inventions for the purpose of developing and submitting information under laws regulating the manufacture, use, or sale of drugs. But the amendment did not address the broader question of when the experimental use defense would be available outside of that very narrow setting.

Other countries have more broadly available experimental use defenses than the US, often explicitly included in the text of foreign patent statutes. But even these defenses typically distinguish between experimenting on a patented invention—that is, using it to study its underlying technology and invent around the patent, which is what the exemption covers—and experimenting *with* a patented invention to study something else, which is not covered by the exemption.

In other words, even outside the US, the defense is not available for researchers who make use of patented research tools in their own work, as opposed to those who study the research tools themselves. It is difficult to imagine how a broader experimental use defense could be formulated that would exempt the use of research tools from infringement liability without effectively eviscerating the value of patents on research tools. The problem is that researchers are ordinary consumers of patented research tools, and that if these consumers were exempt from infringement liability; patent holders would have nowhere else to turn to collect patent royalties. Another way of looking at the problem is that one firm's research tool may be another firm's end product.

This is particularly likely in contemporary molecular biology, in which research is big business and there is money to be made by developing and marketing research tools for use by other firms. An excessively broad research exemption could eliminate incentives for private firms to develop and disseminate new research tools, which could on balance, do more harm than good to the research enterprise.

Case studies

Each of the following cases involves an important research tool in molecular biology, and each was chosen to illustrate a form of protection of intellectual property and a pattern of development involving both the public and the private sector. The ideal strategies for the handling of intellectual property in molecular biology are not always immediately obvious, as these case studies illustrate. For most, final decisions have not been made about how access to these research tools will be controlled. Such decisions might be modified in response to both scientific and legal developments.

Recombinant DNA

The Cohen-Boyer technology for recombinant DNA, often cited as the most-successful patent in university licensing, is actually three patents. One is a process patent for making molecular chimeras and two are product patents—one for proteins produced using recombinant prokaryote DNA and another for proteins from recombinant eukaryote DNA. Recombinant DNA, arguably the defining

technique of modern molecular biology, is the founding technology of the biotechnology industry (19). In 1976, Genentech became the first company to be based on this new technology and the first of the wave of biotechnology companies, which in fifteen years has grown from one to over 2000.

The first patent application was filed by Stanford University in November 1974 in the midst of much soul-searching on the part of the scientific community. Stanley Cohen and Herbert Boyer, who developed the technique together at Stanford and the University of California, San Francisco (UCSF), respectively, were initially hesitant to file the patent. Several years of discussion involving the National Institutes of Health (NIH) and Congress followed. By 1978, NIH decided to support the patenting of recombinant DNA inventions by universities; in December 1980, the process patent for making molecular chimeras was issued. The product patent for prokaryotic DNA was issued in 1984. The patents were jointly awarded to Stanford and UCSF and shared with Herbert Boyer and Stanley Cohen. The first licensee signed agreements with Stanford on December 15, 1981. As of February 13, 1995, licensing agreements had generated $139 million in royalties, which have shown an exponential increase in value since their beginning. In 1990-1995 alone, the licensing fees earned $102 million.

This case has three key elements. First, the technology was inexpensive and easy to use from a purely technical standpoint and there were only minimal impediments to widespread dissemination. Second, there were no alternative technologies. Third, the technology was critical and of broad importance to research in molecular biology.

The technology was developed in universities through publicly funded research. The strategy used to protect the value of the intellectual property was to make licenses inexpensive and attach minimal riders. The tremendous volume of sales made the patent very lucrative. Every molecular biologist uses this technology. However, not all inventions are as universally critical. Only a few university patents in the life sciences, such as warfarin and Vitamin D, have been even nearly as profitable as the Cohen-Boyer patent. Clearly, had this technology not been so pivotal for molecular biology or had an equally useful technology been available, the licenses would not have been sold so widely and the decision to license the technology might have met with more resistance.

The Cohen-Boyer patent is considered by many to be the classic model of technology transfer envisaged by supporters of the Bayh-Dole Act, which was intended to stimulate transfer of university-developed technology into the commercial sector. Ironically, it presents a different model of technology than that presumed by advocates of the Bayh-Dole act.

The biotechnology boom that followed the widespread dissemination of recombinant DNA techniques transformed the way universities manage intellectual property. It also fundamentally changed the financial environment and culture of biological research.

The decision to negotiate nonexclusive, rather than exclusive, licenses was critical to the industry. If the technology had been licensed exclusively to one company and the entire recombinant DNA industry had been controlled by one company, the industry might never have developed. Alternatively, major pharmaceutical firms might have been motivated to commit their resources to challenging the validity of the patent.

PCR and Taq polymerases

Polymerase chain reaction (PCR) technology presents an interesting counterpoint

to the Cohen-Boyer technology. Both are widely used innovations seen by many as critical for research in molecular biology. However, the licensing strategies for the two technologies have been quite different, and they were developed in different contexts.

PCR allows the specific and rapid amplification of targeted DNA or RNA sequences. *Taq* polymerase is the heat-stable DNA polymerase enzyme used in the amplification. PCR technology has had a profound impact on basic research not only because it makes many research tasks more efficient, in time and direct cost, but also because it has made feasible some experimental approaches that were not possible before the development of PCR. PCR allows the previously impossible analysis of genes in biological samples, such as assays of gene expression in individual cells, in specimens from ancient organisms, or in minute quantities of blood in forensic analysis.

In less than a decade, PCR has become a standard technique in almost every molecular biology laboratory, and its versatility as a research tool continues to expand. In 1989, *Science* chose *Taq* polymerase for its first "Molecule of The Year" award. Kary Mullis was the primary inventor of PCR, which he did when he worked at the Cetus Corporation. He won a Nobel Prize for his contributions merely 8 years after the first paper was published in 1985 (20), which attests to its immediate and widely recognized impact.

Whereas recombinant DNA technology resulted from collaboration between university researchers whose immediate goal was to insert foreign genes into bacteria to study basic processes of gene replication, PCR was invented in a corporate environment with a specific application in mind—to improve diagnostics for human genetics. No one anticipated that it would so quickly become such a critical tool with such broad utility for basic research.

Molecular biology underwent considerable change during the decade between the development of recombinant DNA and PCR technologies. The biotechnology industry emerged, laws governing intellectual property changed, there was a substantial increase in university-industry-government alliances, and university patenting in the life sciences increased tenfold. There was virtually no controversy over whether such an important research tool should be patented and no quarrel with the principle of charging licensing fees to researchers. The controversy has been primarily over the amount of the royalty fees.

Cetus Corporation sold the PCR patent to Hoffman-LaRoche for $300 million in 1991. In setting the licensing terms for research use of PCR, Roche found itself in a very different position from Stanford with respect to the Cohen-Boyer patent. First, it was a business, selling products for use in the technology. That made it possible to provide rights to use the technology with the purchase of the products, rather than under direct license agreements, such as Stanford's. This product-license policy was instituted by Cetus, the original owner of the PCR patents. An initial proposal to the scientific community by the president of Cetus for reach-through royalties—royalties on second-generation products derived through use of PCR—was met with strong criticism. Ellen Daniell, director of licensing at Roche Molecular Systems, noted that the dismay caused by the proposal has continued to influence the scientific community's impression of Roche's policy.

Roche's licensing fees have met with cries of foul play from some scientists who claim that public welfare is jeopardized by Roche's goals. Nevertheless, most scientists recognize that

Roche has the right to make business decisions about licensing its patents. The fact that Roche had paid Cetus $300 million for the portfolio of PCR patents led some observers to think that Roche intended to recoup its investment through licensing revenues, a point that Daniell disputed. She pointed out that Roche's business is the sale of products and that licensing revenues are far less than what would be needed to recoup the $300 million over a time period that would be relevant from a business viewpoint. Daniell listed Roche's three primary objectives in licensing technology:

- Expand and encourage the use of the technology.
- Derive financial return from use of the technology by others.
- Preserve the value of the intellectual property and the patents that were issued on it.

Roche has established different categories of licenses related to PCR, depending on the application and the users. They include research applications, such as the Human Genome Project, the discovery of new genes, and studies of gene expression; diagnostic applications, such as human in vitro diagnostics and the detection of disease-linked mutations; the production of large quantities of DNA; and the most extensive PCR licensing program, human diagnostic testing services. Licenses in the last-named category are very broad; there are no up-front fees or annual minimum royalties, and the licensees have options to obtain reagents outside Roche.

Discussion about access to PCR technology centered on the costs of *Taq* polymerase, rather than on the distribution of intellectual property rights. Tom Caskey's view was that "the company has behaved fantastically" with regard to allowing access to PCR technology for research purposes. Bernard Poiesz, professor of medicine at the State University of New York in Syracuse and director of the Central New York Regional Oncology Center, agreed that he knew of no other company that had done as well as Roche in making material available for research purposes. But he also argued that the price of *Taq* polymerase is too high and has slowed the progress of PCR products from the research laboratory to the marketplace. Poiesz stated that the diagnostic service licenses "are some of the highest royalty rates I have personally experienced". He cited the example of highly sensitive diagnostic tests for HIV RNA, which he said are too expensive for widespread use, largely because of the licensing fees charged by Roche. Caskey felt that Roche should have expanded the market by licensing more companies to sell PCR-based diagnostic products and profited from the expansion of the market, rather than from the semi exclusivity that it has maintained.

Ron Sederoff commented that—in contrast to the human genomics field, in which funding levels are much higher than for other fields of molecular biology—many academic researchers do not find easy access to the technology. What is the effect of the Cetus-Roche licensing policy on small companies? Tom Gallegos, intellectual property counsel for OncoPharm, a small biotechnology company, stated that most small companies cannot afford the fees charged by Roche. He noted that the entry fee for a company that wants to sell PCR-based products for certain fields other than diagnostics ranges from $100,000 to $500,000, with a royalty rate of 15%. By comparison, a company pays about $10,000 per year and a royalty fee of 0.5-10% for the Cohen-Boyer license. The effect is an inhibition of the development of PCR-related research tools, with consequent reductions or delays in the total royalty stream and possibly litigation.

In the case of PCR, the research tool is both a commercial product and a discovery tool. As such, it raises questions. Are the PCR patents an example of valuable property that would have been widely disseminated in the absence of patent rights? Is PCR an example of a technology that has been more fully developed because of the existence of patent rights? Daniell stated that Roche has added considerable value to the technology, in part through the mechanism of patent rights. There was vigorous discussion and disagreement as to whether the licensing fees justify the value added by Roche.

Conclusions

Not everyone believes that patenting is worthwhile. Some opponents argue that awarding a monopoly restricts competition, leads to higher prices, curtails new inventions, and favors large corporations at the expense of individual inventors and small firms. Notwithstanding these concerns the patent system is well established and is here to stay. Moreover, patent ownership does not appear to prevent significant research and development by other researchers and companies. Indeed, it might be argued that if patents were serious impediments to innovation, then U.S. patent 4,237,224 which was granted to Stanley Cohen and Herbert Boyer in 1980 for recombinant DNA technology for both the use of viral and plasmid vectors and the cloning of foreign genes, should have seriously constrained the development of rDNA technology. Obviously no such hindrance has occurred.

In the past, patenting and patent enforcement were rarely of interest to researchers working in the biological sciences. Now, however, there is a view in the academic scientific community that patents and the consequences of patenting may be detrimental to establish scientific values .Traditionally, science, especially university- based research, has been an open system with a free exchange of ideas and materials through publications and personal communications. The ideas of others have been respected, and contribution to the technical development of an area of study has, in many instances, been a shared enterprise. However, more recently, some scientists begun to feel that the integrity of traditional scientific enquiry has become secondary to self-interest, in that public recognition and financial gain from innovations are the prime motivations for conducting scientific research. It is argued that research is often carried out secretly and has created elite, non-cooperating research groups. In the past, there was a tendency to avoid secrecy in basic research. The belief was that scientific knowledge would grow if research results were published as articles in journals that could be read by anyone, there by enabling researchers to direct their studies in appropriate direction s and to benefit from the discoveries of the others. With secrecy, time and effort may be wasted on repeating experiments that, unbeknownst to the researcher, have already been done. Now scientists are advised by their patent attorneys to keep their work secret until a patent is filed. Consequently, the lure of patenting has made a large number of scientists reluctant to talk about their work, at least until after the patent application has been filed.

In sum, the enthusiasm for patenting and patent protection has elicited the perception that traditional science may become hostage to patent holders and that research will become less fruitful. Others feel that the traditional way of doing science is an outmoded, inefficient, and indulgent exercise and that patent ownership and the drive for ownership will spur new discoveries. This controversy will not be readily resolved. It is clear that the emergence of molecular biotechnology has raised far-reaching considerations, even including how scientific inquiries ought to be conducted.

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Automated image analysis to observe pellet morphology in liquid cultures of filamentous fungi such as the basidiomycete *Coprinopsis cinerea*

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Abstract

In this study, an image analysis system for fungal pellets was developed using the commercial software system analySIS® and the protocol was evaluated in morphological studies of pellet formation in submerged cultures of the basidiomycete *Coprinopsis cinerea*. Pellets were analysed on large scale (60 to 130 pellets per image, 225 to 400 pellets per culture). Morphologies of pellets were characterised by the parameters grey value, pellet area, convexity, shape factor, sphericity and pellet diameter. Threshold values were defined for all parameters for object filtering. By application of the parameter filter, aggregated hyphal fragments present in larger amounts particularly in cultures grown at higher temperature (37^0C) could be clearly distinguished in image analysis from compact pellets. At a lower growth temperature (25^0C), there was little background of loose hyphal material, fungal pellets were regularly shaped and the pellets remained constant in shape and size over a longer cultivation period.

Key words

Automated image analysis, filamentous organism, pellet morphology, hyphal aggregates, *Coprinopsis cinerea.*

Introduction

Various filamentous fungi are used in biotechnology for production of biomass, secondary metabolites, polysaccharides and/or enzymes and other proteins. Usually, filamentous fungi are cultivated in liquid medium in suitable fermenters. Typically, the fungi will grow in a pelleted form (1, 2). Determination of pelleted growth of filamentous organisms in liquid cultures is mostly done by image analysis, which can be conducted manually (3, 4) or automatically (5, 6). Automatic image analysis implies that pictures of the pellet culture are taken and analysed by a computer based programme regarding specific parameters, which can be e.g. pellet concentration and pellet diameter. Studies exist where such an automatic image analysis was performed on liquid cultures of basidiomycetes (7 - 9).

In an early study, Michel et al. (3) analysed manually just 15 pellets obtained from *Phanerochaete chrysosporium* cultures with respect to pellet diameter. Later on, Márquez-Rocha et al. (6) determined by automated image analysis the diameter of *Pleurotus ostreatus* pellets but only 20 pellets per culture were surveyed. However, also in more recent studies of three *Phellinus* species and of *Ganoderma resinaceum* (8, 9) only the diameter of 50 pellets were analysed per fungal sample. In contrast, Gehrig et al. (7) gave a more complete picture of pellets of *Cyathus striatus* during a fermentation process. The authors analysed in total about 2000

pellets and a broad spectrum of parameters, such as pellet concentration, pellet diameters, total pellet volume (by using average diameter values) and pellet density (from the total biomass dry weight and the total pellet volume).

The studies have in common that all used a CCD camera to obtain photos either on the microscopic scale or on a larger scale with images of areas of up to a few cm^2 [e.g. 2.25 cm^2 in the study by Gehrig et al. (7)]. The photos were required for determination of pellet parameters. For evaluation of the images, different image analysis software was applied, some of which were commercially (6, 8) and some of which were especially written for the analysis of pellet morphology [programme by Defren (10) appointed in Gehrig et al. (7)].

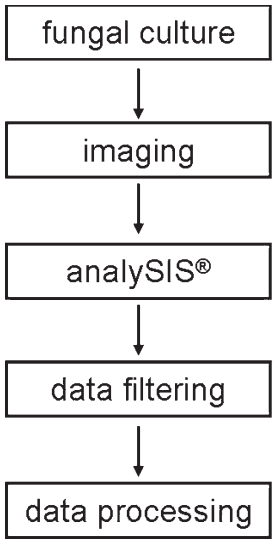


Fig. 1 Flow chart of the steps to be performed during the process of pellet morphology determination.

In the following, using shake flask cultures of the basidiomycete *Coprinopsis cinerea*, a protocol is defined to be applied for morphological studies of fungal pellets and bioreactor cultures. For the analysis of the fungal morphology in liquid cultures, complete shaken flask cultures or samples taken from the bioreactor cultures are poured onto a bordered glass plate. Detailed images are taken with a CCD camera and the software analySIS® (Soft Imaging System GmbH, Münster, Germany) is used to analyse the fungal morphology from obtained images. The so obtained raw data are filtered by defined parameters that unequivocally distinguish pellets from other objects (small hyphal fragments and loosely aggregated hyphal filaments). Data processing obtains afterwards the final data describing the actual fungal morphology (Fig. 1).

Material and Methods

Fungal cultures

A monokaryotic *C. cinerea* strain (FA2222) transformed with the pYSK7 plasmid expressing the laccase gene *lcc1* from *C. cinerea* under control of the *gpdII*-promoter of *Agaricus bisporus* was used (11). The pYSK7-transformant (clone 26) was grown on YMG -agar (per litre: 4 g yeast; 10 g malt extract; 4 g glucose; 10 g agar) plates at 37^0 C until the mycelium reached the edge of the petri dish. Sterile water (ddH_2O) was poured onto the plates and the mycelium with the asexual spores was scraped with a sterile spatula from the agar. Spore solutions were filtered using a sterile funnel filled with glass wool in order to hold back the fungal hyphae. A Thoma counting chamber was used to determine spore concentrations in the solutions.

For shake flask cultures, pre-cultures inoculated with 10^6 spores/ml medium were prepared in 500 ml flasks filled with 50 ml of modified Kjalke medium [(12); per litre: 10 g yeast, 20 g glucose, 0.5 g $CaCl_2$ x 2 H_2O, 2 g KH_2PO_4, 50 mg $MgSO_4$ x 7 H_2O]. Inoculated flasks were incubated for 4 days at 37^0C as stationary (standing) cultures. Pre-cultures were homogenised by an Ultra-Turrax® (IKA Werke GmbH & Co. KG, Staufen, Germany) for 30 sec at 8000 rpm and 30 sec at 9500 rpm (rotations per minute). For main-cultures, 500 ml flasks

with 100 ml of sterile modified Kjalke medium supplemented with 0.1 mM $CuSO_4$ were inoculated with each 5 ml of the homogenised pre-culture. Cultivation took place at 25°C and 37°C on a rotary shaker at 120 rpm for 4 and 10 days, respectively. Per culture day and cultivation temperature, two or three parallel cultures were analysed.

Fungal shaken cultures were poured onto a glass plate (28.5 cm x 38.5 cm, Fig. 2) whose edges were sealed by a silicone border in order to keep the liquid samples on the plate. Water (200 to 300 ml) was added to equally distribute the mycelium as a monolayer of pellets on the glass plate. When necessary, pellets lumped together were manually dispersed with the help of a spatula or forceps.

Total fungal biomass was determined after taking images by filtering complete cultures through a Büchner funnel containing a cellulose filter of known dry weight. The filters together with the wet biomass were dried at 80^0C and the dry weights of the biomass were determined.

Imaging

To record pellet growth, the glass plate with a fungal sample was placed onto the illuminated translucent plate of a camera stand (Kaiser Copylizer eVision ini.tial HF, Kaiser Fototechnik GmbH & Co. KG, Buchen, Germany) with a digital CCD camera (Color View II, Soft Imaging System GmbH, Münster, Germany) installed at a distance of 28 cm to the glass plate. Images were recorded with the CCD camera to which a Lametar 2.8/25 objective (Jenoptik GmbH, Jena, Germany) was fixed. Three non-overlapping photographs (dashed lines in Fig. 2) were taken of each culture.

Analysis of the images

Pictures were evaluated using analySIS® (Soft Imaging System GmbH, Münster, Germany), a software tool for the analysis of microscopic images of biological materials. The magnification, defined by the specific objective at the CCD camera used at a distance of 28 cm to the specimen, was set to be 0.132 (equivalent to 0.0418 mm/pixel).

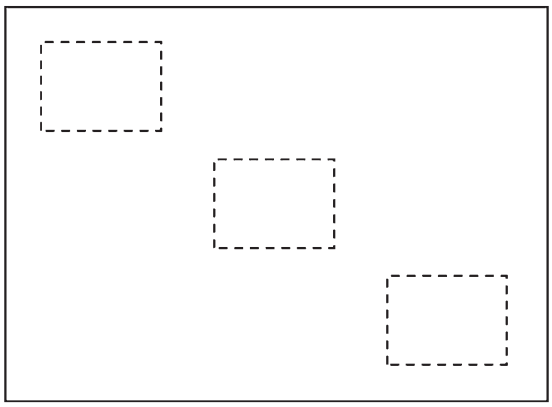


Fig. 2 Scheme of a glass plate (solid line representing the silicone border; size 28.5 cm x 38.5 cm) onto which liquid fungal cultures are poured and the positions of fields (dashed lines; each field size 6.4 cm x 8.5 cm) of which photographs are routinely taken.

The subsequent steps for the process of automated image analysis using predefined functions of the software analySIS® are explained in the following:

- Function RGB reduction: The coloured photograph (Fig. 3A) were reduced to their RGB (red, green and blue) colours. Of the three pictures obtained, the green monochromatic images (Fig. 3B) gave the best contrast between pellets and background. The green monochromatic images were therefore used for further analysis in form of a grey value scale.
- Function Define ROI: A region of interest (ROI, Fig. 3C) had to be defined and loaded into a picture. This ROI defines the area in the image, where objects were analysed. With the magnification as defined above, a ROI of 40.66 cm^2 was used (Fig. 3C).

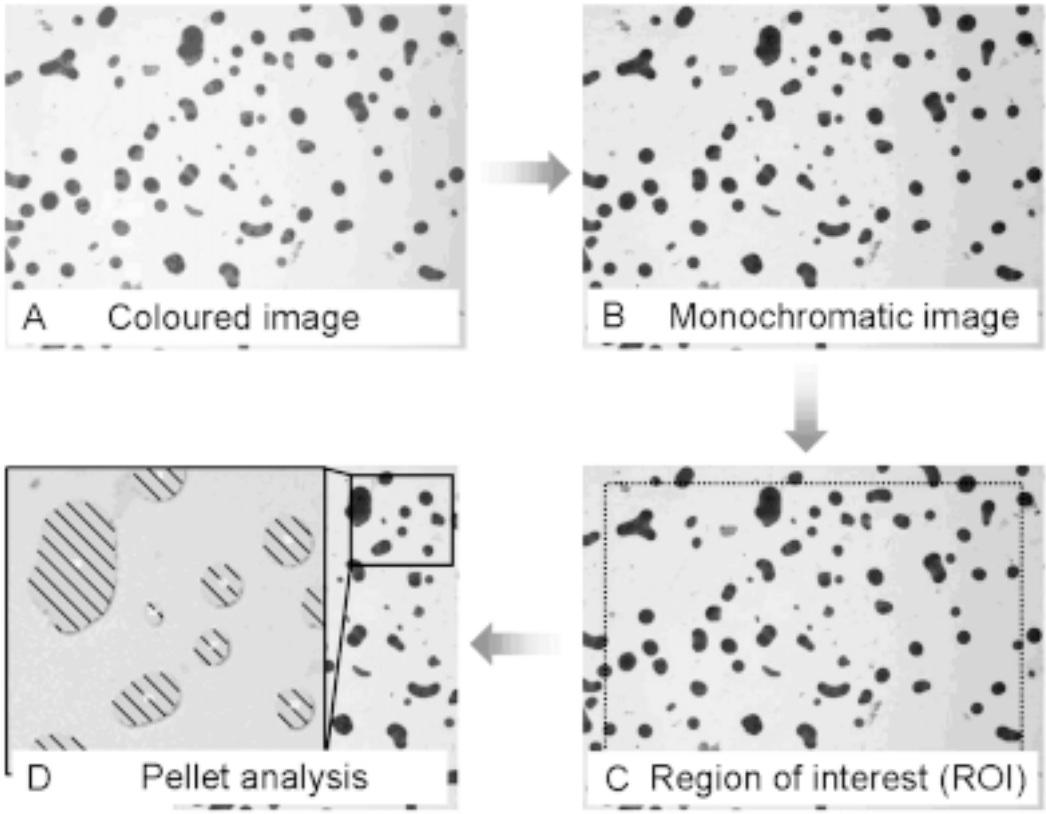


Fig. 3 Processing of photographs of fungal cultures with the analySIS® software. A photograph of the fungal culture (A) is reduced to a monochromatic image (B). A region of interest (ROI) for pellet determination is defined (C) and, after setting a specific threshold for best distinction of pellets and background (see Fig. 4), the pellets are detected by analySIS® (D) as documented in Fig. 5.

- Function Define measurement: In analySIS®, a pre-defined set of object parameters can be chosen of which the following were selected for pellet detection and description (Fig. 3D): grey value mean, area, convexity, shape factor and sphericity. Moreover, the average diameters of the individual pellets were determined in analySIS® from 180 measurements of diameters per object, i.e. one at every angle.
- Function Set threshold: To distinguish the compact mycelial pellets from loose hyphal fragments as background, the settings of the analySIS® software had to be manually adjusted in the programme to a specific threshold determined by the background in a given monochromatic image. Usually, the threshold was set to 180 in order to reject the large peak of smaller objects (hyphal fragments) and other background within the bimodal distribution of greyness level as shown in Fig. 4.
- Function Define Detection: A projected area per object is determined by counting the pixels per object being connected within the object area due to a same grey value contrast. A minimum pixel amount of 10 per object was set to exclude any remaining

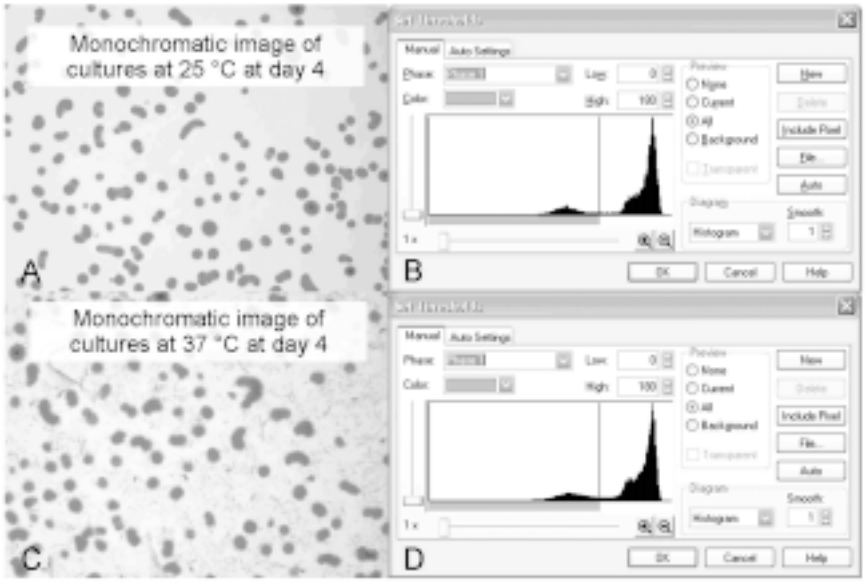


Fig. 4 Differences in the background noise of culture images taken at day 4 of cultivation of *C. cinerea* in modified Kjalke medium at 25°C and 37°C, respectively (A and C). The bimodal distribution of greyness levels (on a scale from 0 = black to 255 = white; black is at the left and white is at the right side of the histograms shown) deduced from these images and the corresponding settings for defining the background threshold for the monochromatic images are presented in B and D, respectively.

smaller loosely aggregated filamentous fragments from further analysis. Detection of the fungal pellets covered by the ROI was done twice: Include and Exclude. In case of the Include function, all pellets inside the ROI plus those that cross the ROI borders were measured. In case of the Exclude function, only pellets localised fully within the ROI were detected (Fig. 5).

- Function Particle Results: Parameters for all pellets were calculated for the Include and for the Exclude setting, respectively, and obtained data were saved in separate spreadsheet files, where each row presents the information for one specific object and each column specific object parameters as defined above.

Data processing

All calculations were done for the raw data set and for filtered data sets. Object data from the three analysed images per culture were combined (Include and Exclude measurements separately) and the amount of objects, the average value of the projected object areas and the average object diameter were calculated with the help of a self-implemented programme constructed with pre-defined analysis objects (average with standard deviation and general histogram used to automatically cluster objects into 19 distinct groups of 0-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8, 0.8-1.0, 1-2, 2-4, 4-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18, 18-20, 21-25, 25-30, 30-50 and 50-500 mm^2 pellet area) of the programming language LabView™. The object data were saved automatically in individual spreadsheet files for

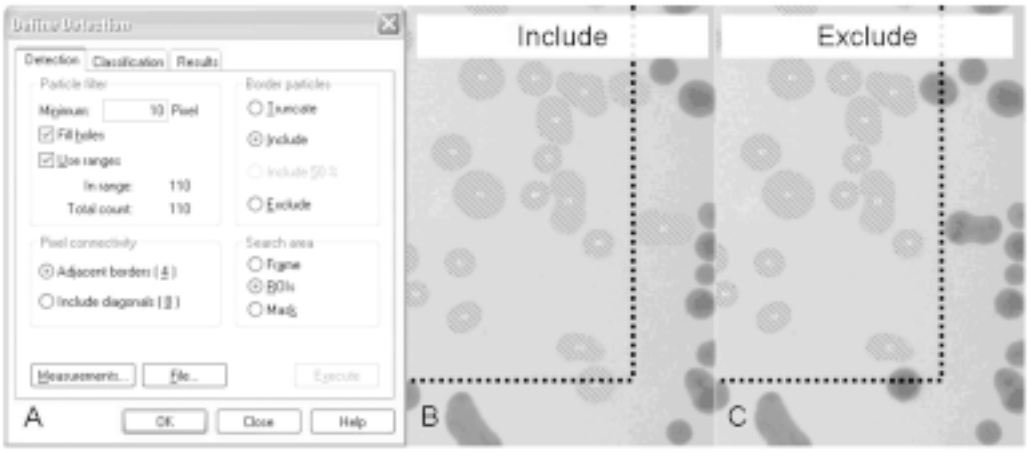


Fig. 5 Each image was analysed twice by analySIS®. The settings for the determination (A) were used for an Include-recording (B) of the ROI (dashed line). For an Exclude-recording (C), the border particles were respectively set onto function Exclude.

further analysis of Include and Exclude data in Excel (Excel 2002, Microsoft). The average Include data and, respectively, the average Exclude data for the projected area and the object diameter from two or three parallel cultures per growth condition were combined and averaged. Next, values derived from Include and Exclude measurements were averaged in order to include a proportionate fraction of objects into the analysis that either touch or cross the ROI border lines so that the complete ROIs are best covered. For calculating the average amounts of objects per culture, the total amounts of objects in the three ROIs analysed per culture were determined and averaged between the cultures of a same growth condition separately for the Include and Exclude data set, respectively. Subsequently, the values derived from Include and Exclude measurements were averaged and this average value was multiplied by a factor of 9.0 resulting in the average number of pellets per 100 ml culture liquid.

Results

Upon inoculation of fresh *C. cinerea* cultures in modified Kjalke medium, compact round to oval pellets dominated at day 4 of cultivation in the shaken cultures, both at 25^0C and 37^0C. In the monochromatic images of the cultures, pellets had a strong grey-shade. However, at the higher cultivation temperature the background including fine hyphal material was generally higher and, in addition, lighter shaded loosely aggregated hyphal fragments were also present (Fig. 6). In older cultures at day 10 of cultivation at 25^0 C, there were no larger differences of pellet structure in the views of the cultures as compared to day 4 of cultivation. In contrast, in addition to dark-shaded compact pellets and loose aggregates of filamentous fragments, pellets of less dense structure and lighter grey shading accumulated in the aging cultures grown for 10 days at 37^0C (Fig. 6).

Particularly in the 37^0C cultures, the projected areas of some of the loosely aggregated filamentous fragments were larger than the minimum size of 10 pixel (projected area = $0.017mm^2$) per object as fixed for pellet recognition in the analysis software (see below and Fig. 7 for further details). Such filamentous fragments will thus interfere with pellet analysis.

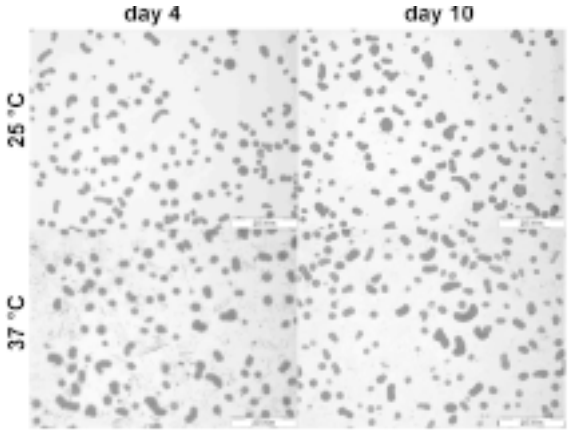
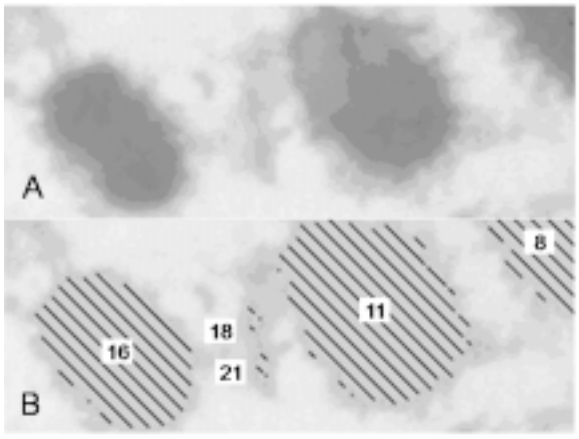


Fig. 6 Monochromatic images of *C. cinerea* cultures grown for different times in modified Kjalke medium at 25°C and 37°C, respectively. The size bars in the images represent 20 mm.

In addition to the mean grey value and the projected area of an object, convexity, shape factor and sphericity (Table 1) were therefore chosen as other parameters in order to better describe pellet morphology and clearly distinguish between fungal pellets and loose filamentous aggregates. Threshold values for filtering as given in Table 1 were empirically defined in order to confidently exclude any loose filamentous aggregates from further pellet analysis.

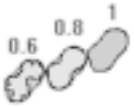
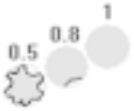
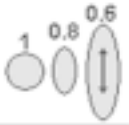
For processing the raw data sets in the spreadsheet files obtained from the function 'Particle Results', at least 3 of the measured values per row (each row defining one detected object) have to pass the defined threshold values for positive filtering of an object as a pellet. If not achieved, a row will automatically be deleted from the raw data set. Fig. 7 demonstrates examples of such positive and negative filtering. Each recognised object in Fig. 7A was identified by a number and overlaid with a shading representing the projected area of an object (Fig. 7B). When comparing the objects in the original monochromatic image (Fig. 7A) with the shades overlaid by the analySIS® programme (Fig. 7B), it becomes clear that the objects with the number 18 and 21 having a projected area above 10 pixel (0.06 and 0.09 mm^2, respectively) are not pellets but loosely aggregated filaments. The filter implemented for the pellet data however remove these objects from the raw data set, because the



Object No	Grey value mean	Area [mm²]	Convexity	Shape factor	Sphericity	Pellets [yes/no]
8	140	12.12	1.05	0.48	0.34	yes
11	149	9.57	0.89	0.51	0.51	yes
16	144	6.47	0.94	0.75	0.43	yes
18	179	0.06	0.75	0.48	0.87	no
21	178	0.09	0.83	0.44	0.43	no
Threshold value	≤162	≥0.1	≥0.7	≥0.5	≥0.3	

Fig. 7 Monochromatic pellet images taken from a *C. cinerea* culture at day 4 of cultivation at 37 °C (A), shades overlaid by analySIS® on present objects defining object areas for calculation (B) and a data sheet of parameters measured for these objects. The data for each object obtained by the software were used for distinguishing pellets and loosely aggregated hyphal fragments, respectively. Values shaded in grey did not reach the defined limits.

Table 1 Parameters for definition of pellet morphology

Parameter provided by the analysis® software		Description as given by the analysis® software	Threshold value for positive filtering as set in this study
	Grey value mean	The arithmetic mean of all grey values of the particle	≤ maximum object grey value in a given image minus 10 % of the maximum grey value *
	Area	The area of a particle is (number of pixels of the particle) times (calibration factors in X and Y direction)	≥ 0.1 mm^2
	Convexity	The fraction of the particle's area and the area of its convex hull	≥ 0.7
	Shape factor	The shape factor provides information about the "roundness" of the particle. For a spherical particle the shape factor is 1, for all other particles it is smaller than 1	≥ 0.5
	Sphericity	Describes the sphericity or 'roundness' of the particle by using central moments	≥ 0.3

* Note that for the grey value mean, the absolute values may vary between different images because of the different background values; e.g. for a maximum grey value of 180 measured in an image, the maximum value for positive filtering is 162 (= 180 - 10% of 180).

threshold values for parameters grey value, area and shape factor were not reached (see Fig. 7 data sheet). In other cases of loosely aggregated filaments, the threshold values e.g. for the combination grey value, shape factor and sphericity or for the combination grey value, convexity and shape factor were not reached (data not shown). In extensive empirical data analysis, a minimum of three parameters were found to be necessary for save pellet filtering from other objects in order not to eliminate very small pellets of low grey shading and/or low convexity from the raw data sets (not further shown).

The defined filter were applied to the raw data sets (Include and Exclude separately), the Include and Exclude data were combined and averaged as described in the methods in order to obtain the average number of pellets per culture. After applying the filter, the number of objects from the raw data sets reduced by 2-24%, respectively, depending on the type of cultures analysed (Table 2). In total, about 225 to 400 pellets (30 to 160 per individual image) were analysed per individual culture. The calculated total pellet numbers per culture varied from about 2300 to 3400 (Table 2).

With the time in 25^{0}C cultures, there was a strong increase in absolute numbers of detected pellets per culture (about 2400 pellets at day 4 to about 3400 pellets at day 10) and a little increase in average pellet area [about 4.8 mm^2 at day 4 to

about 5.0 mm^2 at day 10; both values corresponded well with the peak value in histograms of the individual pellet areas (not shown)], whereas the average pellet diameter decreased slightly from about 2.57 mm at day 4 to about 2.51 mm at day 10. The percentage of non-pellet objects (loose hyphal aggregates) increased from 2% at day 4 of cultivation to 9% at day 10 of cultivation. These data from automated image analysis confirm the impression of the former rough overview that pellets in cultures at 25^{0}C change little in shape and size within the 6 following days of incubation.

The situation in the 37^{0}C cultures was different. Absolute pellet numbers decreased from about 3000 pellets per flask at day 4 of cultivation to 2300 per flask at day 10 of cultivation, but the average pellet area [corresponded again well with the peak value in histograms of the individual pellet areas (not shown)] increased from 5.1 mm^2 at day 4 of cultivation to about 5.5 mm^2 at day 10 of cultivation along with the average pellet diameter (from 2.4 mm at day 4 to 2.7 mm at day 10). As documented in Fig. 8, the 4 day-old cultures at 37^0 C had generally a higher background of small hyphal fragments and a larger fraction of total detected objects (21%; Table 2) were identified in the raw data set as loose filamentous aggregates. At day 10 of cultivation, the amount of hyphal fragments in the background was lower (Fig. 8) and, in parallel, also the amount of loose aggregated filaments detected by the automated image analysis (9%; Table 2). In summary, the data imply that major morphological changes occur at 37^{0}C during the period of cultivation from day 4 to day 10.

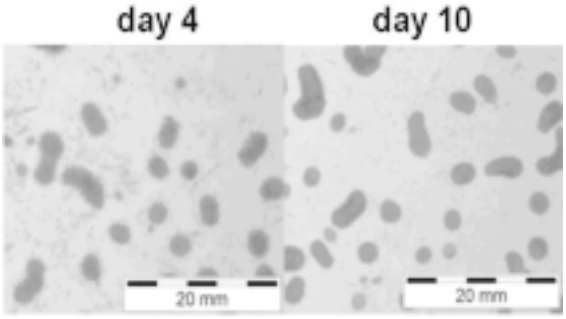


Fig. 8 Enlargement of sectors of photographs from Fig. 6 showing fungal pellets, small hyphal fragments and loose filamentous aggregates in the culture broth of *C. cinerea* cultivated in modified Kjalke medium at 37 °C for 4 and 10 days, respectively.

At both growth temperatures, the differences in average pellet numbers and average pellet areas (Table 2) corresponded well with changes in biomass. The 25^{0}C cultures had a mycelial biomass of 5.6 ± 0.2 g/l and 8.3 ± 0.1 g/l at day 4 and day 10 of cultivation, respectively. The 37^{0}C cultures had a biomass of 8.3 ± 0.2 g/l and 5.5 g/l ± 0.3 g/l at day 4 and day 10 of cultivation, respectively. At 25^{0}C as the suboptimal temperature for growth of *C. cinerea* (14), 10 days are obviously required to achieve highest biomass yields whereas the reduction in total biomass at 37^{0}C implies that the fungus is at day 10 of cultivation already in a major phase of biomass degeneration.

Discussion

Pellet morphology is known to be influenced by culture conditions and, in turn, to affect biomass formation and production yields in submerged fungal fermentations (2, 13). Effects of temperature and age on submerged growth of *Ccinerea* used as an example in this study are obvious from the photographs shown in Fig. 6 and Fig. 8 and from the data presented in Table 2. This fungus has its growth optimum at 37^{0}C (14). Accordingly, we observe that cultures at this temperature have a faster growth rate, reach the highest possible biomass levels earlier and undergo faster biomass degeneration than cultures kept at the lower temperature of 25^0 C (Table 2 and Rühl et al. unpublished results). With the lower growth rate and the delay

Table 2 Pellet distribution in *C. cinerea* cultures at day 4 and day 10 of cultivation in modified Kjalke medium

Culture age	Cultivation temperature	Raw object data				Filtered object data				Reduction in object number per flask [%]
		No of total analysed objects*	Object area [mm^2]	Object diameter [mm]	No of objects per flask	No of total analysed pellets*	Pellet area [mm^2]	Pellet diameter [mm]	No of pellets per flask	
Day 4	25 °C	804	4.85 ± 4.24	2.57 ± 1.32	2411 ± 63	786	4.81 ± 3.20	2.57 ± 1.06	2355 ± 58	2
	37 °C	860	4.13 ± 5.27	2.06 ± 1.88	3866 ± 283	677	5.10 ± 5.35	2.40 ± 1.80	3043 ± 86	21
Day 10	25 °C	1247	4.58 ± 3.89	2.36 ± 1.39	3739 ± 240	1139	4.95 ± 3.67	2.51 ± 1.24	3415 ± 267	9
	37 °C	839	5.24 ± 5.51	2.66 ± 1.81	2516 ± 199	763	5.49 ± 4.72	2.70 ± 1.38	2286 ± 223	9

* For each culture condition, three flasks were analysed except for day 4 of cultivation at 37°C where only two flasks were used. Absolute numbers of objects analysed per culture condition were added from the two or three cultures, respectively.

in biomass degeneration, the overall pellet structures of the cultures grown at 25^{0}C were much more compact and uniformly than the structures of pellets grown at 37^{0}C. Moreover in the 25^{0}C cultures, there was much less background of small hyphal fragments and of loose aggregated hyphal filaments (Fig. 6 and Fig. 8). *C. cinerea* is a filamentous basidiomycete that can easily be manipulated by genetic transformation (15, 16). The fungus has thus found interest for production of glycosylated enzymes from higher basidiomycetes that will not or only poorly be expressed and most possible wrongly be glycosylated in established ascomycete systems for recombinant enzyme production (11, 17, 18). Laccases are for examples enzymes in focus for recombinant production with *C. cinerea* in efficient fermentation processes (11, 19, Rühl et al. unpublished results). Studying fungal pellet morphology will be beneficial for optimising the fermentation processes and enzyme production yields.

An efficient and easy to apply routine technique for observation and large scale pellet analysis for *C. cinerea* but also other filamentous fungi is presented in this work. By taking three images per culture, 225 to 400 pellets per culture are simultaneously and easily characterised by applying specific functions of the commercial analySIS® software. If higher pellet numbers of the same culture are required, further non-overlapping images might easily be taken of other areas of the glass plates onto which the pellets were poured (compare Fig. 2). Pellets for taking photographs are easily spread on the bordered glass plate from cultures with a pellet number of about 2000-2500 per 100 ml. With increasing pellet numbers, in some cases pellets were found packed in densely clusters on the glass plate and had to be separated manually with a spatula or forceps. When wanting most accurate numbers of pellets per culture, simply diluting the cultures is not as advisable since the fungal pellets sink quickly and are difficult to be kept evenly dispersed in solution. Evenly distributing of

pellets from dense cultures on the glass plate was therefore in our hands the most time consuming step in the procedure leading in the worst case to 20 min preparation time per culture until images could be taken. Subsequent usage of the analySIS[Æ] software helped in a fast and, especially, representative determination of pellet morphology, as up to 400 pellets (or more) can be analysed per culture in a few minutes. Considering the parameters grey value, pellet area, convexity, shape factor and sphericity with the implemented filter, fungal pellets and filamentous aggregates could clearly be recognised at the same time and distinguished as such both when relative low and when relative high amounts of these two growth forms were obtained in *C. cinerea* cultures. Such data will allow to quantify the nature of mycelial aggregates, fragmentation of pellets as well as pellet regrowth (20).

Most literature studies on fungal morphology during submerged cultivation consider only small samples of pellets (see examples for basidiomycetes in the introduction). Gehrig et al. (7), in contrast, observed approximately 2000 pellets by image analysis in a *C. striatus* culture by taking pictures of pellets (1-6 mm in diameter) present in a reference area of 15 mm x 15 mm. Total pellet volume was defined by the authors from determination of an average pellet diameter measured from the images of fungal pellets. Moreover, Kelly (21) studied the ascomycete *Aspergillus niger* and similarly determined the pellet diameter of 500 to 1000 pellets per *A. niger* culture. The description of the method used by Kelly (21) is not presented in much detail in the publication but it appears that also this author used a relatively small reference area with maximum 30 to 35 pellets when these were comparably small and laying quite crowded on the area, forcing the researcher to take many different images for obtaining high numbers of different pellets for large scale analysis. In other cases, pellets of *A.niger* were observed on 1 mm-deep cavity slides in case of very small sizes under a microscope or in case of larger pellets on an adjustable camera stand equipped with a CCD camera fitted with a macroscopic zoom lens. Since the size of the images are restricted by the width of the cavity slide, also in these studies numerous pictures have to be taken for analysing high pellet numbers (20, 22, 23).

The protocol presented here allows analysis of much higher numbers of pellets (60-130 pellets) per single image. Moreover, additional parameters of morphology were considered in this study. For simplification of analysis, Gehrig et al. (7) and Kelly (21) assumed a spherical shape of the fungal pellets. The photographs and images in Fig. 3 to 8 show that fungal pellets may be stretched in one dimension and that pellets can be of fringy shape. Therefore, a definition of the projected area calculated from all connected pixels of an object by the software analySIS® will give much more accurate pellet area values than is possible by only considering the pellet diameters. Although not analysed in more detail in this study, the independently collected individual values for the extra parameters convexity, shape factor and sphericity of the pellets are available from the same analysis if a more deep insight into such extra growth parameters for a more detailed description of pellet morphology in *C. cinerea* will be required. Furthermore, analysing the individual grey shades of pellets can serve to define a relative distribution of more compact and less dense pellets in a culture. The usefulness of such data for morphological classification of fungal pellets by automated image analysis has been demonstrated before for *A. niger* (22, 23).

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Variabilty for quality traits in a global germplasm collection of ginger (*Zingiber officinale* R.)

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Abstract

Fourty six ginger accessions originating from India, China, Pakistan, Brazil, Jamaica, Oman, Nepal, Nigeria and Queensland analysed for the quality attributes revealed that the primitive types/land races are rich in oleoresin and essential oil and low in crude fiber content as compared to the improved varieties.

Key words : *Zingiber officinale, germplasm, oleoresin, crude fiber and essential oil*

Introduction

Ginger (*Zingiber officinale* Rosc.) is a major spice and medicine used all over the world. Ginger has been used as a medicine in Chinese, Indian and Arabic herbal traditions since ancient times (1). Characterized by its typical flavour and aroma, ginger is noted for its richness in oleoresin, essential oil and fiber contents. Ginger is commercially available in various forms such as fresh ginger, dry ginger, ginger powder, ginger oil, ginger oleoresin and preserved ginger. China and India are the two major producers of ginger. Cultivar diversity of ginger is very high in China, followed by India (2). The main ginger growing countries other than India and China are Jamaica, Taiwan, Sierra Leone, Nigeria, Fiji, Mauritius, Indonesia, Brazil, Costa Rica, Ghana, Malaysia, Bangladesh, Philippines, Sri Lanka, Thailand, Trinidad, Uganda, Hawaii, Guatemala and many Pacific Ocean Islands (3).

Ginger is known to vary widely for the quality traits besides yield (4). A critical analysis of variability for the important quality traits in the germplasm from different sources grown under uniform conditions/season will help us to know the spectrum of variability for oleoresin, essential oil and crude fiber in this clonally propagated crop without the confounding effects of the micro and macro climatic factors.

Materials and Methods

a) Plant materials

Rhizomes of the ginger accessions were collected from Germplasm Conservatory of Indian Institute of Spices Research, Peruvannamuzhi, Kozhikode, Kerala, India, where the germplasm is grown in cement tubs (45x45x45cm) under partial (50%) shade with half of the recommended fertilizers. This germplasm repository houses one of the world's largest collections of ginger germplasm under *Ex situ* condition. Fourty six ginger (*Zingiber officinale* Rosc.) accessions, which include released varieties, exotic and primitive types were used in the study (Table. 1)

b) Extraction of oleoresin

Oleoresin was extracted from the, dried and powdered rhizome in acetone. Ten grams of the sample was weighed and transferred to a glass column (18 x 450mm) with stopcock. Added 50ml of acetone. Allowed to stand overnight for 16 hrs at 25±2?C. The filtrate extracted through non absorbent cotton was collected in a pre-weighed 100ml beaker. Column was washed with 20ml of acetone. The extracts were pooled and evaporated to dry at 80°C over a water bath. The amount of oleoresin was estimated gravimetrically (5).

c) Estimation of crude fibre

Crude fiber from the powdered ginger sample was estimated by using a Dosi-Fibre apparatus (J.P.Selecta, SPAIN). For determining crude fibre, the organic matter in the dried residue remaining after digesting the sample with distilled sulphuric acid and sodium hydroxide was weighed (5).

d) Essential oil

The steam volatile oil from ginger was extracted by using a modified Clevenger method. The apparatus was assembled using the proper Clevenger trap (5) and was boiled for 2 1/2 hrs.

Results and Discussion

a) Oleoresin

Flavour and pungency of ginger are accumulated in the oleoresin. The oleoresin yield of the 46 ginger accessions are given in Table 2. The primitive types/land races from India registered high oleoresin content. The oleoresin content of the 46 ginger accessions ranged from 3 ('Acc. No. 59') to 82% ('Kozhikkalan'). Other primitive type ginger like 'Kakakkalan', 'Sabarimala' etc. also recorded high oleoresin yield. Higher percentage of oleoresin content in the primitive type ginger 'Kozhikkalan' is reported (6). Some other collections (land races) of ginger such as 'Vizagapatnam-1', 'Pulpally'& 'Neyyar' also showed high oleoresin percentage. Oleoresin content in ginger can range from 3 to 11% (7).

Genotypes, harvesting age, cultivation practices, choice of solvents and method of extraction etc. are known to affect the oleoresin content in ginger (8). In the present study all factors except the genotypes being common, the variability observed for oleoresin may be attributed to the effect of the cultivars. The primitive types/land races ('Kozhikkalan', 'Sabarimala', 'Pulpally', 'Vizagapatnam-1', 'Neyyar', 'Jolpaiguri' etc.) can be a better source for commercial exploitation of oleoresin.

b) Crude fiber

Crude fiber content of the ginger accessions are given in Table 2.Among the 46 ginger accessions, the exotic ginger 'Kintoki' and cultivar 'Nadia' recorded 1.3% and 1.8% crude fiber, respectively. Most of the other ginger accessions recorded about 4-5% crude fiber. High crude fiber(< 7%) was observed in the ginger accessions 'Pulpally', 'Todupuzha' and 'Vizagapattanam'. Crude fiber content in ginger usually ranges from 4.8 to 9% (9).

Fiber content is the most important criteria for assessing the suitability of ginger rhizome for specific products like ginger paste, salted ginger, ginger powder etc. For the manufacture of processed food such as jams, marmalades, cakes and confectionery too less fiber gingers are more suitable, though both low fiber and high fiber gingers are important depending on the end use. In the present study cultivars such as 'Kintoki, and 'Nadia' recorded very less fiber and hence can be exploited for making value added products like slices preserved in syrup, ginger candy (crystallised ginger), jams, cakes etc. whereas the cultivars such as 'Suruchi', 'Oman', 'Pulpally,

'Thodupuzha-2', 'Kottayam, 'Wyanad local', 'Vizagapatnam-1', which recorded high fiber content will be useful for dry ginger making.

c) Essential oil

Cumulative effect of the essential oil components imparts the perfumery smell to ginger/the essential oil**.** Essential oil from dried rhizomes of ginger cultivars/varieties are given in Table 2. The yield of ginger essential oil ranged from 0.9 to 4%. Highest percentage of essential oil content was observed in the 'Pink ginger' (4%) followed by 'Kakakkalan' (3.5%) and 'Kozhikkalan' (3%). 'Pink ginger' is a local land race of Nagaland, India and the tribal folk there prefer it as an ethnic medicine besides in a variety of meat dishes. The lowest percentage of essential oil was recorded in the cultivar 'Mananthodi' (0.9%) and exotic ginger from 'Pakistan' (0.9%). Yield of the essential oil in ginger ranges from 0.2 to 3.0%, depending on the origin and state of the rhizome (10,11). Primitive ginger from Kerala, India, namely 'Kozhikkalan' is reported to be rich in essential oil (6). The high essential oil yielding 'Pink ginger', 'Kozhikkalan' and 'Kakakkaln' can be better suited for the perfume industry.

Though the values of oleoresin, essential oil and fibre content observed in the present study are within the range reported for these constituents (7,9,10,11) some variation in the same acceesions eg 'Kottayam','Wynadu Local','Thodupuzha'and 'Suruchi' from the earlier reported values was observed. This may be due to the growing conditions of the germplasm. Quality of ginger is known to be influenced by the environment, growing conditions and the levels of shade (4,12,13).

The present study thus indicated the suitability of different genotypes namely, 'Kozhikkalan', 'Sabarimala', (primitive types from India)'Pulpally', 'Vizagapatnam-1', 'Neyyar', 'Jolpaiguri'(land races from India) for high oleoresin; 'Kintoki' and 'Nadia '(land races from Japan/India) for less fiber content and 'Pink ginger', 'Kozhikkalan' and 'Kakakkalan' (all primitive types from India) for high essential oil.

In general, the primitive types/land races, though poor yielders (yield data not shown) were better source of the quality attributes as compared to the improved types. Breeding mainly directed for high yield might have had a negative impact on the quality traits. Further, the germplasm from India is found to be more variable, notwithstanding the less number of accessions studied from other countries. This is rather expected as the center of origin of the crop falls in this region.

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Table. 1. Ginger accessions studied

Sl. No	Name	Acc No.	Remark
1	'Varada'	64	Released variety from Indian Institute of Spices Research, Calicut, Kerala, India.
2	'Mahima'	117	Released variety from Indian Institute of Spices Research, Calicut, Kerala, India.
3	'Rejatha'	35	Released variety from Indian Institute of Spices Research, Calicut, Kerala, India.
4	'Suruchi'	714	Released variety from Orissa University of Agriculture & Technology, High Altitude Research Station, Pottangi, Koraput, Oriisa, India.
5	'Suprabha'	293	Released variety from Orissa University of Agriculture & Technology, High Altitude Research Station, Pottangi, Koraput, Orissa, India.
6	'Himachal'	294	Land race from Himachal Pradesh, India.
7	'Maran'	295	Land racefromAssam, India.
8	'Nadia'	27	Land race from West Bengal, India.
9	'Karakkal'	20	Land race from Pondicherry, India.
10	'Mananthody'	244	Land race from Wynadu, Kerala, India.
11	'Sabarimala'	246	Primitive type Collected from Sabarimala forests, Western Ghats Kerala, (slender rhizome), India.
12	'Kozhikkalan'	537	Primitive type Collected from Nedumangad, Kerala (slender rhizome), India.
13	'Ellakallan'	463	Primitive type Collected from Idukki, Kerala (slender rhizome), India.
14	'Kakakkalan'	558	Primitive type Collected from Nedumangad, Kerala (slender rhizome), India.
15	'Pakistan'	733	From Pakistan.
16	'Oman'	734	From Oman.
17	'Brazil'	736	From Brazil.
18	'Jamaica'	17	From Jamaica originally.
19	'Rio-de-Janeiro'	59	From Brazil originally.
20	'Pink ginger'	731	Collected from Meghalaya state, India.
21	'Bakthapur'	563	From Nepal.
22	'Kintoki'	648	From Japan.
23	'Nepal'	575	Collected from Nepal.
24	'China'	9	Originally from China.
25	'Juggigan'	18	Originally from Nigeria.
26	'Acc. No. 50'	50	Kerala, India.
27	'Pulpally'	56	Collected from Pulpally,Kerala, India.
28	'Acc. No.95'	95	Kerala ,India.
29	'Ambalawayalan'	109	Collected from Wynad, Kerala, India.
30	'Kozhikkode'	162	Collected from Kozhikkode, Kerala ,India.
31	'Thodupuzha-1'	204	Collected from Thodupuzha, Kerala, India.
32	'Konni local'	206	Collected from Konni, Kerala, India.
33	'Angamali'	214	Collected from Angamali, Kerala, India.

34	'Thodupuzha -2'	217	Collected from Thodupuzha, Kerala, India.
35	'Kottayam'	225	Collected from Kottayam, Kerala, India.
36	'Palai'	228	Collected from Palai market,Kerala, India.
37	'Silent valley'	240	Collected from Silent valley forests of Western Ghats, India.
38	'Wyanad local'	251	Collected from Wynad, Kerala, India.
39	'Vizagapatnam-1'	411	Collected from Vizagapatnam, Andrapradesh, India.
40	'Vizagapatnam-2'	420	Collected from Vizagapatnam, Andrapradesh, India.
41	'Fiji'	430	From Queensland.
42	'Gorubathani'	515	Collected from Sikkim, India.
43	'Bhaise'	552	Collected from Kalimpong, West Bengal, India.
44	'Naval parasi'	569	Collected from Nepal.
45	'Neyyar '	650	Collected from Neyyar, Kerala, India.
46	'Jolpaiguri'	654	Collected from Jalpaiguri, West Bengal, India.

Table 2. Oleoresin, crude fiber and essential oil percentage from dry rhizomes of ginger

Sl. No	Cultivar/ variety	Oleor esin (%)	Crude fiber (%)	Essential oil (%)	Sl. No.	Cultivar/ variety	Oleor esin (%)	Crude fiber (%)	Essential oil (%)
1	'Varada'	4.0	4.0	1.7	24	'China' '	3.6	3.4	2.0
2	'Mahima'	3.7	4.8	2.1	25	'Juggigan'	4.4	3.5	1.9
3	'Rejatha'	3.6	5.8	1.0	26	'Acc. No.50'	5.3	4.5	1.9
4	'Suruchi'	3.9	6.8	1.7	27	'Pulpally'	7.0	7.2	1.9
5	'Suprabha'	4.0	4.2	1.9	28	'Acc. No.95'	3.6	5.3	1.8
6	'Himachal'	4.3	4.8	2.1	29	'Ambalawayalan'	4.5	5.3	2.1
7	'Maran'	4.3	3.7	1.1	30	'Kozhikkode'	5.1	5.6	1.6
8	'Nadia'	4.2	1.8	2.0	31	'Thodupuzha'	4.2	4.8	1.1
9	'Karakkal'	2.7	4.0	2.0	32	'Konni local'	5.3	5.9	1.8
10	'Mananthodi'	3.7	4.1	0.9	33	'Angamali'	5.1	4.3	1.7
11	'Sabarimala'	6.4	4.3	2.0	34	'Thodupuzha'	5.1	7.5	1.5
12	'Kozhikkalan'	8.2	4.3	3.0	35	'Kottayam'	5.6	8.0	1.7
13	'Ellakkallan'	3.4	4.1	2.4	36	'Palai'	3.4	4.2	1.0
14	'Kakakkalan'	5.6	5.3	3.5	37	'Silent valley'	4.7	2.8	1.8
15	'Pakistan'	4.6	3.8	0.9	38	'Wyanad local'	5.1	6.5	1.0
16	'Oman'	4.8	6.5	1.4	39	'Vizagapatnam'-1	7.1	7.0	2.0
17	'Brazil'	3.3	3.8	1.3	40	Vizagapatnam-2	5.0	4.6	1.3
18	'Jamaica'	3.9	5.5	2.3	41	'Fiji'	5.1	5.2	1.9
19	'Rio-de-Janeiro'	3.0	4.6	1.6	42	'Gorubathani'	4.8	4.1	1.3
20	'Pink ginger'	5.4	4.8	4.0	43	'Bhaise'	3.2	4.3	1.6
21	'Bakthapur'	4.8	4.4	1.7	44	'Naval parasi'	4.2	5.5	1.5
22	'Kintoki'	3.3	1.3	1.5	45	'Neyyar'	6.2	4.2	2.3
23	'Nepal'	5.0	5.3	1.8	46	'Jolpaiguri'	6.0	5.3	2.1

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Antimicrobial activity of *Abelmoschus moschatus* leaf extracts

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Abstract

Hexane, ethyl acetate, methanol and aqueous extracts prepared from the leaves of *Abelmoschus moschatus* were evaluated for their antimicrobial activity against a number of pathogens by using disc diffusion assay method. Clear zones of inhibition were reported for *Staphylococcus aureus, Bacillus megaterium, Shigella flexneri, Proteus mirabilis, Proteus vulgaris* and *Cornebacterium diphtheriae.* After performing a bioassay guided fractionation of the eight hexane fractions, it was revealed that the fraction exhibiting major antibacterial activity against *C. diphtheriae* contained terpenoid oil.

Key words: Antibiotic, bioactivity guided fractionation, crude extracts, disc diffusion assay.

Introduction

It is well known that infectious diseases are responsible for a high proportion of health problems, especially in developing countries. Microorganisms have developed resistance to many antibiotics due to their frequent use. The situation has created immense clinical problems for infectious disease treatment. More scientists are in search for new antimicrobial substances derived from plants. Historically, plants provide us with a good source of anti-infective agents. Emetine, quinine, berberine etc. remain highly effective drugs in the fight against microbial infections (1). In the traditional systems of medicine, plants are used in the form of crude extracts, infusions and powders to treat common infections without scientific evidence of efficacy (2). Therefore it is of great interest to screen these plants to validate their use in traditional medicine and to reveal active principles through isolation and characterization. Once their therapeutic action(s) is/are established, they may serve as an important source for designing new and more effective drugs for chemotherapy.

In the present study the antimicrobial properties of *A. moschatus* have been evaluated. *A. moschatus* is a known medicinal plant used for the cure of bacterial diseases in the Indian traditional system of medicine (3-4). The plant is used for the treatment of gonorrhea, leucoderma, diabetes, cramps, poor circulation, and aching joints (5). The microbes used in the present study for evaluation of antimicrobial activity of *A. moschatus* were *S. aureus (*causative agent of hospital acquired surgical wound infections*), B. megaterium* (causative agent of diseases like meningitis, endocarditis, conjunctivitis and acute gastroenteritis*), S. flexneri* (common pathogen for diarrhea*), Proteus species (*urinary tract and wound infections*), C. diphtheria* etc. The seeds of the plant are known for musk like essential oil. However, these are used as antiseptic, antispasmodic, opthalmic, diuretic, deodorant, and to sweeten the breath (6-

7). Major reports of isolation and identification of secondary metabolites come from the seeds of *A. moschatus* that include: ambrettolic acid, β-sitosterol, β-sitosterol-β-D-glycoside, farnesol, and furfural (8). A brief overview of the traditional medicinal use of *A. moschatus* indicates its effectiveness in the treatment of various bacteriological and fungal pathogenesis. *In vitro* activity of extracts prepared from the leaves were evaluated against a number of pathogenic microorganisms to validate and provide a scientific basis to support these claims.

Materials and methods

Plant material

A. moschatus was grown in the Green House of the School of Biotechnology, Devi Ahilya University, Indore. The plants were used for their supply of the leaves.

Bacterial strains

The test microorganisms included a number of clinical isolates listed in Table 1 procured from Choithram Hospital and Research centre, Indore.

Preparation of crude extracts

Leaves of *A. moschatus* were washed thoroughly under tap water and shade dried at room temperature (25-30^{0}C) until a constant weight was obtained. The dried leaves were pulverized with a mechanical grinder. Seventy grams of pulverized powder was defatted by soaking in 100 ml of hexane. The soaking sample was kept for 48 h on a rotary shaker at room temperature. Hexane was decanted every 48 h and fresh hexane was added. This was repeated until the sample was colorless. The hexane extracts were pooled and concentrated under vacuum until dry product was collected. The residue of the leaf powder left after hexane extraction was further extracted with solvents of increasing polarity such as ethyl acetate, methanol and water. The ethyl acetate, methanol, and aqueous extracts obtained were concentrated under vacuum and stored at 4^{0}C. The percent yield of each extract was calculated (9).

Disc diffusion assay for microbial sensitivity testing

Agar disc diffusion assay for screening the anti-bacterial potential of hexane, ethyl

Table 1. List of test pathogens along with the (+) ve controls used in the disc diffusion assay

Microorganism	Zone (in mm)	Amount (μg/disc)	(+)ve control (antibiotic)
Escherichia coli	17	10	Ampicillin
Escherichia coli	18	10	Ampicillin
Bacillus megaterium	17	50	Chloramphenicol
Bacillus subtilis	18	50	Chloramphenicol
Proteus mirabilis	16	50	Chloramphenicol
Proteus vulgaris	19	15	Erythromycin
Klebsiella pneumoniae	26	50	Chloramphenicol
Cornebacterium diphtheriae	24	50	Chloramphenicol
Candida albicans	24	50	Chloramphenicol
Pseudomonas aeruginosa	28	50	Chloramphenicol
Shigella flexneri	13	50	Chloramphenicol
Salmonella typhii	17	50	Chloramphenicol

acetate, methanol, and aqueous extracts of *A. moschatus* leaf powder was carried out as described by Murray et al. (10). The dried extracts were dissolved in their respective solvents to a final concentration of 0.1, 1 and 2 % and sterilized by filtration through a 0.45 µm membrane syringe filter. The sterile discs (6 mm in diameter) were soaked in their respective extracts for complete saturation. Afterwards, discs were completely dried to ensure complete evaporation of the solvent. The discs were stored under aseptic conditions. A 5µl of each bacterial strain was inoculated in 5 ml of Mueller Hinton broth (Meat infusion 2.0 g/l; casein hydrolyzate 17.5 g/l; starch 1.5 g/l) in a test tube and incubated at 37^0C for 24 h. Thereafter, Mueller Hinton Agar No. 2 (Mueller Hinton broth with agar-agar 13.0 g/l) was prepared and poured in sterile petri plates. The test strain 200 µl (inoculum size 10^8 cells/ml) was evenly spread on agar petri plates with a sterile glass spreader. Sterile discs impregnated with the respective extracts were transferred aseptically to the agar plates containing the inoculum. Seven discs were placed per plate. Each test was repeated three times with a positive control (antibiotic respective for each microorganism) and a negative control (solvent disc). After incubation at 37^0C for 24 h, the plates were examined for development of zones of inhibition. Microbial growth was determined by measuring the diameter of zone of inhibition in millimeter by the antibiotic zone scale.

Bioactivity guided fractionation of active crude extracts

Based on the results of bioassay and Thin Layer Chromatography (TLC) profile, the hexane extract of *A. moschatus* leaf powder was further fractionated by silica gel adsorption column chromatography. Silica gel is a 3–D polymer of tetrahedron groups of silicon oxide ($SiO_2.H_2O$) consisting of exposed silanol groups that act as active centers for formation of H–bonds with compounds being chromatographed. A thick wall glass column size 45 x 4.5 cm was packed with a suspension of silica gel (mesh size 60 -120; Merck) in hexane with a bed height of 20 –30 cm and 40-60 cm head space. A 100 g of silica / g of the crude extract was used for column packing. After equilibration, crude active hexane fraction was absorbed on minimum amount of silica and loaded on top of the column. Solvents of increasing polarity (mixture of hexane – ethyl acetate) were passed through the column for elution of the sample. A linear flow rate of 2 ml/ min was maintained and fractions of 30 ml were collected. The 30 ml fraction samples were then subjected to TLC. The identical fractions were pooled and concentrated to dryness under vacuum. The different fractions obtained through fractionation of *A. moschatus* hexane extract were subjected to disc diffusion assay for identification of bioactive fractions.

Results and Discussion

The percent yield of hexane, ethyl acetate, methanol and aqueous extracts of *A. moschatus* were found to be 4.4 %, 4.2 %, 14 % and 32.66 % respectively. TLC profile of the crude extracts indicated positive tests with Liebermann-Burchard reagent (LB reagent), Noller's reagent and tetranitromethane revealing presence of terpenoids. The samples were also positive for alkaloids and saponins as tested by standard phytochemical methods (11). These compounds have been isolated from numerous plants and are known to exhibit a broad spectrum of anti-microbial activities (12-15).

The results of the antimicrobial activities of the hexane, ethyl acetate, methanol and aqueous extracts of *A. moschatus* leaf powder extracts by the disc diffusion assay method indicated that they were active against a number of tested pathogenic microorganisms (Table 2). There was significant antimicrobial activity of the hexane extract against *C. diphtheriae,* the causative agent of diphtheria. A 2 %

Table 2. Anti-Bacterial disc diffusion assay of *A. moschatus* leaf extracts

Organisms	Hexane Extract (%)			Ethyl acetate extract (%)			Methanol extract (%)			Aqueous extract (%)		
	0.1	1	2	0.1	1	2	0.1	1	2	0.1	1	2
E. coli	-	T	-	-	T	T	-	-	-	-	-	T
S. aureus	-	T	T	-	T	-	-	10±0.2	10±0.2	-	-	-
B. megaterium	-	-	-	-	T	12± 0.5	-	-	-	-	-	-
B. subtilis	-	T	T	-	-	-	-	-	-	-	-	-
P. mirabilis	T	T	14±0.2	-	-	-	-	-	-	-	-	-
P. vulgaris	T	10±0.2	16±0.2	T	T	T	T	16±0.2	16±0.2	-	-	T
K. pneumoneae	-	-	-	-	-	-	T	T	T	T	T	T
C. diptheriae	-	-	19± 0.2	-	-	T	-	15±0.2	15±0.4	-	-	-
C. albicans	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-
S. flexneri	-	-	16±0.2	-	-	-	-	-	-	-	-	-
S.typhii	-	-	-	-	-		-	-	-	-	T	T

All the zones are in 'mm' and each value represents an average of three replications. T- Activity in traces (zone of inhibition < 10 mm); ± is the standard error.

concentration of the hexane extract showed a zone of inhibition of the size 19 mm. Hexane extract also inhibited the growth of *Proteus* species (*P. mirabilis* and *P. vulgaris*) and *Shigella flexneri*. The *P. mirabilis* has the ability to produce high levels of urease making the urine more alkaline. If left untreated, the increased alkalinity can lead to the formation of crystals of struvite, and calcium carbonate. *P. vulgaris* is known to cause urinary tract and wound infections. *S. flexneri* is common pathogen for diarrhea. The ethyl acetate extract at a concentration of 2 % exhibited activity only against *B. megaterium.* This bacterium has ability to form tough, protective endospore allowing the organism to tolerate extreme environmental conditions. It is the causative agent of diseases like meningitis, endocarditis, conjunctivitis and acute gastroenteritis in immuno-compromised patients.

Methanol extract exhibited significant activity against a number of bacteria tested, probably due to the presence of high amounts of flavonoids and alkaloids that are also known to possess antibacterial activity (16). A 1 % concentration of methanol extract inhibited growth of *S. aureus, P. vulgaris* and *C. diphtheria* (Figure 1). The *S. aureus is* a major cause of hospital acquired surgical wound infections. It is a leading cause of various ailments such as soft tissue infections, pneumonia, meningitis, boils, arthritis, osteomyelitis (chronic bone infection),

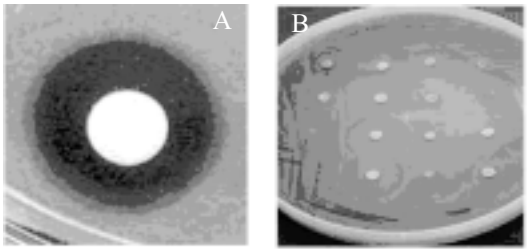

Fig.1 : Zone in inhibition obtained in anti-bacterial disc diffusion assay of A. moschatus extracts. A- Methanol extract against P. valgaris. B-Methanol extract against S. aureus

toxic shock syndrome (TSS) and scalded skin syndrome. Besides, it rapidly develops resistance to many antimicrobial agents (17). Both hexane and ethyl acetate extracts exhibited equal amount of activity against *P. vulgaris* (zone of inhibition 16 mm). Aqueous extract of *A. moschatus* did not exhibit activity against any of the tested microorganisms. Neither of the extracts was active against *Candida albicans, Pseudomonas aeruginosa* and *Salmonella typhii*. The microorganisms were least sensitive to the aqueous crude extracts due to negligible secondary metabolites in it.

Column chromatography of the bioactive hexane fraction of *A. moschatus* yielded 8 different fractions. Results of the bioassays of these fractions concluded that major activity was present in fractions 2-7 eluted by hexane containing 10-70 % ethyl acetate (Table 3). Negligible activity was demonstrated by fractions 1 and 8. The fraction 5 was found responsible for major inhibition of the growth of *C. diphtheriae*. The fractions 2 to 7 exhibited activity against *Proteus* species. The Fraction 7 exhibited strongest activity against *P. mirabilis* (zone of inhibition 28 mm) whereas all other fractions were equally effective against *P. vulgaris*. The results are very promising since *Proteus* species are responsible for 29% of all human urinary tract infections and hospital acquired wound infections. The *S. flexneri* active fractions were 3, 5 and 6. Shiga toxin produced by *Shigella* species is the cause of shigellosis (Bacillary dysentery). However, nearly 3% people with a certain genetic predisposition, namely HLA-B27 infected with *S. flexneri* subsequently develop Reiter's syndrome (pains in their joints, irritation of the eyes, and painful urination) which can last for years and can lead to chronic arthritis which is difficult to treat. The present findings may be exploited for a new drug against *Shigella* species.

The active column fractions resolved into a series of spots when TLC plates were observed under UV light and after spray detection. The fractions 1 to 5 were thick non volatile oils and developed blue green colored spots on spraying with LB reagent indicating presence of terpenoids. Noller's reagent also showed positive reaction with these fractions developing a range

Table 3. Anti-Bacterial disc diffusion assay of *A. moschatus* hexane extract and column fractions

Organism	Crude Hexane extract (%)			Hexane column Fractions (1%)							
	0.1	1	2	1	2	3	4	5	6	7	8
P. mirabilis	T	T	14±0.2	T	12±0.2	16±0.2	12±0.4	18±0.5	14±0.4	28±0.2	T
P. vulgaris	T	10±0.2	16±0.2	T	10±0.4	12±0.2	12±0.2	12±0.4	10±0.2	12±0.2	T
C. diphtheriae	-	-	19± 0.2	-	-	-	21±0.2	-	-	-	-
S. flexneri	-	-	16±0.2	-	T	16±0.2	T	12±0.4	12±0.4	-	T

All the zones are in 'mm' and each value represents an average of three replications. T- Activity in traces (zone of inhibition< 10 mm); ± is the standard error.

of colors from red, magenta to purple further confirming that all these oils are mixtures of terpenes mainly sesquiterpenes (18). Terpenes (oils) from various plants such as *Mentha piperita*, *Mentha spicata*, *Thymus vulgaris*, *Origanum vulgare*, *Origanum applii*, *Aloysia triphylla*, *Ocimum gratissimum*, *Ocimum basilicum* are shown to exhibit considerable inhibitory effects against *Pseudomonas aeruginosa, S. aureus* and many other pathogens (19-23). Efforts are in progress for complete characterization of the oils.

Increasing number of plants are being screened for their anti-microbial activity day by day. According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. *A. moschatus* shows anti-microbial activity against a wide variety of pathogens not a common characteristic as usually a single plant species does not show activity against not so many microbes tested (24-25).

Conclusion

The present study revealed the role of essential oil from leaf extracts of *A. moschatus* as a strong anti-bacterial agent against *Proteus* sp and *C. diphtheriae* under the laboratory conditions. It may be considered as a fruitful approach towards the search of new drugs. The overall results of the antimicrobial activity of the leaf extracts of *A. moschatus* justified the traditional uses of the plant and suggested that the indigenous traditional medicines could be used as a guide in the continuing search of new natural products with potential medicinal properties.

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Retrospective study of FMD serotypes and seasonal analysis of outbreaks using sero-typing strategy in Uttar Pradesh (India)

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Abstract

A laboratory evidence based study on FMD was conducted on the diagnosis of retrospective serotyping and seasonal analysis of outbreaks in cattle, sheep, pigs, goats and buffalo. The sample size was tongue epithelium, feet epithelium and blood serum (n= 40) and the samples were screened for the evidence of outbreak of FMD in Mathura, Pratapgarh, Jaunpur, Bhadoi and Etawa district of this study location. Seasonal analysis of the FMDV outbreaks revealed that out of total nineteen outbreaks, the highest 42.10% occurred in April followed by 26.30% in January and 15.80% in both December and March. The maximum numbers of outbreaks are observed in cattle followed by sheep and buffalo. For serotyping study of virus from five districts of Uttar Pradesh all the forty samples were processed using sandwich ELISA and LAB-ELISA techniques and the two-virus type, serotype A FMDV (84.61%) and serotypes O FMDV (15.39%) were recorded. However, serotype C and Asia-1 FMDV was not recorded from any of the outbreaks during the study year. Further we conducted a retrospective diagnosis study of antibody titre of more than 1.8 $\log_{10}$ against FMDV samples of serotype A were recorded 32 sera samples, 7 sera samples were found showing high titre against serotype O FMDV, 4 sera samples were found showing higher titre against FMDV type Asia-1. The present research work of FMDV outbreaks, April (beginning of summer season) and September (end of long rainy season), two times per, year can be suggested as the most suitable time for vaccination against FMD in the study area and imperative that findings of this work might be helpful in to validate the knowledge on diagnosis of FMD.

Keywords: FMDV, serotyping, virus types O, A and asia-1, outbreaks, seasonal analysis and retrospective diagnostic studies.

Introduction

Foot and mouth disease is an acute and highly contagious febrile disease affecting cattle, sheep, pigs, goats, buffalo and many species of cloven-hoofed wild life. FMD caused by a single stranded RNA virus belonging to the genus *Aphthovirus* in the family *Picornaviridae*. Identification of the FMD virus (FMDV) the causative agent of the disease, posed problems because of the occurrence of many types and subtype of the virus. Foot and mouth disease cause restriction to the trade of live animals and livestock products internationally (11). It is characterized by fever, loss of appetite, salivation and vesicular eruptions on the feet, mouth and teats (23). FMD is a global disease that through the years has affected most of the countries. It occurs throughout the world, most commonly in

Asia, Africa, the Middle East, and parts of South America. North America, Central America, Australia, New Zealand, Chile, Japan, and most of European countries have been recognized as free, and Uruguay and Argentina have not had an outbreak since April 1994 (20). Due to poor reporting from the African continent, FMD is considered endemic in most of the African Countries with only Morocco (based on serological survey), Swaziland, Lesotho, Zimbabwe, Namibia, Botswana and the Republic of south Africa being considered free of the disease by the OIE in 1999 (12). Infections in humans are very rare and minor clinical significance (2, 18). The disease is characterized by the formation of vesicles (fluid-filled blisters) and erosions in the mouth, nose, teats and fee tare.

Foot and mouth disease is endemic in India since many centuries. It is present almost in all parts of the country and occur round the year. Approximately 470 million domestic livestock are susceptible to FMD apart from the free living and captive wild ungulates. During centuries of evolution of FMD in the field, repeated opportunities for variation have led to the viral diversification which is, now a days, reflected in the co-existence of seven serotypes: A, O, C, SAT-1, SAT-2, SAT-3 and Asia-1 in the world (20). However in India only O, A, C and Asia-1 have been reported. Serotype 'C' too has not been recorded in the country since 1995. Serotype O is the most prevalent of the seven serotype and occurs in many parts of the world. Within type O, genetic lineages fall into geographically distinct groups known as topotypes. The Middle East, South Asian (ME-SA) topotype comprises a grouping of genetically similar viruses that is endemic to the region, from which the Pan Asia strain appears to have emerged (21). Although the exact origin of the Pan Asia strain is uncertain, the virus was first identified in northern India around 1990. FMD is endemic on the Indian subcontinent and approximately 90% of outbreaks caused by type-O virus (9). Vaccination against FMD is grossly inadequate in the country. The annual loss due to FMD in India is roughly estimated US dollar 800 million.

Currently there are seven serotypes of foot and mouth disease virus (FMDV), namely O, A, C, Southern African Territories (SAT) 1, 2 and 3, and Asia 1, which infect cloven-hoofed animals. Within these serotypes, over 60 subtypes have also been described using biochemical and immunological tests; and new subtypes occasionally arise spontaneously. However, at a specific time, there are only a few subtypes causing disease throughout FMD endemic areas. The importance of subtypes is that a vaccine may have to be tailored to the subtype present in the area in which the vaccine is being used (OIE, 2004). At present, a sequencing of FMD virus is increasingly being used to establish intratypic variations of FMD viruses and classifying viruses in to genotypes and lineages (20).

FMD is probably one of the most important livestock diseases in the world in terms of economic impact. The economic importance of the disease is not only due to the ability of the disease to cause losses of production, but also related to the reaction of veterinary services to the presence of the disease and to the restrictions on the trade of animals both locally and internationally (11). Despite the wide spread and enormous economic importance of FMD in India, clinical and serological studies to characterize the disease, under local Indian conditions have never been exhaustive and the endemic level has not been established. The extent to which a disease is recognized as a problem is often dependent on the efficacy of the means for diagnosing it and observing its occurrence (15). Therefore the main objectives of this study to validate the knowledge on diagnosis of FMD, note the earliest signs of affected animals, identify the serotypes of FMD

virus circulating in the study areas of Uttar Pradesh and their retrospective diagnostic studies using sandwich ELISA and LPB ELISA method.

Materials and Methods

Diagnosis of FMD infected animals

Clinical diagnosis based on lesion identification, in the early stage of infection, FMD virus or viral antigens can be detected using several techniques. However, different serological methods are used to detect antibody against FMD virus and is the main indication that infection has taken place. The diagnosis of FMD infected animals can be characterized into two categories.

Field Diagnosis

In cattle and buffalo, FMD should be considered whenever salivation and lameness occur simultaneously and a when a vesicular lesion is seen or suspected. Fever often precedes other clinical signs; therefore, febrile animals should be carefully examined. Early diagnostic lesions may be found before animals start to salivate, have a nasal discharge, or become lame. Field diagnosis can present many difficulties due to viral infections of the mucous membrane, which produce similar clinical signs. Differential diagnosis for FMD should include vesicular stomatitis, rinderpest, malignant catharal fever, the bovine herpes infections, swine vesicular disease, vesicular exanthema of swine and bluetongue (3).

Laboratory Diagnosis

Due to the highly contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a virus-secure laboratory (17). Appropriate samples for FMD laboratory diagnosis are; Vesicular fluid usually contains the highest quantity of virus. Epitheliums from early vesicles and from recently ruptured vesicles are tissue of choice for virus isolation (17). When epithelium tissue is not available from ruminant animals e.g. in advance or convalescent cases and infection is suspected in the absence of clinical sign, samples of oesophageal-pharyngeal fluids (OP) is collected by means of a probang and used for virus isolation (1). Other samples such as, blood with anticoagulant, Serum, and lymph nodes, thyroid gland, adrenal gland, kidney, and heart are good sources of specimens from postmortem.

Serum sample collection

The sample size was tongue epithelium, feet epithelium and blood serum (n= 40) were collected from FMD affected cattle, buffalo, goat and sheep population in five districts of Uttar Pradesh namely: Mathura, Pratapgarh, Jaunpur, Bhadoi and Etawa. The whole blood was collected from a jugular vein of randomly selected animals into 10 ml sterile vacationer tubes and stored overnight at room temperature for serum separation. The serum was then transferred into a single sterile cryovial, bearing the names of the animal species with sample number and transported in an icebox, to laboratory under cold condition. Serum samples preserved in 50% phosphate buffer saline (PBS), glycerol, pH 7.4 and stored at -20^{0}C until laboratory investigation.

Experimental Procedures

Liquid phase blocking ELISA

Antibody detection by liquid phase blocking ELISA detects and quantifies FMDV antibodies in serum of both infected and vaccinated animals (8). Plates were coated with 50µl trapping rabbit antibody stock (Rabbit anti-FMDV serotypes O, A, C and Asia-1) diluted 1:1000 in coating buffer (carbonate/bicarbonate) into 96 wells of ELISA plate reader (Hindustan Electronics) microplate and incubated at 40^{0}C

over night. Simultaneously, 50µl of test and control sera (C++, C+ and C-), diluted 1:16 in diluents buffer A (PBS and Tween 20) were added into wells of flat-bottomed microplates and 50µl of FMDV antigen (serotypes O, A, C and Asia-1) diluted at suggested working dilutions were added into all 96 wells of the perspective polypropylene flat-bottomed microplates. Sera and antigen were mixed and incubated at 40C over night, washed with dilution of PBS at pH 7.4, three times; then, 50µl serum-antigen mixture was transferred from flat-bottomed microplates to the appropriate wells of ELISA plate reader plate and incubated at 37^0C for one hour, with continuous shaking. After microplates were washed, 50µl detecting antibody (Guinea pig anti-FMDV serotypes O, A, C and Asia-1), diluted 1: 1000 in diluent buffer B (PBS, Tween 20 and skimmed milk powder) was added into all 96 wells of the respective microplates and incubated at 37^0C for one hour with continuous shaking. After washing the plates, 50µl of conjugate (Horseradish peroxides conjugated rabbit anti-guinea pig immunoglobulin) diluted 1:200 in diluent buffer B was added into 96 wells of each microplate and incubated at 37^0C, for one hour with continuous shaking. Finally, the plates were washed and 50µl of substrate/chromogen (hydrogen peroxide (H_2O_2)/Ortho-Phenylene-diamine (OPD) solution was added and incubated at ambient temperature for 15 minutes (briefly placed on the shaker to ensure even mixing) before 50µl of stopping solution sulphuric acid H_2SO_4 was added into all 96 wells of the microplates. The ELISA reader was connected to the computer loaded with ELISA Data Information (EDI) Software, which is used to automate the reading of OD value and calculate the percentage inhibition (PI).

Antigen titration

Antigen titration procedures were used (7) to check the working dilution of each FMD antigen used in serotyping. Plates were washed three times between each stapes except after substrate added.

Estimation of titres

The percent inhibition in each well was calculated in reaction to antigen control using the formula:

$$\text{Percent inhibition} = 100 - \frac{\text{OD of test well} - \text{background OD}}{\text{OD of Ag control well - background}}$$

The reciprocal of $\log_{10}$ dilution corresponding to 50% inhibition was considered to be the titre of the serum.

Data collection and analysis

The participatory and laboratory investigation results were analyzed using Statistical Package for Social Sciences (22) and Statistical software (STASOFT version 6.0), respectively.

Results and Discussion

Retrospective study of FMD serotypes disease outbreak using sero-typing strategy the two approaches was used e.g. field analysis and laboratory analysis of FMD. Firstly, the field analysis to diagnoses the FMD affected animals and note their earliest signs, Secondly, in laboratory analysis the seasonal variations of FMDV outbreaks and sero-typing retrospective studies using diagnostic tools and techniques LAB-ELISA.

Field analysis of FMD

Diagnoses the earliest clinical signs

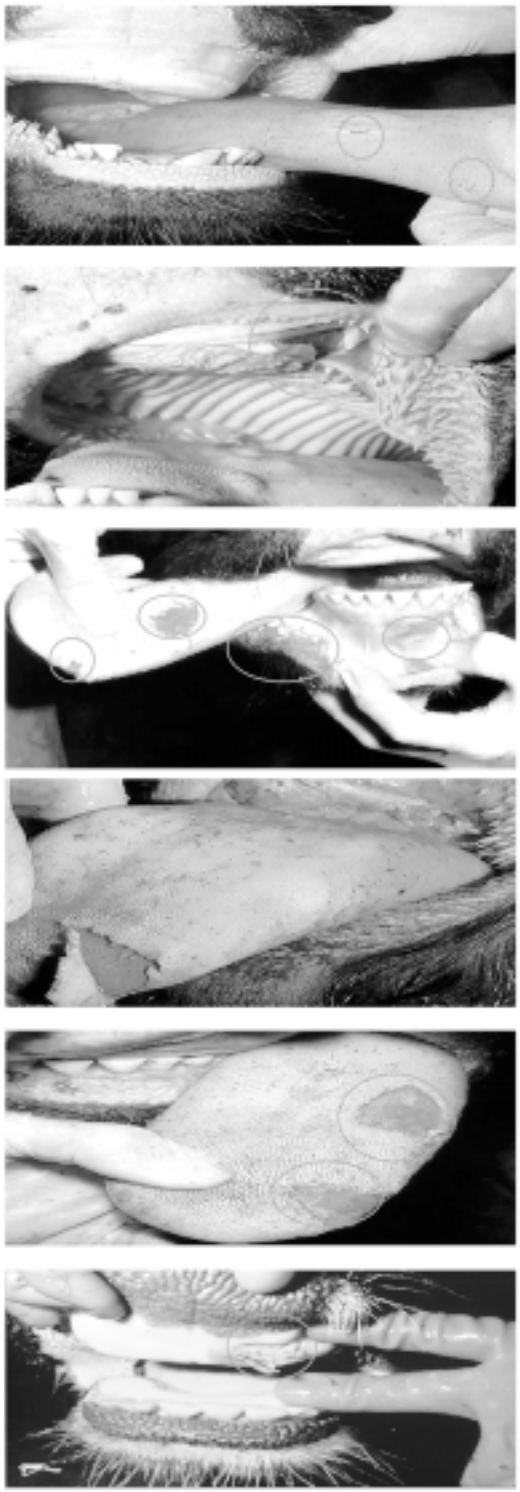
When susceptible animals are in contact with clinically infected animals, clinical signs usually develops in 3 to 5 days (13), although in natural infection, the incubation period may range from 2-14 days. The severity of clinical signs of the disease varies with the strain of the virus, the exposure dose, the age, and breed of the animal, the host species, and its degree of immunity. The signs can range from a mild or in apparent in

sheep and goats to a severe disease occurring in cattle and pigs (17). FMD should be suspected wherever; vesicles are seen in cloven-hoofed. Vesicles begin as small white fluid filled areas that quickly grow to a blister about 3cm in diameter. Two or more blisters may join to form a large one. The blisters usually burst leaving a raw surface. These heal over a few days (Fig. 1a and 1b).

In Cattle and Buffalo: The earliest sign observed in the FMD affected animals are fever of 103-105^{o}F, dullness, poor appetite and fall in milk production. These signs are followed by excessive salivation, smacking of the lips, grading of the teeth, drooling, serous nasal discharge; shaking, kicking of the feet or lameness; and vesicle (blister) formation. The predilection sites for vesicles are areas where there is friction such as on the tongue, dental pad, gums, soft palate, nostrils, muzzle, interdigital space, coronary band, and teats (20, 24). After vesicle formation, drooling may be more marked, and nasal discharge, lameness, or both may increase. Pregnant cows may abort, and young calves may die suddenly without developing any vesicle because of inflammation of the heart (Myocarditis) (3). Morbidity can approach 100%, but Mortality in adult animals is rare, although in young animals death can occur due to myocarditis and mortality can exceed 50% (24). Pregnant cows may abort (3). The course of an FMD infection is 2 to 3 weeks although infection may delay recovery of mouth, feet and teat lesions, resulting in hoof deformation, mastitis, low milk production, failure to gain weight, and breeding problems. A lactating animal may not recover to pre infection production because of

Fig. 1a. Thin ropey saliva (demarcating with circle) in cattle affected early with foot and mouth disease.

Fig.1b. Pictures showing the clinical signs of FMD Virus in Foot lesions (demarcating with circle) in Cattles and Buffalos.



1. Tongue of a steer with 1-day-old vesicle. Which, ruptured when the tongue was drawn from the mouth.

2. Steer with 2 days old ruptured vesicle along upper gum and several 1-day-old unruptured vesicles on the tongue.

3. Two days old ruptured vesicles on the tongue, lower gum and lower lip of a steer.

4. A further examination of 2-days-old lesions in the mouth of a steer, sharp showing margins of lesions and red raw appearance of exposed dermis.

5. Tongue of a steer with 3 days old lesions. Sero-fibrinous exudation into the lesions has resulted in a loss of earlier red raw appearance and also sharpness of margination.

6. Mouth of a steer showing ruptured vesicles with exposed dermis on the upper gum.

Fig. 2. Pictures showing the clinical signs of FMD Virus(demarcating with circle) in Cattles and Buffalos.

damage to the secretory tissue. A chronic Panting syndrome characterized by dyspnoea, anaemia, hair overgrowth and heat intolerance has been reported as a sequel of cattle recovered from FMD associated with pituitary gland damage (4). If at pasture, the animal will be away from the rest of the heard and probably lying down. Loss of condition is marked because of the fever and because the mouth is so painful that the animal is afraid to eat Fig 2.

In Sheep and Goat

In sheep and goats, if the clinical signs occur, it tends to be very mild, and may include dullness, fever; and small vesicles or erosions on the dental pad, lips, gums, and tongue. Mild lameness may be the only sign Fig 3. . In lame animals, there may be vesicles or erosion on the coronary band or in the interdigital space. Infected animals may abort and nursing lambs may die without showing any clinical sign (10). Most of the signs listed for FMD were consistent which is indicated in veterinary literatures (5, 6, 19).

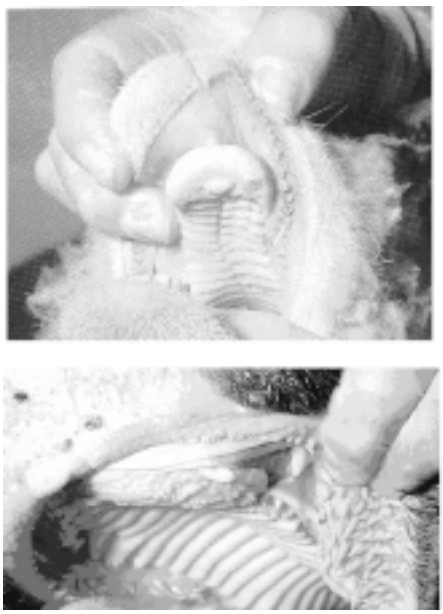


Fig. 3. Pictures showing the clinical sign of FMD Virus (demarcating with circle) in dental pad of Sheep.

Laboratory analysis of FMD

Serotyping retrospective studies based on ELISA and LAB-ELISA

The study of seasonal variations of FMDV outbreaks and sero-typing retrospective studies the techniques were used sandwich ELISA and LPB (Liquid Phase Blocking) ELISA. A total of n=40 samples were collected and investigated in nineteen outbreaks from five districts of Uttar Pradesh during the month of January to June, 2006. Seasonal analysis of the FMDV outbreaks (Fig. 4) revealed that out of total nineteen outbreaks, the highest 42.10% occurred in April (Eight outbreaks) followed by 26.30% in January (Five outbreaks) and 15.80% in both December and March (Three outbreaks). The maximum numbers of outbreaks are observed in cattle followed by sheep and buffalo (Table. 1). According to the annual report of Animal Health Division of Ministry of Agriculture in 2000, the incidence of FMD outbreaks has increased by 1.3-1.5 folds since 1990 (20). Extensive movement of livestock, the high rate of contact among animals at commercial markets, in communal grazing areas and at watering points, were among the reasons forwarded for the

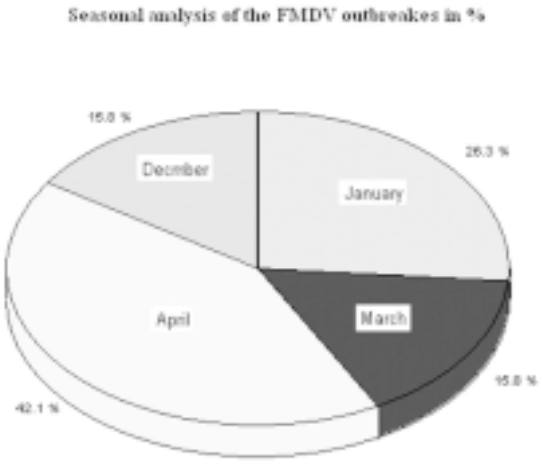


Fig. 4. Relationship between seasonal variations of FMDV and outbreaks percentage

Table 1: Identification the virus type and distribution of FMDV in the affected ***Animals.***

Species	Number of Specimens	Virus types				
		"O"	"A"	"C"	Asia-1	*VNR*
Cattle	10	1	9	-	-	-
Buffalo	27	1	-	-	-	26
Goat	1	-	-	-	-	1
Sheep	2	-	2	-	-	-
Total	**40**	**2**	**11**	**-**	**-**	**27**

increasing incidence of the disease in recent years (14). Disease was started from Mathura, Pratapgarh, Bhadoi, Etawa and Jaunpur districts of U.P. in the month of April (Fig. 4). For serotyping study of virus from five districts of Uttar Pradesh all the forty samples were processed using sandwich ELISA and LAB-ELISA techniques and the two virus type, serotype A FMDV (84.61%) and serotypes O FMDV (15.39%) were recorded. However, serotype C and Asia-1 FMDV was not recorded from any of the outbreaks during the year 2006 (Table. 1). The retrospective studies revealed the antibody titre of post infected animals (unvaccinated) against virus type O, A, C and Asia-1 (Table 2). In addition to 40 clinical samples from 19 outbreaks mentioned above 43 sera samples were also collected from affected / convalescing animals from another five FMD outbreaks and analyzed for antibody titre against FMD types O, A and Asia-1 for retrospective FMD diagnosis. Of these, antibody titres more than 1.8 $\log_{10}$ against FMDV serotype A were recorded 32 sera samples, 7 sera samples were found showing high titre against serotype O FMDV, 4 sera samples were found showing higher titre against FMDV type Asia-1 (Table 3). These retrospective studies could be used in addition to routine FMD typing as a definitive indication for FMD virus typing work. It is imperative that findings of this work might be helpful in formulating FMD emergency vaccination, within an infected area, has gained more preference in recent years, in an attempt to reduce the amount of virus circulating and spreading beyond the restricted area. The use of emergency FMD vaccines has two clear objectives. Firstly, to provide protective immunity, as rapidly as, possible to susceptible stock, and secondly, to reduce the amount of virus released and thereby limit the spread of disease and vaccination strategies in light of the concept of 'FMD free zone programme. Therefore, based on these findings, the following is recommended; the participatory epidemiological and conventional veterinary methods are complementary and they should be used side by side during animal health research, especially in FMD affected areas. An extensive regular serological survey, virus isolation, and characterizations of FMDV need to be conducted for a possible development of polyvalent vaccine. Based on the present study of FMDV outbreak, April (beginning of summer season) and September (end of long rainy season), two times per, year can be suggested as the most suitable time for vaccination against FMD in Utter Pradesh.

Table 2: Antibody titre ($\log_{10}$) of sera samples tested for retrospective study

S.no.	Sample type	Sample no.	District	Species/ Breed	Sex	O	A	Asia-1
1	Serum	S-01/MTH/05	Mathura	B	F	1.5	1.5	1.2
2	Serum	S-02/PTG/05	Pratapgarh	B	F	>2.1	1.8	1.8
3	Serum	S-03PTG/05	Pratapgarh	B	F	1.2	1.3	1.5
4	Serum	S-04/PTG/05	Pratapgarh	B	F	1.2	1.5	1.2
5	Serum	S-05/JNP/05	Jaunpur	S	F	1.8	1.5	1.5
6	Serum	S-06/JNP/05	Jaunpur	S	F	>2.1	1.8	1.2
7	Serum	S-07/JNP/05	Jaunpur	S	F	1.8	1.8	1.8
8	Serum	S-08/JNP/05	Jaunpur	S	F	1.8	1.5	1.5
9	Serum	S-09/BHD/05	Bhadoi	C	F	>2.1	1.5	1.5
10	Serum	S-10/BHD/05	Bhadoi	B	F	>2.1	1.8	>2.1
11	Serum	S-71/ETW/05	Etawa	C	F	<1.5	>1.8	<1.5
12	Serum	S-72/ETW/05	Etawa	C	F	1.2	2.1	1.2
13	Serum	S-73/ETW/05	Etawa	C	F	<1.5	>1.8	1.5
14	Serum	S-74/ETW/05	Etawa	C	F	1.2	2.1	<1.5
15	Serum	S-75/ETW/05	Etawa	C	F	1.6	1.6	1.6
16	Serum	S-76/ETW/05	Etawa	C	F	<1.5	>2.1	<1.5
17	Serum	S-77/ETW/05	Etawa	C	F	1.2	2.1	1.2
18	Serum	S-78/ETW/05	Etawa	C	F	1.2	1.8	<1.5
19	Serum	S-79/ETW/05	Etawa	C	F	0.9	2.1	1.2
20	Serum	S-80/ETW/05	Etawa	C	F	0.9	>1.8	1.2
21	Serum	S-89/ETW/05	Etawa	C	F	1.5	1.5	<1.5
22	Serum	S-99/ETW/05	Etawa	C	F	<1.5	>1.8	1.5
23	Serum	S100-/ETW/05	Etawa	C	F	1.2	2.1	<1.5
24	Serum	S-101/ETW/05	Etawa	C	F	1.2	>1.8	<1.5
25	Serum	S-102/ETW/05	Etawa	C	F	<1.5	2.1	1.2
26	Serum	S-105/ETW/05	Etawa	C	F	1.4	2.1	<1.5
27	Serum	S-106/ETW/05	Etawa	C	F	<1.8	>1.8	1.5
28	Serum	S-107/ETW/05	Etawa	C	F	<1.5	2.1	1.8
29	Serum	S-109/ETW/05	Etawa	C	F	1.2	2.1	1.2
30	Serum	S-110/ETW/05	Etawa	C	F	1.2	2.1	<1.8
31	Serum	S-111/ETW/05	Etawa	C	F	1.2	>1.8	<1.5
32	Serum	S-112/ETW/05	Etawa	C	F	<1.5	>1.8	<1.5
33	Serum	S-113/ETW/05	Etawa	C	F	<1.5	2.1	<1.8
34	Serum	S-114/ETW/05	Etawa	C	F	1.2	2.1	<1.5
35	Serum	S-115/ETW/05	Etawa	C	F	0.9	2.1	1.2
36	Serum	S-116/ETW/05	Etawa	C	F	1.4	1.6	1.5
37	Serum	S-117/ETW/05	Etawa	C	F	1.6	1.2	1.2
38	Serum	S-118/ETW/05	Etawa	C	F	1.6	1.6	1.5
39	Serum	S-119/ETW/05	Etawa	C	F	1.2	1.8	1.2
40	Serum	S-120/ETW/05	Etawa	C	F	<1.5	2.1	<1.5
41	Serum	S-121/ETW/05	Etawa	C	F	1.2	>1.8	1.2
42	Serum	S-122/ETW/05	Etawa	C	F	1.2	<2.1	1.5
43	Serum	S-123/ETW/05	Etawa	C	F	1.5	<2.1	<1.5

B – Buffalo, C – Cattle, S – Sheep, F – Female

Table 3: Serum antibody status of post infected animals (Unvaccinated) against type O, A and As-1 in sera collected during 2006.

S. No.	District	No. of sera sample tested	Anti body titre Log_{10}					
			Titre < 1.8			Titre >1.8		
			O	A	Asia-1	O	A	Asia-1
1.	Etawa	33	33	5	32	-	28	1
2.	Bhadoi	2	-	1	1	2	1	1
3.	Jaunpur	4	-	2	3	4	2	1
4.	Mathura	1	1	1	1	-	-	-
5.	Pratapgarh	3	2	2	2	1	1	1
Total		43	36	11	39	7	32	4

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Growth inhibition and induction of apoptosis in estrogen receptor-positive and negative human breast carcinoma cells by *Adenocalymma alliaceum* flowers

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Abstract

Adenocalymma alliaceum (*A. alliaceum*) is well known for its traditional medicinal uses and as a substitute for garlic. The methanol extract from *A. alliaceum* flowers (*AAF*) was investigated for its growth inhibitory activity on the estrogen receptor positive MCF-7 and estrogen receptor negative MDA-MB231 breast cancer cells by MTT assay. Treatment of breast cancer cells with different concentrations of *AAF* resulted in dose dependent growth inhibition with a growth inhibitory concentration (GI_{50}) of 53.1±4.1 µg/mL in MCF-7 and 23.9±3.7 µg/mL in MDA-MB-231 cells. Treatment of breast cancer cells with *AAF* resulted in time-dependent sequence of events marked by apoptosis, as shown by translocation of phosphatidylserine and activation of caspase-3. Analysis of data suggests that *AAF* exerts growth inhibition on both breast cancer cells through apoptosis induction, and that it may contain potent anticancer secondary metabolites valuable for application in drug products.

Keywords : *Adenocalymma alliaceum*, breast cancer cells, apoptosis, enrichment factor, sulphur compounds

1. Introduction

Breast cancer is the second leading cause of cancer-related deaths (1). The treatments include surgery, radiation, and in some cases, drugs that have a specific target such as tamoxifen in estrogen-dependent tumours (2). However, the majority of cases, especially those that result in metastasis, are still treated with conventional chemotherapy. The problem in drug resistance is a major obstacle in chemotherapeutic treatment, therefore, there is a great need for the development of new therapeutic drugs that will be more efficient or will synergise with existing ones.

There has been a growing interest in the use of herbs as a potent source of new therapeutic anticancer drugs. Plants contain a wide variety of secondary metabolites that have potent biological effects, including anticancer activity (3). In this research we focused on the growth inhibitory effect of the *Adenocalymma alliaceum* flowers on breast cancer cells and its mode of action.

Adenocalymma alliaceum Miers. (family: Bignoniaceae), commonly known as 'garlic creeper', is native to the Amazon rain forests of South America. The leaves and flowers are widely consumed by Brazilians as a substitute for garlic (4). The plant has a number of traditional medicinal properties such as antimycotic, analgesic, antiarthritic, anti-inflammatory, antipyretic, antirheumatic, antitussive, depurative, purgative, and vermifuge

properties (5). It contains nonacosane, oct-1-en-3-ol, octacosan-1-ol, pentatriacont-1-en-17-ol, hentriacontane, hexacosan-1-ol, hexatriacontane, trithiacyclohexene, n-triacontane, triacontan-1-ol, 24-ethyl-cholest-7-en-3β-ol, 3β-hydroxy-urs-18-en-27-oic acid, β-sitosterol, stigmasterol, fucosterol, glycyrrhetol, daucosterol, dotriacontan-1-ol, β-amyrin, β-peltoboykinolic acid, ursolic acid, α-4-hydroxy-9-methoxy-lapachone, α-9-methoxy-lapachone, apigenin, cosmosiin, cyanidin-3-O-β-D-rutinoside, luteolin, n-scutellarein-7-O-β-D-glucuronide, aspartic acid, glutamic acid, leucine, 1-2:3-vinyl-dithi-4-ene, 1-2:3-vinly-dithi-5-ene, dithiacyclo-pentene, allin, allyl sulfides, diallyl sulfides and triallyl sulfides (5-11).

As part of our continuing search for bioactive natural products, in the present study we examined the effect of methanol extract from *A. alliaceum* flowers (*AAF*) on estrogen receptor positive (ER +ve) MCF-7 and estrogen receptor negative (ER -ve) MDA-MB-231 human breast cancer cells, including the mode of cell death. Results of the present study show that *AAF* has potent growth inhibitory effect on both cells. Its activity is through apoptosis as detected by the adhesion of annexin V to phosphatidylserine on the outer leaflet of the cell membrane and activation of caspase-3.

2. Material and Methods

2.1 Plant material

Fresh flowers of *Adenocalymma alliaceum* was collected in the city of Tirupati, Andhra Pradesh state and identified by Dr. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati. The flowers were dried in hot air oven at a temperature not more than 50ϒC. The dried flowers were powdered using an electric blender. Powdered flowers (50 g) were extracted with methanol using soxhlet extractor. The methanol extract was concentrated in rotary evaporator at a temperature not more than 50ϒC. The concentrated methanol extract was dried using freeze dryer at -33ϒC.

According to the National Cancer Institute (NCI), USA; a crude extract may be considered as potent cytotoxic if its $IC_{50} \leq 20\mu g/mL$ (12). So, in the present study, the highest concentration of extract used was 150µg/mL. Dried extract was dissolved in 50% (v/v) methanol in ultra pure water to obtain the final concentrations of 150, 140, 130, 120, 110, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 2.5, 1.25 and 0.625µg/mL. For proliferation and apoptosis assays, the solutions of different concentrations of extract were sterilised by passing them through 0.22 µm membrane filters.

2.2 Reagents

MTT assay kit and Dual Apoptosis assay kit were purchased from Biotium, USA. Cell Death Detection ELISA PLUS kit was purchased from Roche Applied Sciences, Germany.

2.3 Cells and culture condition

Breast-adenocarcinoma cells MCF-7 (ER +ve) and MDA-MB-231 (ER –ve) from ATCC (Rockville, MD) were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% FBS and 2 mM L-glutamine, 1% penicillin/streptomycin (PenStrep) under a fully humidified atmosphere, 5% CO_2 at 37ϒC. For experiments, cells were collected from subconfluent monolayers with accutase. The studies were carried out using cells from passages 3-7.

2.4 Proliferation assay

The effect of *AAF* on the viability of the cancer cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. Briefly, 99 µL of the cell suspensions were plated in 96-well flat-bottomed tissue culture plates (Nunc, Denmark) at a concentration of $1 \leftrightarrow 10^4$ cells/well. After incubating the plates for 24 hours at 37ϒC in a

humidified incubator, different concentrations of sterilised *AAF* solutions were added to the respective wells of the plate. The final volume in each well was 100 µL. Each concentration of *AAF* was repeated in minimum of three wells in each plate and the assay was repeated in at least two plates. The plates were further incubated for 48 hours. Five microliters MTT reagent was added to each well and incubated for 4 h after which the plates were centrifuged at 600g for 5 min at 4ϒC. MTT solution and medium were aspirated from the wells and100 µL of buffered DMSO was added to each well. The plates were shaken for five minutes and the absorbance (OD) was recorded on a microplate reader at the wavelength of 570 nm and a reference wavelength of 630 nm. The effect of *AAF* on growth inhibition was assessed as percent cell viability where vehicle (0.5%v/v methanol in water)-treated cells were taken as 100% viable. Percentage of cell viability in each well was calculated using the formula:

$$Percentage\ of\ viables\ cells = \frac{-OD\ of\ the\ extract - OD\ medium\ control}{OD\ vehicle\ control - OD\ medium\ control} \sqrt{\leftarrow 100}$$

The GI_{50} value, the concentration of *AAF* required to reduce the cell growth by 50%, was evaluated from the dose-response curve.

Untreated and vehicle treated cells were incubated as controls. The final concentration of methanol in each well did not exceed 0.5% (v/v). This concentration did not affect the apoptosis or cell proliferation of the investigated cells.

2.5 Apoptotic death assays

Apoptotic death assays were carried out at only one concentration of *AAF*, GI_{50}, on MCF-7 and MDA-MB-231 cancer cells. Mode of cell death (apoptosis) was qualitatively determined using Dual Apoptosis assay and quantified using Cell death detection by enzyme-linked immunosorbent assay (ELISA).

2.5.1 Dual apoptosis assay

This assay was carried out using Dual Apoptosis Assay Kit with NucView™ 488 caspase-3 substrate & sulforhodamine 101-annexin V (Texas Red⟩-annexinV) according to instructions in the product protocol. This kit detects two important apoptosis events, caspase-3 activation and phosphatidylserine (PS) translocation in a single experiment. The MCF-7 and MDA-MB-231 cells were incubated for 24 hours in a humidified CO_2 incubator on coverslips, which were previously coated with poly-L-lysine. The cells were challenged with the extract and the negative control cells were challenged with the 0.5% (v/v) methanol in water. The cells were incubated further for 6 hours and 12 hours to observe the apoptotic changes. After the respective incubation periods, the culture medium was aspirated and the cells were washed with annexin V binding buffer. Then annexin binding buffer (100 µL), 0.2 mM NucView™ 488 caspase-3-substrate (5 µL) and sulforhodamine 101-annexin V (5 µL) were added to each cover slip and incubated for another 45 minutes. The coverslips were washed with annexin V binding buffer and mounted in annexin V binding buffer on to slides. The apoptotic events in the stained cells were observed under a fluorescence microscope using FITC and Texas-Red filters. The positively stained apoptotic cells were counted and the apoptotic index was calculated as the number of apoptotic cells relative to the total number of cells.

2.5.2 Cell death detection by enzyme-linked immunosorbent assay (ELISA)

The mechanism of cell death, i.e. apoptosis or necrosis was quantitatively determined using the Cell Death Detection ELISA^{PLUS} assay (13) as recommended by the manufacturer. This kit can detect and quantify both apoptosis and necrosis. Briefly, 99 µL of the cells suspensions (MCF-7 and MDA-MB-

231) were plated in 96-well flat-bottomed tissue culture plates at a concentration of $1 \leftrightarrow 10^4$ cells/well. After incubating for 24 hours at 37ϒC in a humidified incubator, sterilised solutions of *AAF* were added to the respective wells of the plate. The final volume in each well was 100 µL. Each solution was repeated in minimum of three wells. The plate was centrifuged at 600*g* at 4ϒC for 10 minutes. The DNA fragments released from the cells due to necrosis were present in the supernatant layer. The supernatant was carefully transferred without disturbing the cell pellets into a glass vial and stored in a refrigerator at 4ϒC until further analysis for necrosis. The cell pellet containing the apoptotic bodies was resuspended in lysis buffer and incubated for 30 minutes at room temperature. The plate was centrifuged and cell lysate was transferred into a glass vial and stored in a refrigerator at 4ϒC until further analysis for apoptosis.

Supernatant and cell lysate solutions (20 µL) were placed in triplicate into wells of streptavidin coated microplate and added 80 µL of the immunoreagent, containing a mixture of anti-histone-biotin and anti-DNA-POD. The plate was covered with an adhesive cover foil and incubated for 2 hours at 25ϒC in a shaking incubator at 300 rpm. During the incubation period, the anti-histone antibody binds to the histone-component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin-coated microplate via its biotinylation. At the same time, the anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes. The unbound antibodies were washed with incubation buffer. The amount of nucleosomes retained by the POD in the immunocomplex, corresponding to the extent of apoptosis and necrosis, was quantitatively determined photometrically with ABTS as substrate using microplate reader at a wavelength 405 nm and reference wavelength of 490 nm.

2.6 Statistical analysis

All the data represented were mean ± S.D (standard deviation) of triplicate. Statistical analyses were conducted using SPSS ver. 15.0 software. The significance between control and treated groups was performed by student's t-test and p values less than 0.05 were taken as significant.

3. Results

To evaluate the activity of *AAF* on the growth of mammary cancer cells, the MCF-7 cells, a well established model for the in vitro-investigation of estrogenic activities and MDA-MB-231 (ER –ve) cells were employed.

3.1 Growth inhibitory activites of A. alliaceum flowers

The effect of *AAF* was studied as a dose-response experiment for 48 h at the dilutions of 0.25-150 µg/mL. Both cells exhibited significant *AAF*-induced suppression of growth. A dose-dependent inhibition of cell growth was observed between 4-80µg/mL (Fig. 1). The growth inhibitory effect of *AAF* was more pronounced in MDA-MB-231 cells, which was mirrored in its GI_{50} concentrations (Fig. 1). The 50% inhibitory

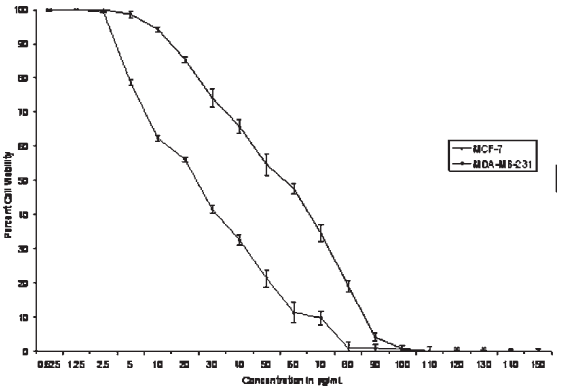

Fig. 1 Growth inhibitory activities of methanol extract from *A. alliaceum* flowers on MCF-7 and MDA-MB-231 breast cancer cells. The percentage cell viability was determined in triplicate. Data represent the mean±SD. The percent cell viability was significantly ($P<0.05$) different from control at concentrations; ⊕ 5 µg/ml in MDA-MB-231 and ⊕ 10 µg/ml in MCF-7.

concentration of *AAF* was 53.1±4.1µg/mL (MCF-7) and 23.9±3.7µg/mL (MDA-MB-231). The growth of both cells was inhibited almost completely by 100 µg/mL of *AAF*. At concentration of extract of up to 2µg/mL no effect was observed on the proliferation of both cells.

3.2 Adenocalymma alliaceum flowers induced apoptosis in MCF-7 and MDA MB 231 cells

In principle, a reduction of cell growth can reflect either a decreased proliferation rate or an enhanced cell death by either necrosis or apoptosis or a combination of these two

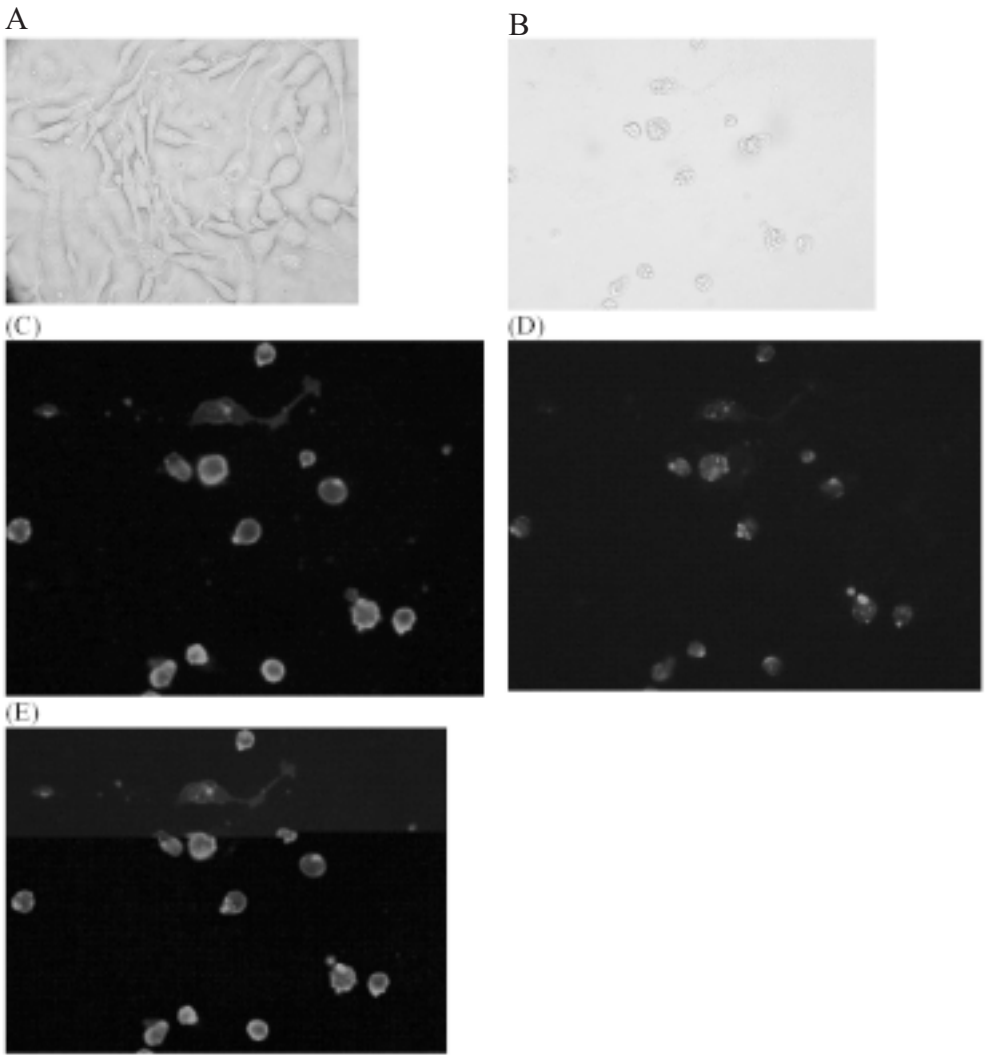

Fig. 2 Morphological changes of the MDA-MB-231 cells after 12 h treatment (A) solvent (0.5% v/v methanol) only; (B) with 25 µg/mL of *AAF* viewed under bright field and stained cells viewed under FITC & Texas-red filters using fluorescent microscope (C) red border around the cell indicating PS translocation; (D) green nucleus in the cell indicating capase-3 activation; (E) green nucleus surrounded by red border indicating the apoptotic cell.

mechanisms. We also investigated whether the extracts can induce apoptosis by monitoring the two important apoptosis markers: phosphatidylserine (PS) translocation on cell membrane (identified by sulforhodamine 101-Annexin V, 14) and caspase-3 activation (identified by NucView™ 488 caspase-3 substrate, 15). The morphological changes were inspected by microscopy. Some cells were beginning to detach from the plate and becoming rounded after 6 h treatment of *AAF* (50µg/mL for MCF-7 and 25µg/mL for MDA-MB-231). Because loss of adhesion to the culture dishes of tumoral epithelial cells has been described as an apoptosis-related event (16), we examined the morphological apoptotic changes on slides under bright field after 6 h and 12 h. In opposite to good spreaded cells in the negative control, a morphological change with cell shrinkage was detected in cells treated with *AAF* extract. Necrosis was less prominent than apoptosis. In Fig. 2 the representative photomicrographs of MDA-MB-231 cells after treatment with 25 µg/mL *AAF* extract in comparison to solvent treated control are shown.

To further substantiate the growth inhibitory effects of *AAF*, the apoptotic cells were monitored by Annexin V adherence and caspase-3 activation. In viable cells, PS is located on the cytoplasmic surface of the cell membrane; in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS. Annexin V is a Ca^{2+} dependent phospholipids-binding protein with high affinity for PS. The binding of sophorodamine101-AnnexinV probe to PS that has translocated to the outer membrane cell produces red border around the cell under fluorescent microscope using red filter (Fig. 2). Caspase-3 (CPP32) is a cytosolic protein that normally exists as a 32-kDa inactive precursor. It is cleaved proteolytically into a heterodimer when the cell undergoes apoptosis (17). The cleavage of NucView™488 caspase-3 substrate by activated caspase-3 stains the cell nucleus green (Fig. 2). The induction of apoptosis by *AAF* was time-dependent (Fig. 3). In MCF-7, there were fewer apoptotic cells, *AAF* at concentration of 50µg/mL inducing apoptosis in 18.3 and 30.7% after 6 and 12 h treatment. In MDA-MB-231 cells an elevation in apoptosis

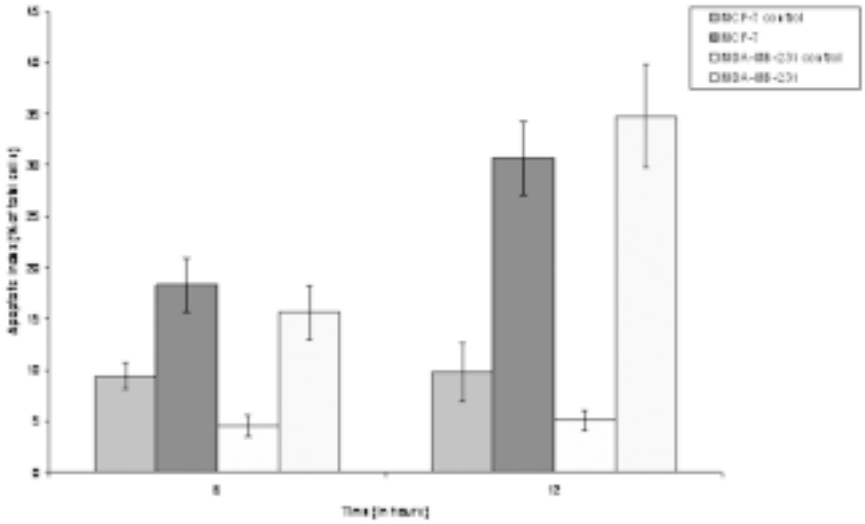

Fig. 3 Time-dependent apoptosis of MCF-7 and MDA-MB-231 breast cancer cells induced by methanol extract from A. alliaceum flowers. The apoptotic indices were determined in triplicate. * indicates that the results are significantly different ($P<0.05$) from respective controls.

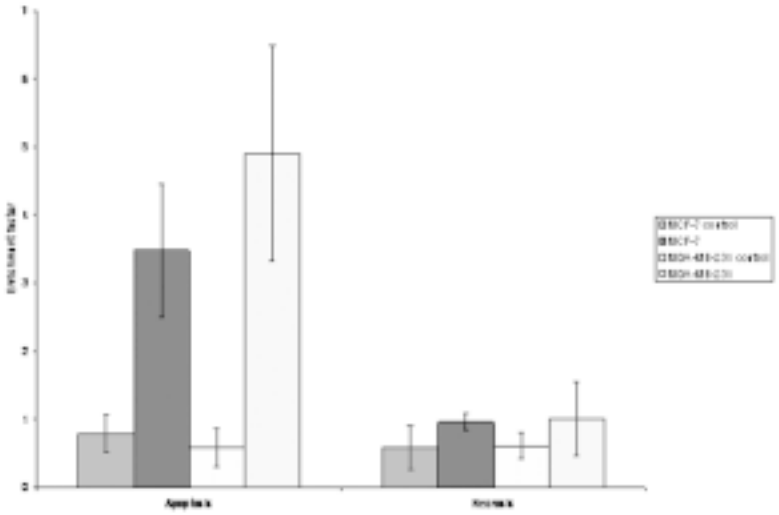

Fig. 4 Quantification of apoptosis and necrosis in MCF-7 and MDA-MB-231 breast cancer cells induced by methanol extract from *A. alliaceum* flowers. The enrichment factor was determined in triplicate. The results represent mean ± SD. * indicates that the results are significantly (P<0.05) different from respective controls. NS, indicates the results are not statistically significant.

positive cells up to 15.6% was found after 6 h and reached 34.8% after 12 h exposure to 25µg/mL *AAF*. The solvent controls did not increase the spontaneous apoptotic rate in the two malignant cells tested.

To quantitate and further support the finding that *AAF* exposure causes apoptosis in cancer cells we performed cell death detection by ELISA. Compared to solvent treated control, exposure of MCF-7 and MDA-MB-231 cells to *AAF* at 50 and 25 µg/mL concentrations resulted in 3.5- and 4.9-fold increases in induction of apoptosis while necrosis induced by *AAF* is negligible (Fig. 4).

4. Discussion

The results reported herein reveal that the methanol extract of *A. alliaceum* exerts growth inhibitory action on MCF-7 and MDA-MB-231 breast cancer cells. The dying cells exhibit the ultrastructural and biochemical features that characterise apoptosis, as shown by the loss of viability, PS translocation and capase-3 activation. Like many other plants, *AAF* extract is a multicomponent mixture with pharmacologically active substances. The growth inhibitory activity may be attributed to a number of sulphur containing compounds.

We first established the 50% growth inhibitory concentration using MTT assay. The MDA-MB-231 (ER –ve) cells were significantly more sensitive than MCF-7 (ER +ve) cells with an approximately 2-fold variation in the GI_{50} concentration of the *AAF*. The growth inhibitory action of *AAF* on breast cancer cells is dose-dependent and probably evoked by ER-mediated and non-ER-mediated mechanisms because of the various physico-chemical properties of individual components of *AAF*. Discovery of active compounds from natural products with apoptosis-inducing ability rather than cytotoxic ability is of great interest for cancer treatment. Screening for anti-cancer substances is commonly

conducted using viability assays. An inherent problem with this approach is that all compounds that are toxic and growth inhibitory, irrespective of their concentration-dependent mechanism of action, will score positive. Apoptosis is essential for normal physiological development but is also critical in eliminating any abnormal cells after exposure to genotoxic or DNA-damaging agents.

Therefore, we investigated the apoptotic changes in mammary cancer cells induced by *AAF*, using dual apoptosis assay kit. The morphological changes occurred early, after 6 h treatment with, with loss of adhesion. The apoptotic changes characterised by PS exposure (detected by Annexin V adherence) and caspase-3 activation, as shown in Fig. 2 on MDA-MB-231 cells are representative for both cells. The initial apoptotic rate of MCF-7 cells in comparison to MDA-MB-231 was higher (Fig. 3) probably due to the estrogen deprived test conditions. About 31% of ER +ve cells and about 38% ER –ve cells were undergone apoptosis after 12 h treatment with 50 and 25 µg/mL *AAF* respectively. These findings correspond with the results from proliferation assays and suggest that *AAF* cytotoxicity appears to be explained in part by the induction of apoptosis. Further, it was interesting to note that there was a significant increase in the level of apoptosis compared to necrosis in both mammary cancer cells induced by *AAF* (Fig. 4). To our knowledge this is the first report showing the growth inhibitory activity of *A. alliaceum* on cancer cells.

5. Conclusion

In conclusion, to our knowledge this is the first report showing that *A. alliaceum* flowers exhibits an growth inhibitory effect by induction of apoptosis that is associated with phosphatidyl serine translocation and caspase-3 activation in MCF-7 and MDA-MB-231 cancer cells. As apoptosis has become a promising therapeutic target in cancer research, these results confirm the potential of *A. alliaceum* flowers as an agent of chemotherapeutic and cytostatic activity in human breast cancer cells. However, more detailed studies are required to determine the exact mechanism(s) of action of *A. alliaceum*, specifically evaluating its effects on epigenetic and signal transduction pathways.

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Micropropagation of *Crataeva religiosa* Hook. f. & Thoms

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Abstract

A protocol was developed for *in vitro* propagation by multiple shoot induction of *Crataeva religiosa* Hook. f. & Thoms, a medicinal tree having high medicinal values belonging to the family Capparidaceae. High frequencies of multiple shoot regeneration were achieved from apical bud on MS medium fortified with 8 mg/L BAP alone. Five to seven shoots per explant were obtained. The elongated shoots were subcultured for rooting on half strength MS supplemented with various concentrations of IBA and IAA. The *in vitro* raised plantlets were acclimatized in green house and successfully transplanted to natural condition with 72% survival.

Key words: *Crataeva religiosa*, Medicinal plant, Micropropagation, Acclimatization.

Abbreviations: MS-Murashige Skoog medium, NAA-Napthalene Acetic Acid, BAP-Benzyl Amino Purine, IAA-Indole Acetic Acid, IBA-Indole Butric Acid.

Introduction

Tissue culture techniques are being increasingly exploited for clonal multiplication and *in vitro* conservation of valuable indigenous germ-plasm threatened with extinction. Greater demand for these plants especially for the purpose of food and medicines which is one of the causes of their rapid depletion from primary habitats. Micro-propagation offers a great potential for large scale multiplication of such useful species and subsequent exploitation (1). *Crataeva religiosa* Hook. f. & Thoms., non Frost. F. belongs to the family Capparidaceae is a moderate-sized deciduous tree (Tamil: Mavilankai, Telugu: Magalingam, Malayalam: Nirmatalam, Hindi: Barun). *C. religiosa* is characterized with astringent, bitter, acrid, diuretic, anthelmintic, carminative, laxative and stomachic. Root and bark promote appetite, and increase biliary secretion. Leaves are stomachic and tonic. Juice of leaves is given internally to cure rheumatism. Bark is demulcent, alterative, tonic, stomachic, laxative, diuretic, antipyretic, and useful in calculus affections and for disorders of urinary organs. Powdered bark is useful in urinary and renal troubles, gastro-intestinal and uterine infections (2). There has been progress in tissue culture studies in many Capparidaceae members such as *C. nurvala* (3), *C. magna* (4) and *Capparis decidua* (5) to propage them. But no such *in vitro* culture studies have been carried out in this valuable medicinal tree, *C. religiosa*. The present investigation elucidates *in vitro* multiple shoot regeneration through apical bud segments of *C. religiosa* for better exploitation and also preservation of this valuable germ-plasm which has already undergone a severe biotic pressure.

Materials and Methods

Apical buds of *C. religiosa* were collected from the campus of Thiagarajar College, Madurai, washed thoroughly in running tap water and treated with detergent solution. The apical buds were initially disinfected by rinsing it in 90% ethanol for 15 seconds followed by surface sterilization in an 0.1% (w/v) $HgCl_2$ aqueous solution of 0.1% (w/v) $HgCl_2$ for 2-3 minutes. The explants were once again rinsed thrice with sterile distilled water. The sterilized explants were inoculated in MS medium containing 3% sucrose and 0.8% agar with supplemented with BAP alone (0.5 – 10.0 mg/L) and different concentrations (1.0 – 5.0 mg/L) of BAP along with NAA (0.25 mg/L). The pH of the media was adjusted to 5.8 and autoclaved at 1.06 kg/cm^2 pressure and 121º C temperature for 15 min. For root induction, the well developed shoots were transferred in to half strength MS medium with different concentrations of IAA (3.0 mg/L) and IBA (3.0 mg/L). All subsequent subculturinge were performed at four week of interval to fresh medium. The cultures were incubated in a culture room maintained at 25 ± 2ºC under 16 hours photoperiod with light intensity of 3000 lux. For each treatment 14 replicate cultures were maintained and all the experiments were replicated thrice. The data were statistically analyzed using one way analysis of variance and means were compared using the Duncan's Multiple Rank Test at the 0.05% level of significance.

Results and Discussion

The use of pre-existing buds for propagation reduces the possibility of variation among the progeny and therefore can be safely applied for rapid propagation of *Crataeva religiosa*. We optimized shoot multiplication conditions and novel rooting techniques for mass clonal propagation without interference of callus. This method is quite common for the propagation of *Fragaria indica* (6), and *Acacia mearnsii* (7) and *Sandalam album* (8). The apical bud explants showed slight swelling prior to the emergence of shoot buds developing from the pre-existing material 20 days after inoculation. Initially two to four shoot buds per explant emerged 30 days after inoculation and gradually the number of shoot buds per explant increased up to 5 – 7 (Table 1; Plate 1a) on MS media fortified with 8 mg/L BAP. But low number of buds developed in the concentration of 0.5 mg/L BAP. Superiority of BAP over other cytokinin has been reported and discussed in relation to shoot proliferation in cultures of trees (9, 10) and the regeneration of shoots from nodal explants has also been encountered in Capparidaceae plants like *C. nurvala* (3), *C. magna* (4) *C. adansonii* (11) and *Capparis deciduas* (5). Sometimes callus formation from the basal cut ends of the apical bud explant was also observed (Plate 1b).

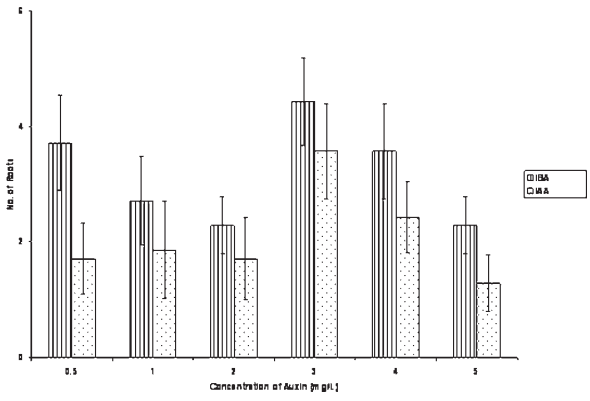
The *in vitro* developed shoots from the apical bud cultures were harvested and transferred to half strength MS medium added with various concentrations of IBA and IAA. A maximum number of 4 - 5 roots were observed when the medium was supplemented with 3.0 mg/ L IBA or IAA after 5 weeks, irrespective of the type of auxins used (Fig. 1). Similarly, pulse treatments of IBA were given for root induction in shoots produced in cultures from nodal explants of adult plants of *Camellia sinensis* (12), *Maytenus emayginata* (13) and *Prosopis cineraria* (14) and *Sandalum album* (8). IBA is the most commonly used auxin for root formation from shoots of woody trees (15). The *in vitro* regenerated rooted plantlets were washed with sterile water and transferred to small plastic pots containing sand, garden soil and digested coir pith (1:1:1) mixture (Plate 1c). Then the plants were maintained under shade and partial shade for one more week. The regenerated plants were transferred to the soil with 72% survival.

Table1: Effect of NAA and BAP on *in vitro* shoot formation from apical shoot bud explants of *Crataeva religiosa* after 30 days of culture

Growth Regulators mg/L		Mean number	Morphogenic
NAA	BAP	of Shoots/explant	Response
0	0.5	2.571 ± 0.327^{h}	CS
0	2.0	3.570 ± 0.589^{g}	CS
0	4.0	4.420 ± 1.496^{e}	S
0	6.0	5.710 ± 0.822^{b}	S
0	8.0	6.850 ± 0.839^{a}	S
0	10.0	5.710 ± 0.822^{b}	S
0.25	0.5	3.714 ± 0.826^{g}	CS
0.25	1.0	4.290 ± 0.629^{e}	CS
0.25	2.0	4.290 ± 0.509^{e}	S
0.25	3.0	4.290 ± 0.496^{e}	CS
0.25	4.0	5.420 ± 0.639^{c}	S
0.25	5.0	4.857 ± 0.841^{d}	S

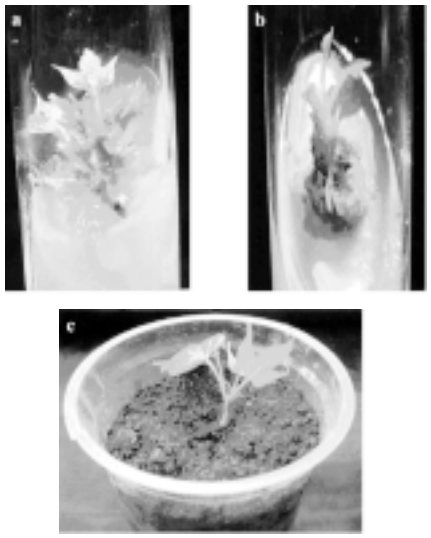
± - Standard Error. Means followed by the same letter not significantly different by the Duncan's Multiple Rank Test at P< 0.05 level of significance.
Where CS – Callus + Shoot, S - Shoot

Fig. 1: Effect of various auxins on rooting response from *in vitro* regenerated shoots of *Crataeva religiosa* cultured on half strength MS medium after 5 weeks of culture.



Vertical line on the bar indicates Standard Error. Means followed by the same letter not significantly different by the Duncan's Multiple Rank Test at P< 0.05 level of significance.

Fig. 2: Plate one



a) Shoot proliferation from apical bud on MS media fortified with 8 mg/L BAP
b) Direct shoot formation from callus on MS media supplemented with 0.25 mg/L NAA and 1.0 mg/L BAP
c) Successful establishment of rooted plants in plastic cup.

The present investigation has demonstrated a potentially efficient technique for the large scale micropropagation of *C. religiosa* from apical bud explant. The data indicated that BAP at 8.0 mg/L in MS medium is more effective for shoot multiplication from the shoot apical bud. Half strength MS medium supplemented with 3.0 mg/L IBA or IAA is best for root induction.

The protocol described is an efficient and could be used as a means of propagation and multiplication of *Crataeva religiosa* – a potential medicinal plant for commercial exploitation while previously published protocols are having some complications like incubation in dark for 6 days (5).

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Protective effects of *in vitro* supplementation of ascorbic acid on plasma membrane, acrosomal membrane and mitochondrial activity index of human spermatozoa

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Abstract

Spermatozoa were the first type of cells reported to produce free radicals. Reactive oxygen species (ROS) mediated damage to sperm is a significant contributing factor to male infertility. Impaired motility, impaired fertilization and oxidative DNA damage are three inter-related mechanisms that account for oxidative stress mediated male infertility. Spermatozoa lack cytoplasmic antioxidant defense due to exclusion of cytoplasm and therefore rely upon antioxidants present in the seminal plasma. Centrifugation of a semen sample prior to its use for intra-uterine insemination (ICI) and *in vitro* Fertilization (IVF) induce oxidative stress. Therefore there is need to supplement the semen with antioxidants. In the present investigation attempts were made to study the effects of *in vitro* supplementation of non-enzymatic antioxidant ascorbic acid on sperm plasma membrane integrity, acrosome intactness and mitochondrial activity index. There was highly significant ($p<0.001$) improvement in these parameters that relate to healthy state of the spermatozoa.

Key words: Spermatozoa, Oxidative stress, Male infertility, Antioxidants, Ascorbic acid

Introduction

Spermatozoa is equipped with poor antioxidant defense system as compared to other cells. Among environmental, genetic and physiological factors responsible for the poor sperm function, free radical induced oxidative stress gained much attention, due to its deleterious effects on sperm plasma membrane and DNA damage leading to infertility (1-3). Though having essential role in sperm physiological processes such as capacitation, hyperactivation and sperm-oocyte fusion (4-5) spermatozoa are sensitive to Reactive Oxygen Species (ROS) because they lack cytoplasmic defenses (6), Moreover sperm plasma membrane is rich in polyunsaturated fatty acids, which are vulnerable oxidative damage (7). Contaminating leucocytes & immature spermatozoa are the major sources of ROS in the semen. (3, 8-11). Oxidative damage to plasma membrane, acrosomal and mitochondrial membrane in the form of lipid peroxidation results in the loss of functional membrane integrity and decreased production of ATP, which leads to infertility. Along with membrane damage ROS have found to be responsible for nuclear and mitochondrial DNA damage offering a damaged genome to the next generation at the onset of fertilization (12-13). Antioxidants are the scavenger molecules, neutralizing the damaging effects of free radicals. Ascorbic acid is a well known, non-enzymatic, water soluble natural antioxidant having a capacity to minimize the ROS damage by its chain breaking activity (14).

The present investigation was carried out with the hypothesis that *in vitro* supplementation of ascorbic acid can provide extra protection to spermatozoan plasma membrane, acrosomal membrane, mitochondrial activity index against the ROS in semen and therefore can improve motility and viability. This is an attempt to provide the direction to Assisted Reproductive Techniques (ARTs) in minimizing the failures resulting from ROS.

Material and methods:Collection of the semen sample

Human semen samples were used in the present investigation. Semen samples from twenty-five healthy volunteers of the age group 22-25 years old were collected by masturbation after sexual abstinence for 2-3 days as per the WHO guidelines 1999 (15). Immediately after liquefaction each semen sample was divided into two groups.

1. Control group: 0.5mL of the liquefied semen was mixed with 0.5mL of the Ringer Tyrode solution (NaCl 0.8g, KCl 0.02g, $CaCl_2$ 0.02g, $NaHCO_3$ 0.1g, NaH_2PO_4 0.005g, $MgCl_2$ 0.01g, Glucose 0.1g in 100mL double distilled water) and incubated at 37^0C for 60 min.
2. Experimental group: 0.5mL of the liquefied semen was mixed with 0.5mL of the Ringer Tyrode solution containing 1mM ascorbic acid (Merck India Ltd). The suspension was incubated at 37^0C for 60 min.

Motility: Semen sample from both the groups was analyzed for % motility as per the WHO guidelines 1999 (15).

Viability (16)**:** A drop of semen sample was mixed with a drop of 0.1% Trypan blue on a clean grease free slide and observed for stained and unstained spermatozoa under 400 X magnification. Plasma membrane of the live spermatozoa is impermeable to Trypan blue. Therefore, live spermatozoa remain colourless while dead spermatozoa get stained blue in colour.

Hypoosmotic swelling test (HOS) (1)

0.1 mL of the semen from each group was mixed with 0.9 mL of 150mosmol solution of fructose and sodium citrate. The mixture was incubated at 37^0C for 30 min in a tightly stoppered vial. A drop of incubated mixture was placed on a Neubauer's chamber and allowed to stand for 1 min after placing a cover-slip and observed under dark field microscope at 40 X objective for spermatozoa with coiled tails.

Viability in Hypoosmotic Solution (VHOS) (17)

0.1mL of the semen from each group was mixed with 0.9 mL of 150 mosmol solution of fructose and sodium citrate containing 0.1% Trypan blue and the percentage of unstained spermatozoa with coiled tails was calculated.

Gelatinolysis test for acrosome intactness (18)

10 'L of the semen sample was diluted with 190 ìL of Phosphate buffered saline (PBS) (pH 7.8) containing 3% D-glucose solution. 20 ìL of this diluted semen was smeared on 5% aqueous gelatin coated slides and incubated at 37^0C for 2 hours in moist chamber. The halo diameter was measured under dark field microscope using 40 X objective and with an eyepiece micrometer. The halo formation rate was calculated per slide as the percentage of spermatozoa showing a halo. One hundred spermatozoa were evaluated. An acrosin activity index was calculated by multiplying the halo diameter by the halo formation rate.

Sperm mitochondrial activity index (19)

15 'L of semen sample from each group was mixed with 15 ìL of 0.1% Nitro Blue Tetrazolium (NBT) in Tris-HCl buffer (pH 7.4), mixed well and incubated at 37^0C for 30 min.

After incubation, a wet smear of these contents was prepared on a pre cleaned slide. Allowed it to dry and fixed in neutral buffered formalin and observed under 100 X objective for NBT precipitation. On the basis of NBT precipitation in the middle piece of the sperm the spermatozoa were scored for mitochondrial activity index.

Statistical analysis: Results were interpreted by using paired t test.

Results and Discussion

There was highly significant ($p<0.001$) increase in percentage of motile and viable spermatozoa in ascorbic acid treated group than the control (Figure 1). Like wise there was highly significant ($p<0.001$) improvement in percentage of hypoosmotically-swollen spermatozoa, percentage of viable and hypoosmotically-swollen spermatozoa (Figure 2), sperm mitochondrial activity index (Figure 1) and acrosin activity index (Figure 3) in ascorbic acid treated group than the control (Table 1).

The imbalance between generation of free radicals and their inactivation results in oxidative stress a condition resulting in increased rate of cellular damage (7). Damaging effects of ROS as a factor contributing to male infertility have been documented earlier (20). At cellular level, membrane fluidity is the crucial factor responsible for sperm-oocyte fusion reflecting the success of fertilization (21,22). Oxidative damage to mitochondrial membrane lowers the production of ATP (23) ultimately affecting the motility, as there is a positive correlation between ATP production and motility (24). The overall effect of membrane damage might be responsible for continuous decease in motility and viability of spermatozoa after ejaculation (25).

Though having antioxidant property, Rolf et al (26) did not find any significant change in semen parameters in aesthenozoospermic patients with the dietary supplementation of ascorbic acid at the concentration of 1000 mg/ day. Fernandez-Santos et al. (27) also found that

Table 1. Effect of ascorbic acid on various sperm function tests after 60 min of incubation at 37^0C (Results are mean $\pm$ SEM n=25)

Sperm function tests	Ringer Tyrode group	Ringer Tyrode with 1mM ascorbic acid treated group
% motility	23.84 $\pm$ 0.60	40.64 $\pm$ 0.63 ***
% viability	79.44 $\pm$ 0.69	86.56 $\pm$ 0.71 ***
Sperm mitochondrial activity index	74.12 $\pm$ 0.57	81.76 $\pm$ 0.80 ***
% of swollen tail spermatozoa (HOS)	64.20 $\pm$ 0.53	69.04 $\pm$ 0.63 ***
% of viable spermatozoa in hypoosmotic solution	62.32 $\pm$ 0.62	67.04 $\pm$ 0.48 ***
Halo diameter in ìm	12.95 $\pm$ 0.3	14.77$\pm$ 0.11***
Halo formation rate	80.20 $\pm$ 0.49	86.08 $\pm$ 0.50 ***
Acrosin activity index	10.39	12.71

***indicates $p<0.001$

in vitro treatment of vitamin C is ineffective to protect spermatozoa in freeze-thaw cycle as compared to enzymatic antioxidants. In the present investigation *in vitro* treatment of ascorbic acid at the concentration of 1 mM, have found to protect the spermatozoan membrane against ROS induced damage. Increased motility along with viability definitely helps the more number of spermatozoa to reach at the site of fertilization especially in aesthenozoospermic and oligospermic patients. Increased HOS and VHOS are clear indicators of healthy status of membrane structure and function of sperm head and tail, respectively. Increased acrosin activity index promises the spermatozoa to penetrate through zona pellucida. Increase in SMAI shows healthy status of mitochondrial membrane with less generation of ROS and increased production of ATP. Earlier we have demonstrated protective effect role of alpha tocopherol on structural and functional integrity of human spermatozoa (28). Sagun et al. (29) have demonstrated that vitamin C enters mitochondria through facilitated glucose transporter (glut 1) and protect mitochondria from oxidative injury. Fraga et al. (30) and Mahmoud et al. (31) have shown the protective role of vitamin C against endogenous oxidative DNA damage. Thiele et al. (32) have found a positive correlation between vitamin C concentration in seminal plasma and number of morphologically normal spermatozoa. Prolonged *in vitro* incubation and repeated centrifugation of spermatozoa results in increased oxidative damage and addition of vitamin C to cryopreservation medium (33- 34) resulted in increased motility and fertilizing ability as evident by obtaining embryos from cryopreserved spermatozoa. (35). Thus present investigation demonstrates that treatment of ascorbic acid to spermatozoa after ejaculation helps protect the spermatozoa from oxidative damage and improves sperm functions.

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Tumor-targeted drug delivery by folate conjugated amphiphiles

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Abstract

A series of amphiphilic conjugates of folic acid and aliphatic fatty acids was designed to serve as targeted drug delivery carriers for anticancer agents. The effectiveness of targeted delivery of amphiphile was evaluated using folate receptor positive HeLa cells and Caco-2 cells that do not express folate receptor. Wild type HeLa cells were found to have 40- fold greater folate receptor - (FRα) expression than Caco-2 cells. Amphiphile uptake was studied by the internalization of 7-amino-4carboxymethyl coumarin labeled fluorescent amphiphilic conjugate in FR- knockdown HeLa, and Caco-2 cells at 37°C and 4°C, respectively. siRNA specific for FRα was used to knockdown the receptor in HeLa cells by 75% and these modified cells were used as the control in determining the specificity of amphiphiles uptake. Wild type HeLa cells internalized twice as much fluorescent amphiphiles as compared to all other treatment groups at 37°C. Paclitaxel, a lipophilic anti-tumor agent was used as a model compound to evaluate the efficacy of three homologous series of amphiphile conjugates as targeted carriers in HeLa and Caco-2 cells. The amphiphiles were non-toxic to both cell lines at the concentrations lower than 100μM. Amphiphilic micelle containing paclitaxel exhibited significantly lower IC_{50} values in HeLa cells when compared to free drug and untargeted amphiphile micelles. The data from the current studies demonstrated the feasibility of using folic acid conjugated amphiphiles to selectively deliver drugs to FRα positive cancer cells.

Keywords : Folate receptor, targeted delivery, amphiphiles, paclitaxel

Introduction

Treatment using anticancer drugs is limited due to their intolerable side effects. Conventional chemotherapeutic agents kill cells by disrupting the cell division. Their action is non-specific and cause serious damage to even non-tumor cells. An ideal cancer therapy should specifically direct drugs to target cancer cells and kill them preferentially or selectively while avoiding damage to the normal cells (1). One approach to achieving this goal is by targeted drug therapy.

Targeting can be categorized as either passive or active based on the absence or presence of site-directing ligands (2). Passive targeting generally takes advantage of natural anatomical structures or physiological processes to direct drugs to the site of action. Active targeting requires site-directing ligands to bind and interact with target site. Examples of targeted biopharmaceuticals include antibody conjugates and folate-bearing conjugates which utilize ligand-receptor interactions (3).

The folate receptor - (FRα) is a 38.5 kD glycophospatidyl inositol anchored protein with a high affinity [$k_D = 10^{-9}$ M] for folic acid (4). The folate receptors are diffusely distributed on the cell surface but are functional when concentrated in the caveolae (5). After binding to folic acid, the receptor is internalized by a non-endocytic process and is recycled back to the cell surface after dissociation from the substrate (6-8). The receptor mediated pathway of folate uptake and the expression levels of the receptor itself is regulated by intracellular levels of folic acid (9). The FRα is overexpressed consistently in nonmucinous ovarian carcinomas and tumors of epithelial lineage in endometrium, lung, breast, renal cells and brain metastases (10). Thus the therapeutic advantage of targeting the folate receptors is due to their overexpression, often twenty times more, in these types of malignancies than in epithelial cells or fibroblasts (11). Folate receptor targeting can be achieved with monoclonal antibodies or folate itself (12). Folic acid conjugated radioisotopes have been successfully targeted to ovarian cancer, the second most common gynecologic cancer, for the purposes of prognosis and imaging (13-14). Folate receptor targeting has also been performed by conjugating folic acid to drug delivery systems like liposomes and nanoparticles whereby higher payloads of therapeautic agents could be delivered to the tumor cells (15-18).

Self-assembled amphiphilic molecules in the form of micelles have gained recognition as drug carriers due to their small size, tendency to evade phagocytosis by mononuclear phagocyte system, and prolonged circulation time (19-20). Other advantages of micellar drug carrier include ease of formulation, increased solubility of poorly soluble drugs, enhanced penetration of drug through cell membrane, and passive accumulation in regions of leaky vasculature (19). Drugs encapsulated inside the micelle are protected from inactivation by biological surrounding. Micellar drug carrier can be made to specifically target cancer cells by attaching specific ligands on outer layer of micelles. The shell of the micelle containing folate binding moiety will exhibit enhanced binding, due to multivalent "cluster effect", to several folate receptors localized in caveolae to help deliver the drug molecule to the site of action (21).

In this study, amphiphiles synthesized using folic acid were used to deliver a model lipophilic anticancer molecule, paclitaxel, to folate receptor expressing HeLa cells. The amphiphile mediated delivery allows a simple formulation approach and may be extended to other lipophilic antitumor molecules intended for folate expressing tumors.

Materials and Methods

Materials

Dialyzed and heat inactivated fetal bovine serum, folate free Dulbecco's minimum essential medium (DMEM), chemicals for polyacrylamide gel electrophoresis and alkaline phosphatase coupled rabbit anti goat secondary antibody were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Polyvinylidene difluoride (PVDF) membrane for blotting, Immobilon P, was purchased from Millipore Corp. Goat polyclonal anti human FRá antibody and siRNA for human FRá were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal anti glyceraldehyde phosphate dehydrogenase (GAPDH) antibody was purchased from Ambion. Bicinchonic acid protein assay kit and stripping buffer were purchased from Pierce. Spectrofluorimetry was performed on RF5301 Shimadzu spectro-fluorimeter (Shimadzu Corp. Japan). A Nikon Eclipse TE 200 epifluorescent microscope (Nikon Corp. Japan) was used in visualization of cells treated with aminocoumarin labeled amphiphiles. The folate labeled amphiphiles and the fluorophore labeled amphiphile were

synthesized based on the synthetic strategy of Luo et al (Fig. 1) (22).

Cell culture

HeLa and Caco-2 cells were maintained in folate free Dulbecco's minimum essential medium (DMEM) (Sigma) supplemented with 10% fetal calf serum which resulted in a final concentration of 5-methyl-tetrahydrofolate of 9nM (23). The cells were incubated in low folate media at 37°C in 5% CO_2.

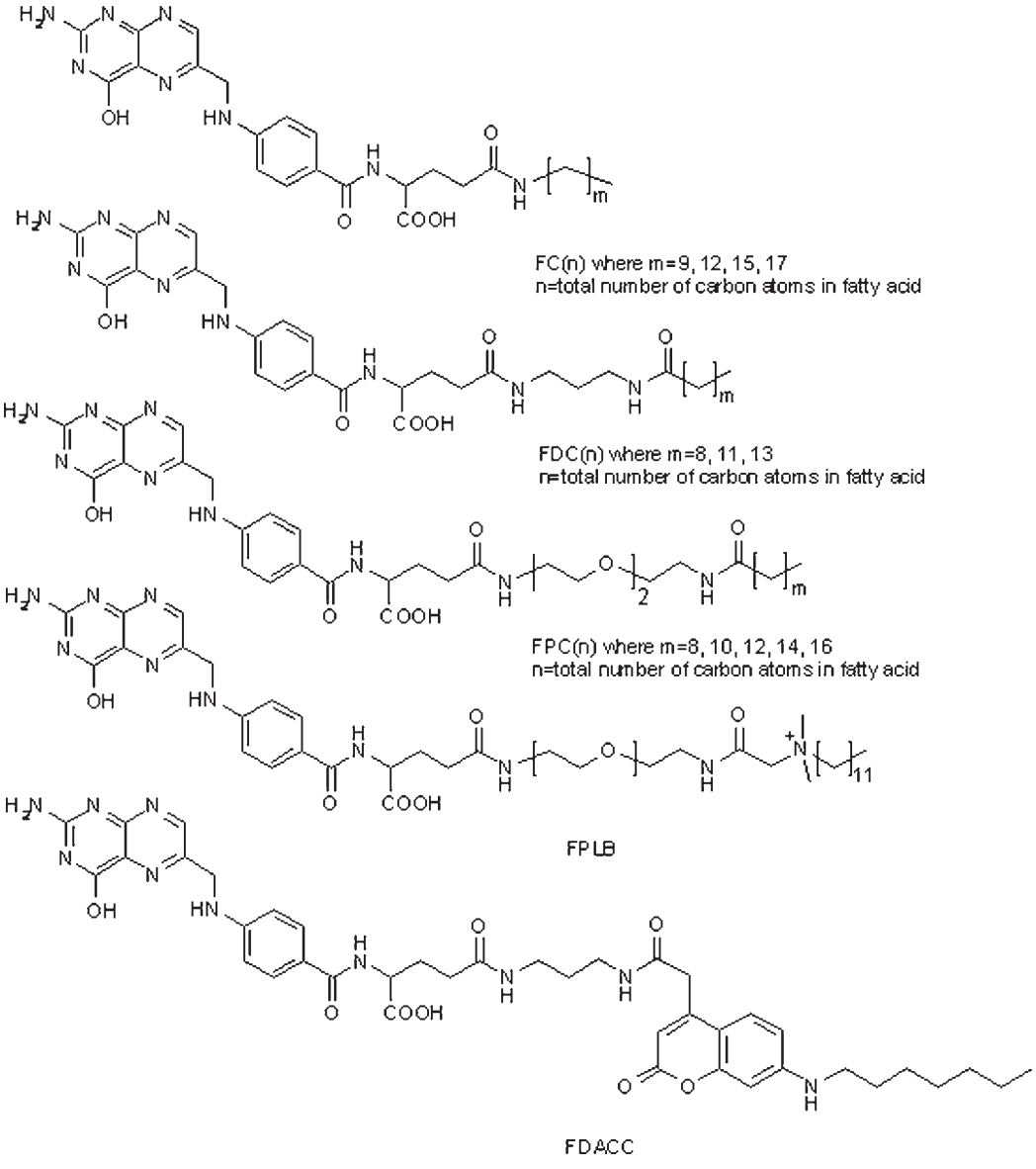

Figure 1. Chemical structures of different series of amphiphilic conjugates of folic acid with aliphatic carboxylic acids.

Western Blot analysis

Cells were lysed in a buffer containing 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2mM EDTA, dithiothreitol (DTT), 10% (v/v) glycerol, 1mg/ml leupeptin and 1 mM phenylmethanesulphonylfluoride (PMSF) by four freeze thaw cycles followed by three 10sec pulses of sonication. One hundred micrograms of total protein was resolved on 8% SDS PAGE. The protein blotted on to a polyvinylidene fluoride (PVDF) membrane was first incubated with goat polyclonal anti–human FR antibody overnight at 4°C, followed by incubation for 1 hour with alkaline phosphatase coupled rabbit anti-goat antibody. The Lumiphos substrate was then washed off and the membrane was treated with stripping buffer for 15 minutes at room temperature. The membrane was then probed with mouse monoclonal antibodies to glyceraldehyde phosphate dehydrogenase (GAPDH) followed by goat anti-mouse alkaline phosphatase labeled secondary antibody and detected by autoradiography. The receptor protein signal was normalized with the GAPDH signal from the corresponding lanes.

Folic acid receptor (FR-) knockdown by siRNA

HeLa cells (3×10^5) were seeded in each well of a 6-well plate and were grown for 24h. Cells were either transfected with FR- siRNA or scrambled siRNA using Lipofectamine Plus reagent, or left untreated. Cells were harvested after 2 days of growth and folate receptor levels were quantified using Western Blot analysis. The transfected cells were passed into fresh 60 mm plates and the folate receptor levels were quantified after 3 days of growth.

Internalization of fluorescent tagged amphiphiles

Internalization of fluorescent tagged amphiphilic molecule FDACC (Fig. 1) was used to study the uptake of these amphiphilic conjugates into folate receptor overexpressing cells. Wild type HeLa cells, FRá specific siRNA transfected HeLa cells (48h after transfection) and Caco-2 cells were plated in six well plates with 3×10^5 cells per well. After 24h of growth in folate depleted DMEM, the cells were washed twice in HBSS and treated with 20 ìM of FDACC for 30 minutes. A triplicate set of each cell type was maintained at 37°C and 4°C, respectively. The cells were scraped off the plates and lysed in HEDG buffer with three freeze-thaw cycles. The lysates were read in a spectrofluorimeter with exitation/emission 350/450 nm. Protein content of the lysates was measured by BCA assay and the fluorescence from the treated groups was normalized with their respective protein concentrations. For taking fluorescence microscopic images, HeLa and Caco-2 cells were also grown on acid treated (1N HCl for 1h) cover slips and treated with 20µM FDACC in HBSS for 30 minutes. The cells were then washed twice in HBSS and fixed in 4% formalin. They were then observed under an epifluorescent microscope with UV excitation.

Cytotoxicity studies

In vitro cytotoxicity of the amphiphilic carriers in HeLa and Caco-2 cells was assayed by sulforhodamine B (SRB) assay. In a typical assay procedure 5000 cells are plated in each well of a 96-well plate and incubated for 24h. The cells were then treated with various concentrations of amphiphilic conjugates and further incubated for 48h. The cells were then fixed in 10% trichloroacetic acid for 45 minutes at 4 °C, washed, stained with 0.4% SRB in 1% acetic acid for 30 minutes then rinsed with 1% acetic acid until the washings are colorless. The stained cells are then treated with 200 ìL of 10 mM Tris base and the release of cell bound dye was measured at 540 nm in a microplate reader. To evaluate the efficacy of the amphiphilic conjugates in targeting tumor cells, paclitaxel was chosen as a model anticancer agent. In a similar setup as described earlier, the cells were treated

with the drug in a micellar solution of targeted amphiphilic conjugates or treated with the drug in water or treated with the drug in a micellar solution of untargeted amphiphiles. A control group of cells were also grown without any treatment. Percent viability of different treatments relative to control at the end of 48h was calculated using following equation :

$$Percent\ Viability = \frac{Abs.Test - blank}{Abs.Control - blank} \rightarrow 100$$

where Abs. Test = mean of absorbance from triplicate wells at a single dose

Abs. Control = mean of absorbance from untreated cells

The %viability was plotted with respect to drug concentration and it was fitted to four parameter logistic equation using Sigmaplot® to calculate the drug concentration required for 50% viability in test samples (24).

$$y = a + \frac{b - a}{1 + 10^{(\log x - \log 7C50)*d}}$$

where a = maximal value of %viability that is attained

b = minimal value of viability% that is attained

d = Hillslope

$\log IC_{50}$ = logarithm of the dose at which 50% of the cells are viable

This concentration, IC_{50}, was taken as a measure of efficacy of the micellar drug delivery system. The IC_{50} parameter calculated from each treatment was then compared to that obtained from free drug treatment and statistical differences between the test and control groups were evaluated by Students t test at an a level of 0.05.

Results and Discussion

Folate receptor - (FRα) expression and knockdown in HeLa cells

FRα expression is known to be dependant on the intracellular levels of folic acid (9). Cells grown in DMEM containing 4 mg/L folate often have undetectable level of FRá expression (25). Thus HeLa cells were cultured in folate-free DMEM supplemented with 10% fetal calf serum for three months and the expression level of FRα was compared to Caco-2 cells grown under the same conditions. Level of FRα in HeLa cells was found to be forty times more than the levels in Caco-2 cells (Fig. 2). Therefore, HeLa cells cultured in folate-free DMEM were used for further experiments. HeLa cells were transiently transfected with siRNA specific for FRα with Lipofectamine Plus in OptiMEM for 5h. The transfection medium was replaced with folate-free DMEM and the cells were allowed to grow for 48h. Cells were then passed and assayed for FRα expression by Western Blot analysis after 1 day, 2 days and 3 days of growth. A 75% receptor knockdown was noted following 24h of growth after a single passage of the transfected cells (Fig. 3). In the consecutive time points the receptor knockdown levels decreased to 62% at the end of 3 days of growth after a single passage of the transfected cells (Fig. 3). Lack of significant reduction in GAPDH in the transfected cells suggested that the FRα knockdown in HeLa cells was specific for the receptor.

Cellular uptake of fluorescent labeled amphiphiles

7-Aminocoumarin-4-acetic acid labeled fluorescent amphiphiles FDACC were prepared to study the internalization of the delivery system into target cells. Uptake of the fluorescent labeled amphiphiles was measured at 37 °C and at 4 °C in wild type HeLa cells, FRα knockdown HeLa cells and Caco-2 cells. Perinuclear distribution of the fluorescent probe was observed in wild type HeLa cells (Fig. 4). This observation was

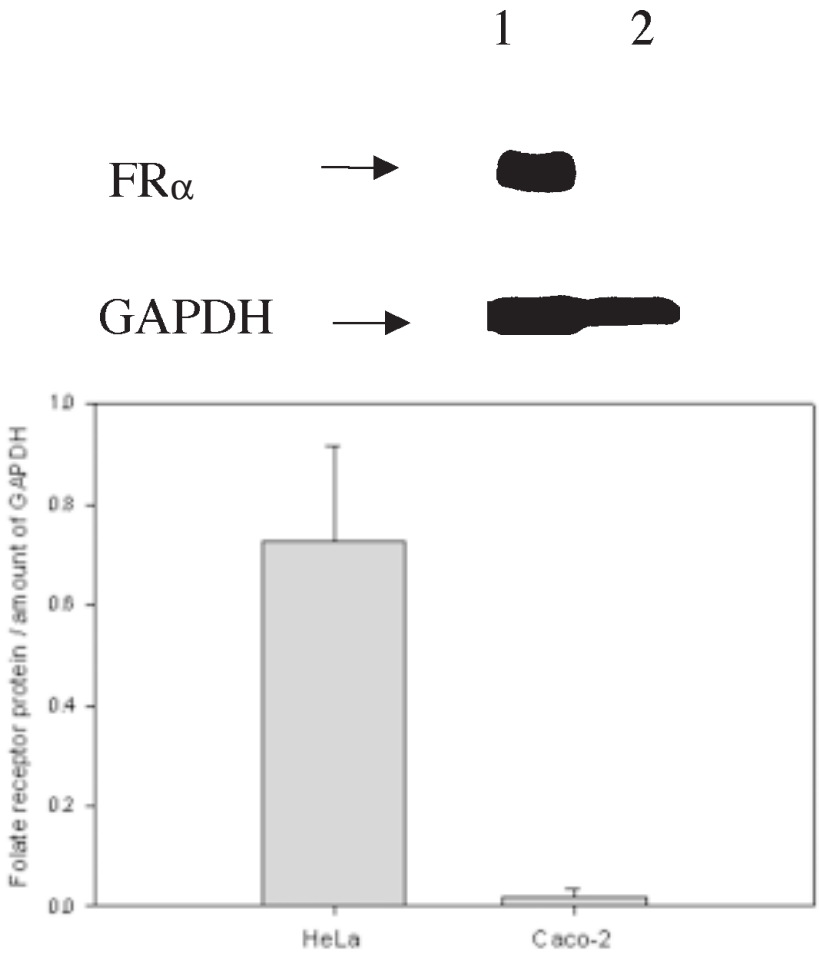

Figure 2. Western blot analysis of the expression of FRα protein in HeLa and Caco-2 cells propagated for 3 months in folate free media. Each data point represents the mean ± SD of three independent evaluations. The Western blot data shown represents one of the three studies. Lanes 1 and 2 refer to cell lysates from HeLa and Caco-2 respectively. $p<0.05$ between lanes 1 and 2

in agreement with the intracellular distribution of FRα as previously shown by Doucette et al (26). The fluorescent tagged amphiphile accumulates more in the HeLa cells than in the Caco-2 cells indicating that the folate receptor targeted drug delivery system can preferentially internalize into folate receptor expressing tumor cells. HeLa cells at 37 °C showed significantly higher amount of fluorescent amphiphile internalization when compared to other groups (Fig. 5). The transport is thus ATP dependant and is expected to slowdown at lower temperatures. In the FRα knockdown cells and Caco-2, small yet significant differences existed between uptake at 37 °C with uptake at 4 °C (Fig. 5). This may be due to a small amount of receptor protein that was present in these cell lines or due to the presence of reduced folate carrier, a trans membrane spanning protein of the solute family class 1 which is often expressed in Caco-2 (27).

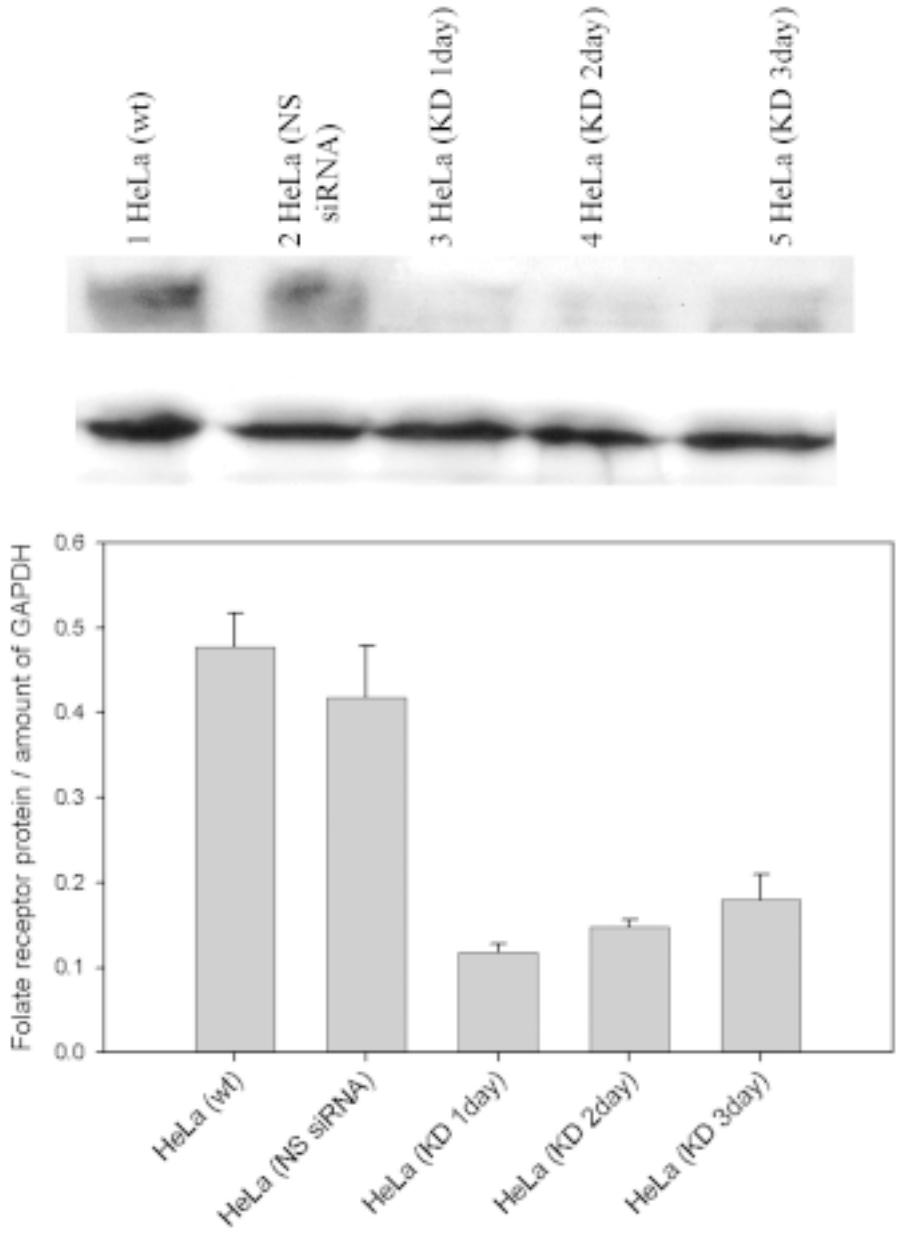

Figure 3. Western blot analysis of siRNA mediated knockdown of FRα in HeLa cells. Each data point represents the mean ± SD of three independent evaluations. The Western blot data shown represents one of the three studies. Lanes 1 and 2 refer to cell lysates from wild type HeLa (HeLa(wt)) and nonsense siRNA transfected HeLa cells (HeLa (NS siRNA)). Lanes 3, 4 and 5 contain cell lysates from FRα specific siRNA transfected HeLa cells at 1 day (HeLa KD 1day), 2 days (HeLa KD 2day), 3 days (HeLa KD 3day) of growth after a single passage following 48h incubation after siRNA transfection. $p>0.05$ between lanes 1 and 2. $p<0.05$ between lanes 3,4,5 and 1

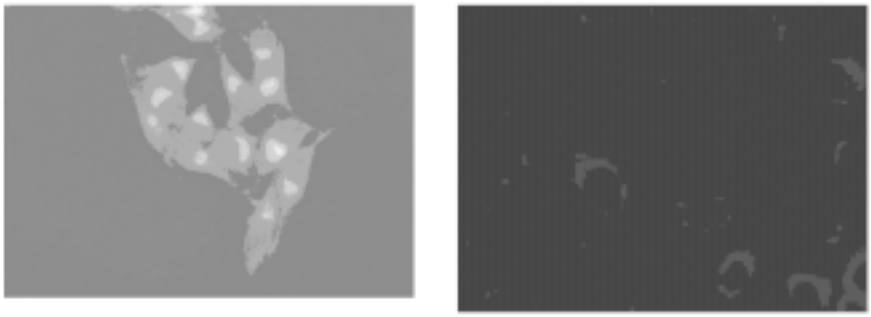

Figure 4. Fluorescent micrographs of cells treated with 20μM FDACC showing internalization of the fluorescent labeled amphiphile at the end of 30 mins. incubation at 37ºC. - A) wild type HeLa and B) Caco-2

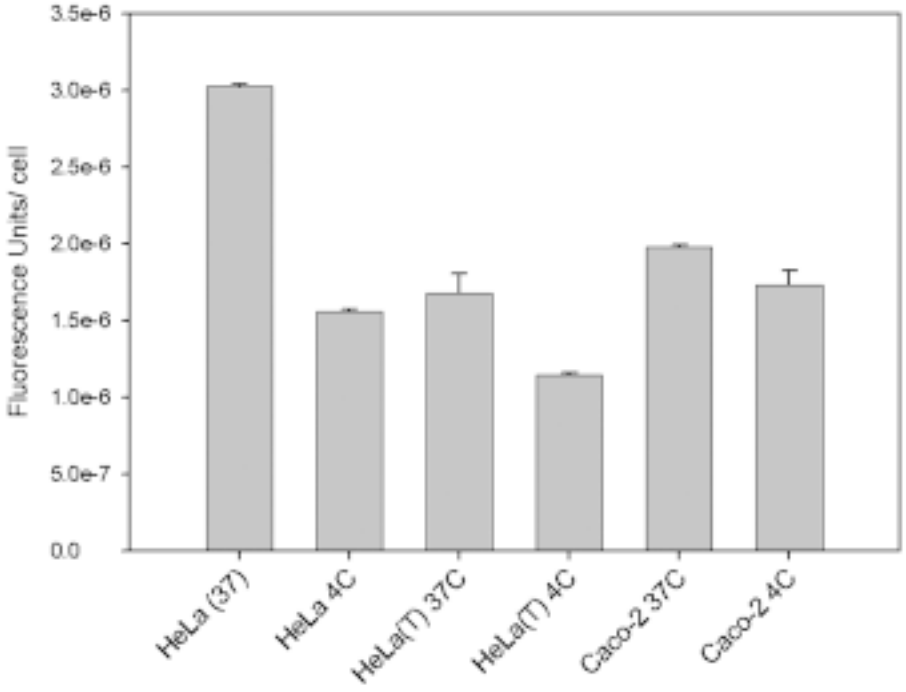

Figure 5. Spectrofluorimetric assay for the amount of FDACC internalized in wild type HeLa, FRα knockdown HeLa and Caco-2 cells at 37˚C and at 4˚C respectively. For all the groups n=4, $p<0.001$ between HeLa (37) and all other groups, $p<0.05$ between treatments at two different temperatures for each cell line.

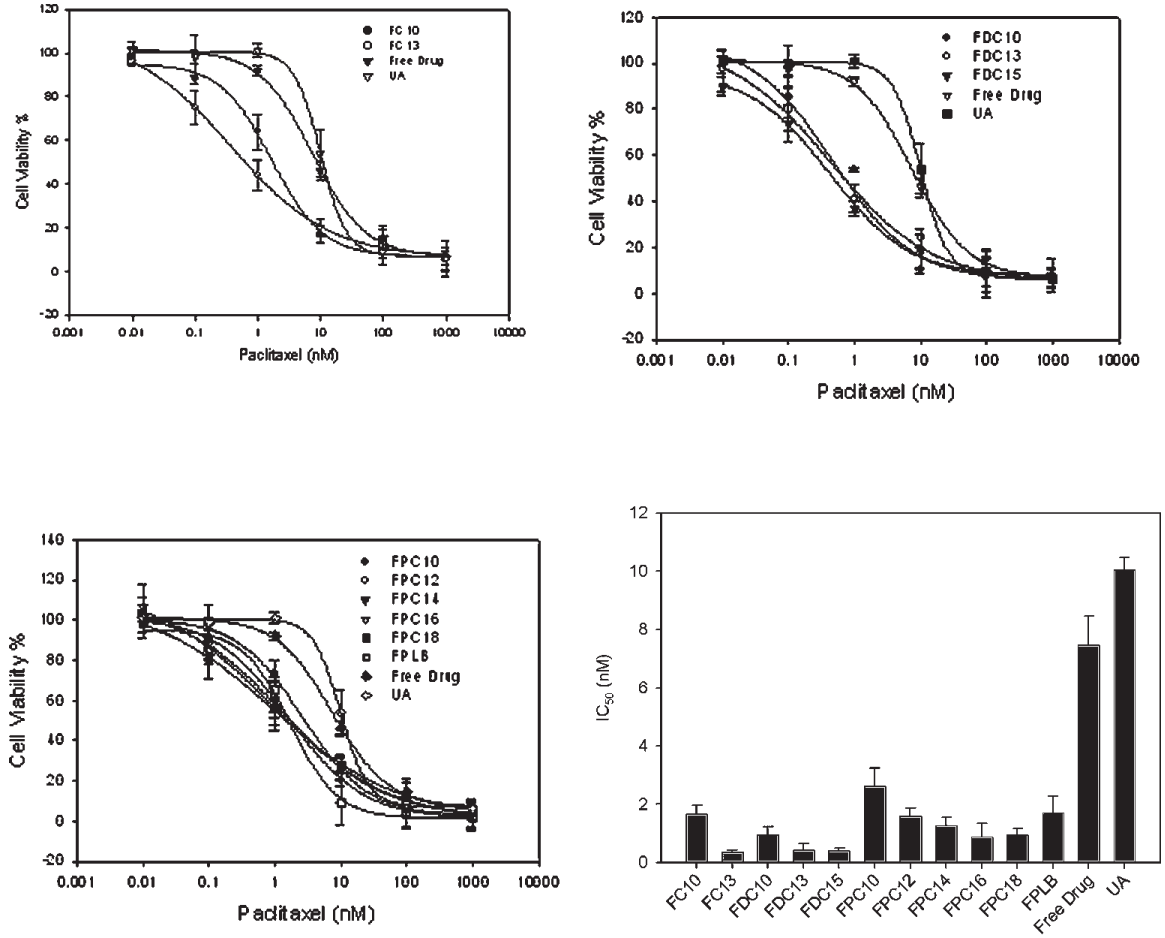

Figure 6. Cytotoxicity profiles of drug treatments in wild type HeLa cells with amphiphilic conjugates, with free drug and with untargeted amphiphile (UA). A) FC(n) series B) FDC(n) series C) FPC(n) series D) IC_{50} values in nM estimated from curve fits of the respective cytotoxicity profiles from each treatment. Each data point in A, B and C is represented as mean±CV% with n=3 and $r^2 > 0.98$ for all the fitted curves. In D, $p<0.05$ between IC_{50} of free drug treatment or IC_{50} of drug treatment in presence of UA and IC_{50} (s) of drug treatment in presence of amphiphilic conjugates. The error bars signify standard error of estimate of IC_{50} from the curve fit.

Internalization of folate tagged delivery systems is known to follow a caveolar pathway (28). The GPI anchored FR- receptors occur diffusely on the cell membrane but are functional only when associated with caveolae in cholesterol rich domains often referred to as lipid rafts (28). The caveolae form flask shaped invaginations on the cell surface and transform into vesicles when transporting folic acid into the cells (28).

Effect of the targeted delivery system on median lethal dose

The major goal of any tumor targeted delivery system is to achieve cell kill at a lower dose than required for a nontargeted system. It is also important that the delivery system be safe and should not be toxic at the range of concentration that will be used in a delivery system. HeLa cells were used as an in vitro test

model for tumors expressing folate receptors, whereas, Caco-2 was used as a control. The folate labeled amphiphilic conjugates and the untargeted amphiphiles were tested for their toxicity in HeLa and Caco-2 cells. An average of 20-30% mortality in HeLa and Caco-2 cells were noted after 48h of exposure to 100 ìM of any of the folate labeled amphiphilic solutions. Visual observation of the treated cells did not reveal any immediate lysis of the cells within 2-3h of treatment as observed for strong surfactants and detergents (29). HeLa cells, when treated with paclitaxel in a micellar solution of the targeted amphiphiles, exhibited significantly lower IC_{50} values than when treated with free drug or with an untargeted amphiphile (Fig. 6 a-d). Similar observations with targeted delivery of anticancer agents have also been demonstrated (30-33). FC(n) series of amphiphilic conjugates resulted in a sixteen fold decrease in the amount of drug required to elicit the IC_{50} response as compared with the free drug. Higher homologues of this series of amphiphiles viz FC16 and FC18 could not be used due to their low solubility. Use of FDC(n), FPC(n) and FPLB classes of amphiphiles resulted in an average reduction of IC_{50} dose by sixteen, eight and six folds, respectively. Within a homologous series of amphiphiles, an increase in the chain length of the aliphatic acid resulted in a decrease in the IC_{50} indicating their involvement in drug permeation into the HeLa cells (Fig. 7). Amphiphilic conjugates with larger aliphatic chain length have lower critical micellization concentration resulting in more number of micelles for the same concentration when compared to one with a smaller aliphatic chain length. For Caco-2 cells the IC_{50} of the targeted delivery had no significant difference with the untargeted delivery signifying no contribution of the amphiphiles in intracellular delivery of the drug (Fig. 8 a-d).

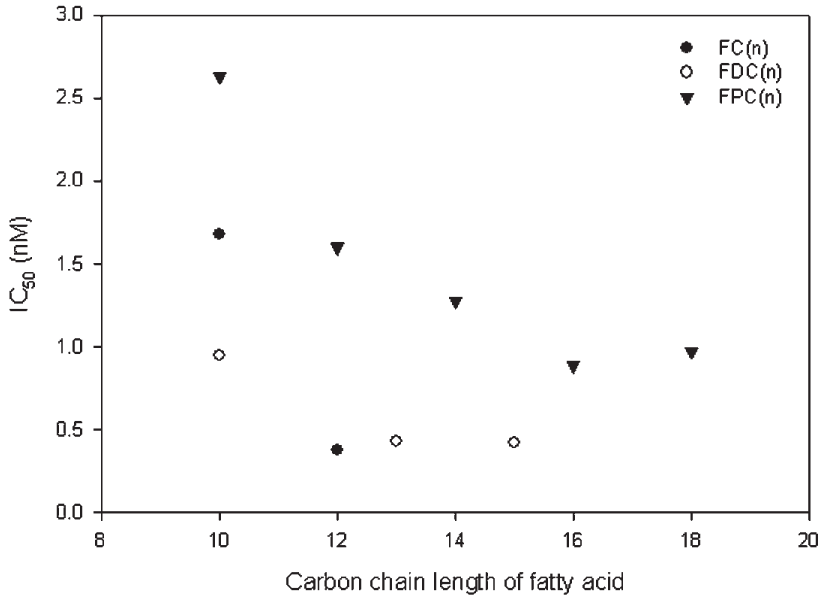

Figure 7. IC_{50} values plotted as a function of size of aliphatic chain length of the amphiphilic conjugates.

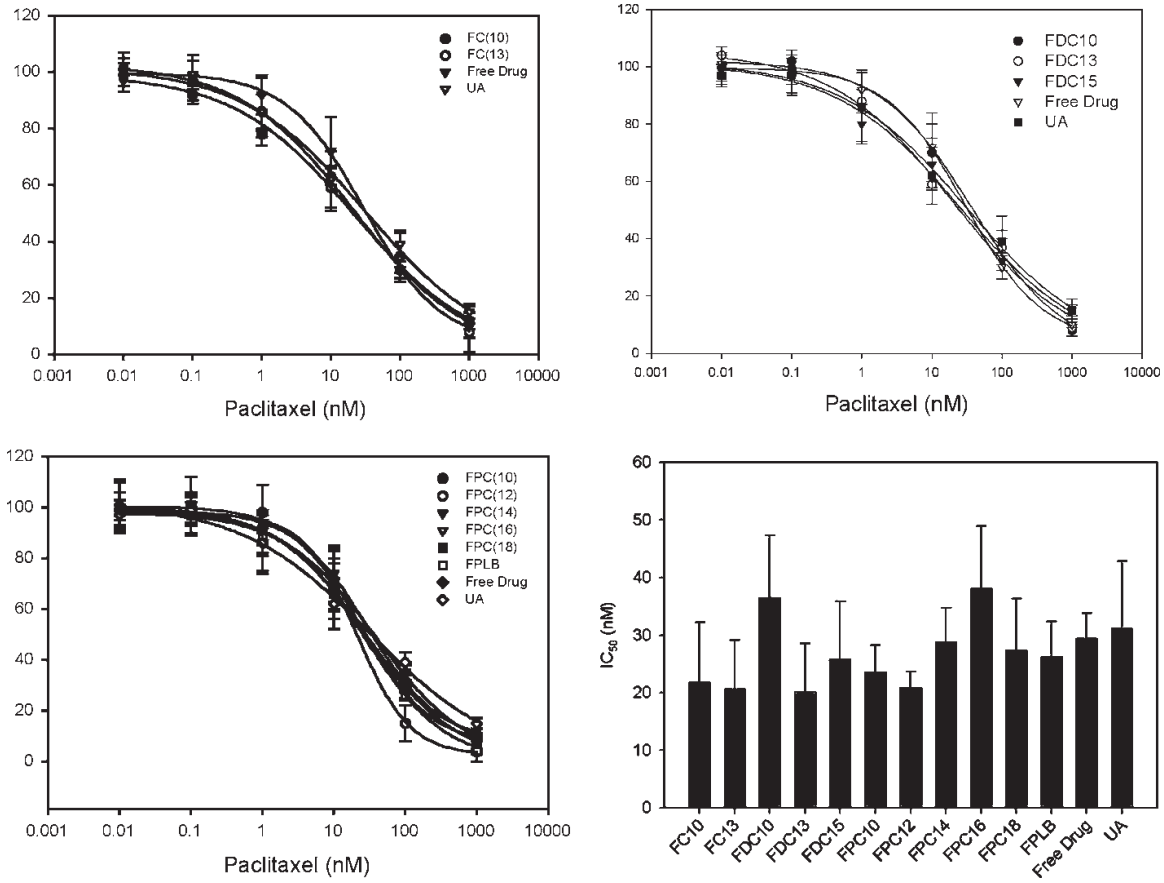

Figure 8. Cytotoxicity profiles of drug treatments in Caco-2 cells with amphiphilic conjugates, with free drug and with untargeted amphiphile (UA). A) FC(n) series B) FDC(n) series C) FPC(n) series D) IC_{50} values in nM estimated from curve fits of the respective cytotoxicity profiles from each treatment. Each data point in A, B and C is represented as mean±CV% with n=3 and $r^2 > 0.98$ for all the fitted curves. In D, $p>0.05$ between IC_{50} of free drug treatment or IC_{50} of drug treatment in presence of UA and IC_{50} (s) of drug treatment in presence of amphiphilic conjugates. The error bars signify standard error of estimate of IC_{50} from the curve fit.

Conclusion

The feasibility of amphiphilic conjugates of folic acid with aliphatic carboxylic acid as micellar carriers for targeted delivery of drugs to tumor cells is demonstrated. The amphiphilic conjugates were actively internalized by a receptor mediated process proving the specificity of this folate tagged delivery system towards the folate overexpressed cells. The amphiphilic conjugates were shown to internalize into the cells and elicited cytotoxic response at a lower concentration and did not show any toxicity within the tested concentration range.

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Effect of various immobilization matrices on *Lactobacillus delbrucekii* cells for optically pure L (+) lactic acid production

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Abstract

The Present study reveals the effect of Lactobacillus delbrucekii cells immobilized in various matrices for the production of optically pure L(+) lactic acid. Functionalized alginate matrices were effective and suitable for higher L (+) lactic acid yields compared to other matrices. Repeated batch fermentation showed productivity of 1.74, 1.44, 1.48 and 1.52 Y p/s with functionalized alginate, Ca-alginate, K-Carrageenan, and glass beads, respectively. *L. delbrucekii* cells were immobilized in natural and functionalized alginate beads. The scanning electron microscopic studies showed increase in entrapped microbial cell biomass in modified immobilized beads compared to other matrices. These modified alginate beads showed enhanced stability and selectivity towards L (+) lactic acid production in higher yields with an enantiomeric selectivity of 99% and low by-product production.

Key words: Alginate, Enantiomeric selectivity, Fermentation, Immobilization, L(+) lactic acid, *Lactobacillus delbrucekii*

Introduction

Optically pure lactic acid is gaining importance in the current biotechnological era as it has vast application potential especially as feedstock for biodegradable polymers, oxygenated chemicals, plant growth regulators, synthesis of environmental friendly green solvents and specially chemical intermediates (1). Hence, it became a large volume (one lakh tones per annum) chemical commodity with an annual growth rate of 15 % in global market with an estimate of 3.9 million tones in the year 2008 (2). Though lactic acid could be synthesized either by chemical means or by fermentation methods using specialized microbial strain, the fermentation methodology is becoming prominent (50 % of the world supply is by conventional free cell fermentation) due to the production potential of optically pure lactic acid (3). In fact, the efficiency and economics of microbial product production has always been a concern with respect to fermentation medium development (4, 5, 6), microbial strain selection (7), substrate economics (8), biomass development and downstream processing (9,10). In order to improve the lactic acid productivity, several microbial strains were isolated and studied in detail to understand their potential in industrial scale. However, it achieved a limited success mainly due to low productivity associated with a change of fermentation environment (increased acidity of the fermentation medium) which resulted in reduced growth of microbial strain and productivity. Efforts have been made to enhance the biomass concentration, online removal of product, reduction of by-product

formation and improve the metabolism and most of the work has been focused by using starch, beet molasses, whey and cane sugar as the fermentation media including glucose containing other wastes (11). In this context, immobilization technology has shown promising role as most of above problems such as cell-retaining capacity, reduced susceptibility to contamination, and reuse of the biocatalyst with higher product conversion capability over free-cell fermentations could be solved without much alteration in the fermentation conditions (12-14). Variety of immobilization matrices including natural (alginate, carrageenan, agar-agar, glass beads, etc) and synthetic (polyvinyl alcohol, poly acrylamide, etc) containing sodium alginate, calcium pectate gels, chemically modified chitosan and alginate beads were evaluated (15-18). Among all immobilization matrices studied, sodium alginate attracted scientific attention due to its eco-friendly nature, cost-effectiveness, the mild conditions required for immobilization, its simplicity and non-toxic nature (17). Studies with *Lactobacillus delbrucekii* strain entrapped in the alginate bead matrix indicated that the mass transfer of substrates and products diffuse in and out easily. However, stability of the beads was important to maintain high conversion of substrate to product as this matrix has certain demerits like losing its stability under extreme pH and certain ionic concentration (14, 20, 21), which was influenced by the concentration of sodium alginate and bead diameter (18, 19, 22). Studies made by different scientific communities using different immobilization matrices, fermentation media, different microbial strains under different fermentation environments caused difficulty in comparison of the results and their subsequent use at process evaluation steps. Hence, in the present investigation, an effort has been made to compare the growth pattern of *L. delbrucekii* under different immobilization environments (natural and functionalized alginate, carrageenan and glass bead). Also evaluate its substrate utilization and obtain optically pure lactic acid production pattern under prescribed fermentation conditions along with its by-product formation.

Materials and Methods

Organism and Medium

The organism, *L. delbrucekii* (NCIM 2365) was used in this study. The culture was maintained on deMan–Rogosa–Sharpe (MRS) agar (Hi-media) slabs and sub cultured twice in a month. Sodium alginate (A2158; viscosity approximately 250 cps at 25 °C) was procured from Sigma-Aldrich, USA. Spherical porous sintered glass beads having SIKUG04 matrix type consisting of 0.4-1.0 mm dimension, 55-60 % pore volume, 120 µm pore diameter and 600 g/l density were purchased from SIRAN (Schott Glassware, MainZ, Germany) and was used for immobilization. All chemicals and solvents used in this study were of analytical grade and procured from standard firms.

Derivatization of Alginic Acid and Analysis

Alginate succinylation and palmitoylation was performed according to modified method of Phillips et al. (21) and Le-Tein et al. (24), respectively. The degree of succinylation was determined by the titration method as described by Wurzburg (25), while the degree of palmitoylation of alginate was determined by ninhydrin method (detecting free amino groups) as described by Phillips et al. (21).

Cell Immobilization

L. delbrucekii cells were immobilized in ê-carrageenan, sodium alginate and succinylated alginate beads using 2 % (v/v) inoculum (10^5 CFU/ml) under sterile conditions. To 4% sodium alginate/succinylated alginate solution, an equal volume of 24 h grown *L. delbrucekii* cell culture was added and mixed thoroughly. The resultant cell suspension was dropped as droplets into 2% calcium chloride solution to get the calcium

alginate/ succinylated/ palmitoylated alginate-immobilized beads. In ê-carrageenan immobilization, 4% solution was prepared at 70°C, cooled to room temperature, and equal volumes of bacterial suspension and ê-carrageenan solution were mixed and immobilized by dropping into 2% KCl solution at 10°C. The resultant immobilized beads were washed with sterile distilled water, and the resultand immobilized cell beads were then incubated at constant shaking condition at 150 rpm at 37°C. The stability of immobilized beads was measured in terms of time taken for dissolution of five beads from each matrix in 3 M phosphate buffer (pH 5.0).

Fermentations

Lactic acid production with both the free and immobilized cell fermentations was performed at 37 °C using the production medium (glucose, 100 g/l; corn steep liquor, 68 ml/l; trace mineral solution, 1 ml/l; and $CaCO_3$, 100 g/l adjusted to pH 6.4) and 24-h grown inoculum (5%, v/v) having an optical density as 1.0 at 600 nm. The cell-free samples were then collected at predetermined time intervals and were analyzed using high-performance liquid chromatography (HPLC). Repeated batch fermentations were carried out regularly in a fresh medium after every 144 h using the immobilized cell beads. The process was repeated till the lactic acid production was continued by viable cells present in the immobilized matrix. The data presented in this study were the average values of three repeated experiments.

Cell mass estimation

Cell growth was determined spectrophotometrically at 600 nm and converted to cell count using a conversion factor (one unit at 600 nm was equivalent to 10^5 CFU/ml, which corresponded to 32 µg protein that was calculated by plotting standard graphs). In case of all immobilized conditions except glass bead immobilization, 5 beads were dissolved in 2ml of phosphate buffer (3M; pH 5.0). Cells were collected by centrifugation at 5000 rpm at room temperature and used for cell mass measurement by serial dilution plate count method using agar-based growth medium. The total number of released cells was determined by standard plate count method using agar plates after incubating at 37 °C for 24 h. At the end of each batch, the cell densities in the beads were enumerated using similar method to study the total cell loss upon repeated use. Viable cell counts were performed in duplicate and expressed in CFU/ml of immobilized beads. While, cells immobilized on glass beads were measured indirectly by determination of total protein content with a bicinchoninic acid (BCA) assay (26, 27). Carrier samples were washed with 1 ml phosphate buffer (17 mM; pH 7.6), followed by addition of 1 ml lyses-buffer (0.1 M TRICIN (N-Tris (Hydroxymethyl)-methyl) glycine, 0.2% Triton X-100, pH 7.6). The resultant solution was subjected to strong overtaxing for 15 min. The BCA-assay was performed with the supernatant of the lyses solution. For this 50 µl sample was mixed with 1 ml standard working reagent and then incubated at 60 °C for 30 min. After incubation, the samples were cooled and its absorbance was measured at 562 nm. This was converted to CFU according to a standard curve, which was constructed before, by applying the BCA-assay with cell suspension of known cell density.

Scanning Electron Microscopy

For microscopic studies, immobilized beads containing *L. delbrucekii* cells were transferred to vials and fixed in 3.5% gluteraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 h at 4 °C. These cells were then post fixed by incubating in 2 % aqueous osmium tetroxide in the same buffer for 2 h. The samples were then dehydrated by gradient alcohol series and dried to the critical point by incubating in an

Electron Microscopy Science CPD unit. The dried samples were then mounted over the stubs with double-sided conductivity tape. Finally, a thin layer of platinum metal was applied over the sample using an automated sputter coater (JEOL JFC 1600) for about 90 sec. The samples were then scanned under scanning electron microscope (model: JOEL-JSM 5600) at various magnifications using 5 kV (accelerating voltage) at RUSKA Lab, college of Veterinary Sciences, ANGRAU, Hyderabad, India.

HPLC Analysis

Concentrations of glucose and organic acids (lactic, formic, propionic and acetic acids) present in filtered fermentation culture broth were determined by HPLC using GROM Resin ZH column (250∞8 mm) using mobile phase 5 mM H_2SO_4 and absorbance at 210 nm. The optical purity of the lactic acid was analyzed using a chiral column [chiral pak MA (+) obtained from Daicel Chemical] using 2 mM $CuSO_4$ as an eluent and absorbance at 250 nm. All the experiments were carried out in three replicates and the results given were the mean values.

Results and Discussion

Lactic acid production pattern was studied under submerged fermentation environment using production medium with different immobilized *Lactobacillus delbrucekii* beads. The acid production pattern differed with the type of immobilization matrix used for *L. delbreckii* cell indicating the selected matrix material's impact on metabolism related to the production of lactic acid in this *L. delbrucekii* with similar fermentation environment. Maximum lactic acid production was noticed with derivatized alginate immobilized beads and minimum production was observed with free cell fermentation. A 57 % improvement in lactic acid production was noticed with immobilized beads compared to free cell fermentation. Lactic acid production was observed to be 51.2 and 80.5 g/l with free and immobilized cells, respectively. Among immobilized cell fermentations, natural polymer immobilized cells (alginate - 63.5 g/l and carrageenan - 68.9 g/l) showed less lactic acid productivity compared with glass bead (72.5 g/l), succinylated (76.2 g/l) and palmitoylated (80.5 g/l) alginate immobilized. A 30 % of productivity was improved with palmitoylated alginate immobilized cells compared to alginate immobilized *L. delbrucekii* cells suggesting that the chemical variation of matrix material had influenced the metabolism of *L. delbrucekii* cells. This enhanced lactic acid production under immobilized conditions may be attributed to improved buffering activity of fermentation medium. Such increased production profile as well as the associated metabolic affects on cell growth and subsequent product formation was well documented in literature (18, 19, 28). Li Ten et al., (24) observed an improved pH stability and better survival of succinylated alginate immobilized *L. delbrucekii* cells during intestinal passage. Further analysis of fermentation broth was performed to investigate the optical purity of produced lactic acid under different immobilized environments. It was observed that the optical purity L (+) lactic acid was 99% in both functionalized (both the succinylated and palmitoylated) alginate beads, compared to other immobilization matrices where it varied from 88 to 92 % (results were not shown).

Immobilization dependent improved product productivity in different microbial strains were attributed for metabolic shift towards product production rather than microbial strain growth in addition to improved biomass density in immobilized bead environment and low cell wash off as compared to free cell fermentation (14, 17, 29). Hence, the cell morphology under different immobilized conditions were studied by analyzing the scanning electron micrographs of surface and cross sections of different matrices

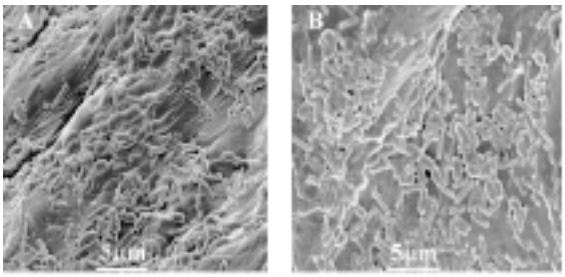

Fig 1: Scanning electron micrographs of *L. delbrucekii* cells immobilized using carrageenan A) surface B) section

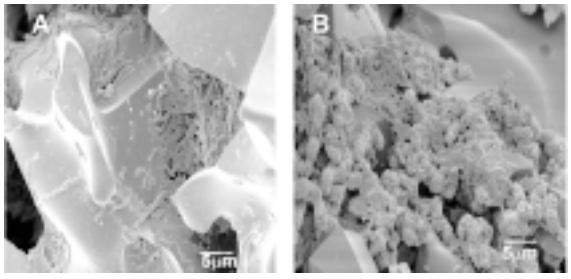

Fig 2: Scanning electron micrographs of *L. delbrucekii* cells immobilized using porous glass beads A) surface B) section

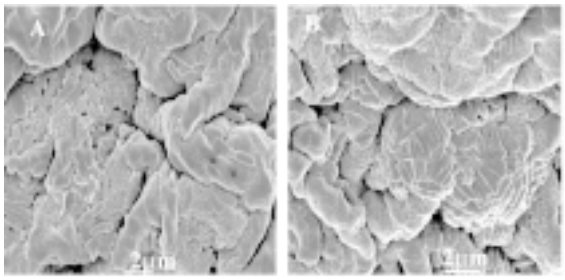

Fig 3: Scanning electron micrographs of *L. delbrucekii* cells immobilized using palmitoylated alginate A) surface B) section

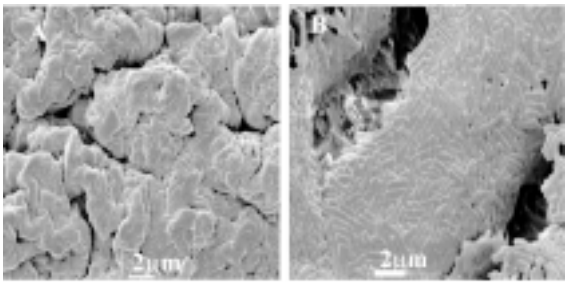

Fig 4: Scanning electron micrographs of *L. delbrucekii* cells immobilized using Succinylated alginate A) surface B) section

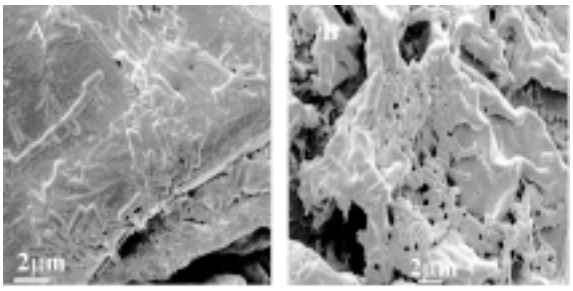

Fig 5: Scanning electron micrographs of *L. delbrucekii* cells immobilized using alginate A) surface B) section

immobilized cell beads and reported in (Fig 1-5). The cell density was differed in different immobilized beads (with respect to matrix material) through the same cell concentration was used for all immobilizations indicating the impact of chemical nature of immobilization matrix on support of microbial cell growth and its retention during fermentation conditions. Maximum cell density was observed with succinylated and palmitoylated alginate immobilized beads with pocketed distribution on /or in the bead (Fig 3 and 4). In case of glass beads the cells were mostly located in pores along with calcium carbonate crystals (Fig 2). A more or less uniform distribution was observed in case of carrageenan and alginate immobilization indicating functionalization has an impact on cell distribution under immobilized environment (Fig 1 and 5).

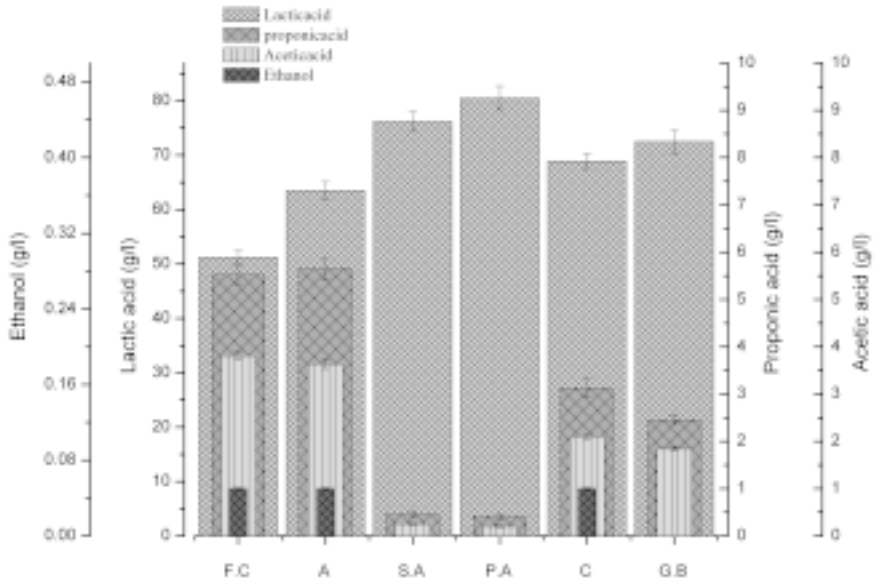

Fig 6: lactic acid and other impurities production profile during fermentation by free cells and different immobilized cells of *L. delbrucekii* F.C. –free cells, A-alginate S.A- Succinylated alginate, P.A – palmitoylated alginate, C-carrageenan, G.B- glass beads

Comparative evaluation of lactic acid production values with respect to cell biomass under immobilized conditions reveals that properties of immobilization matrix has an influence on *L. delbrucekii* metabolism associated lactic acid production. This was concluded based on observation that there was variation of lactic acid production and observed difference in cell density under different immobilized environments by *L. delbrucekii* cells under similar fermentation conditions (Fig 1 and 2). These results were in accordance with observed metabolite production values by same microbial strain with immobilization matrix variation (17).

In most of the lactic producing microbial strains, lactic acid production is associated with other by-products such as ethanol, acetic and propionic acid production (30, 31). These by-products have major impact on production of biodegradable polymer production during polymerization (3). Production of these lactic acid associated by-products depends on culture conditions, fermentation medium composition and microbial genetic nature. Analysis of by-product profile in the cell free fermentation broth of different immobilized *L. delbrucekii* cells indicated that ethanol, acetic and propionic acid profile varies with the chemical nature of the matrix (Fig 6). Among all tested immobilization matrices, palmitoylated alginate showed less impurity profile followed by succinylated and glass bead immobilization. Natural alginate immobilized cells produced more amount of by-products compared to carrageenan immobilized cells. Ethanol impurity was not observed in derivatized alginate and glass bead immobilized cells.

Stability studies indicated that palmitoylated alginate immobilized beads were

more stable as compared to natural alginate, succinylated and carrageenan immobilized during fermentation. Our fermentation studies also revealed that palmitoylated alginate beads could be used more than eight fermentation cycles compared to six cycles in case of alginate. Though glass bead immobilized cells revealed higher fermentation cycles than palmitoylated, the productivity values decreased drastically after its 6th fermentation cycle (results not shown).

Conclusion

Lactic acid along with its by-products production were studied with *L. delbrucekii* cells using immobilized natural polymers (alginate and carrageenan), functionalized polymers (succinylated and pamitoylted) and glass beads, and these were compared with free cell fermentation results. Palmitoylated alginate immobilized cell beads showed improved L (+) lactic acid with less by-product formation, higher stability and more fermentation cycles compared to other fermentation studies. Improved cell density was also observed in derivatized alginate immobilized beads compared to alginate. Overall, effective optically pure L (+) lactic acid production with less by-product (ethanol, acetic and Propionic acid) production were observed with palmitoylated alginate as an immobilization matrix using *L. delbrucekii* cells indicating its commercial significance.

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An assessment of genetic stability in micropropagated plants of *Ochreinauclea missionis* by RAPD markers

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Abstract

Present study reports the use of randomly amplified polymorphic DNA (RAPD) markers, to verify the clonal fidelity between the micropropagated plants and the mother plant of *Ochreinauclea missionis,* an ethnomedicinal and endemic tree in Western Ghats of India. Thirty eight RAPD primers were screened, out of which thirty two primers generated a total of 245 clear, distinct and reproducible bands. Out of 245 total bands, 227 bands were monomorphic with 92.67 % and 18 bands showed polymorphic banding pattern with 7.34 %. Thus, a total of 5390 bands were generated showing uniform banding patterns for each primer that are comparable to the mother plant from which the cultures were established. Cluster analysis based on unweighted pair group method with arithmetic averaging (UPGMA) showed 97% similarity between the mother plant and micropropagated plants. The developed RAPD profiles confirmed the clonal fidelity of tissue culture raised plantlets which were reintroduced to its original habitat for conservation.

Keywords *Ochreinauclea missionis*, Medicinal tree, Genetic fidelity, Polymerase chain reaction, RAPD analysis.

Introduction

Ochreinauclea missionis (Wall. ex G. Don) Ridsd. locally known as 'Jalamdasa', belongs to the family Rubiaceae. It is a medium sized evergreen threatened medicinal tree and is endemic to Central and Southern Western Ghats of peninsular India (1). The powdered bark and its decoction are used for curing cutaneous diseases like leprosy, ulcers and as an effective purgative (2). Root and root bark are employed in treating rheumatism, paralysis, skin diseases, dropsy, eye diseases, constipation, piles, jaundice, fever, edema, hepatic and haemophilic disorders (1). *O. missionis* is reported as rare due to dwindling of natural forest as a result of construction of dams, hydroelectric projects and roads for agricultural purposes. To our knowledge, there have been no reports on the comparative genomic stability or variation in regenerated plants and mother plant of *O. missionis.* However, the vegetative propagation and *in vitro* regeneration through nodal explants were previously described (3, 4). *In vitro* clonal propagation of trees is an attractive method for obtaining high number of elite genotypes. The somaclonal variation of the micropropagated elite genotypes can be a potential draw back. However, true-to-type clonal fidelity is one of the most important prerequisites in the micropropagation of forest tree species (5).

In recent years, several DNA markers have been successfully employed to assess the genomic stability in regenerated plants including

those with no obvious phenotypic alternations (6). Among the markers, RAPD technique being simple and cost effective has been used in numerous studies. RAPD analysis is particularly well suited to high-output systems required for plant breeding because it is easy to perform, fast, reliable and of relatively low cost (7). Nevertheless, RAPD technique has some limitations concerning reproducibility and an uncertain homology of co-migrating fragments in gel electrophoresis (8). But, most of these limitations can be minimized by carefully adjusting the reaction and detection conditions (9). RAPD analysis can be applied to assess the genetic fidelity of plants derived *in vitro* on an industrial scale as part of crop improvement programs (10). This method might be useful for monitoring the stability of *in vitro* germplasm collections and cryopreserved material. RAPD analysis in plants has also been widely used to detect genetic and somaclonal variations (11-14). RAPD technique does not require DNA sequence information and species specificity and hence it is being conveniently used for assessing genetic stability and clonal fidelity of micropropagated plants in a number of genera. The RAPD markers are referred to as an appropriate tool to get rapid information about genetic similarities or dissimilarities in micropropagules. Thus, in the present study we report the assessment of genetic integrity in tissue cultured *O. missionis* plants with their mother plant using RAPD markers.

Materials and Methods

Plant material and *in vitro* regeneration

Tender branches were excised from 8-10 year old mature trees of *O. missionis* growing along the river bank side of Seethanadhi in Udupi district, Karnataka, India. *In vitro* regeneration was achieved using the earlier standardized protocol (4). Multiple shoots were initiated from nodal explants on Murashige and Skoog's (MS) medium (15) incorporated with 2 mg/l 6-benzylaminopurine (BA) and 0.3% (w/v) activated charcoal (AC). After 5 weeks, *in vitro* developed shoots were subcultured onto MS medium containing BA (0.5 mg/l) and naphthaleneacetic acid (NAA 1 mg/l) and AC (0.3%) for shoot elongation. For *ex vitro* rooting, the base of *in vitro* shoots was dipped in different concentrations of IBA solution for different time durations and immediately transferred into bottles and plastic pots containing sterile soilrite (equal proportions of decomposed coir and peat moss, Karnataka Explosives, Bangalore, India) and the plantlets were hardened in greenhouse for a period of six months before transferring to field conditions.

DNA extraction

Twenty one tagged regenerated plants from hardening stage were randomly selected along with single mother plant for screening their genetic integrity. Total DNA was extracted from fresh young leaves of micropropagated plants and field grown mother plant using cetyl trimethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (16) with minor modifications. Quality and quantity of DNA were inspected both by gel electrophoresis and spectrometric assays using UV-Visible Double Beam PC Scanning spectrophotometer (LABOMED, Culver city, USA).

RAPD analysis

PCR amplification was performed with 38 arbitrary decamer RAPD primers (Sigma Aldrich chemicals Pvt. Ltd., Bangalore, India). A total of thirty eight RAPD primers were screened initially and 32 primers were selected in the present study. Amplifications were performed as described in earlier report (7) in a total volume of 20 µl reaction mixture containing 2 µl of 1´ assay buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl and 0.01 % gelatin, pH 9.0), 250 µM dNTPs, 200 µM primer, 1.5 unit (U) Taq DNA polymerase (Bangalore Genei, Bangalore,

India) and 50 ng genomic DNA. PCR amplification was performed using thermocycler UNO II (Biometra, Goettingen, Germany) with hot lid according to the following programme with initial denaturation of 94 ^{0}C for 3 min, followed by 40 cycles for 60s at 94 ^{0}C for denaturation, 1 min at annealing temperature, 2 min extension step at 72 ^{0}C and a final extension step at 72^{0}C for 10 min. The annealing temperature was adjusted according to the primers used in the PCR. The amplification products were resolved by electrophoresis on 1.5 % (w/v) agarose gel (Amersham, Uppsala, Sweden) in 1x TBE buffer (Tris–Borate–EDTA buffer) at 75-100 volts. The gels were stained with ethidium bromide solution. The amplified products were visualized and photographed under UV transilluminator and documented using Bioprofile Image Analysis System (Vilber Lourmat, France). Molecular marker λ DNA / EcoR I -Hind III double digest (Bangalore Genei, Bangalore, India) was used to estimate the size of amplification products. In order to have reproducible and clear banding patterns, PCR amplifications were repeated for atleast twice.

Data analysis

The presence and absence of bands between samples was scored and data were transcribed into binary format (1, 0 respectively) in each plant at a particular position, which was treated as an independent character regardless of its intensity. PCR amplified bands in the size range of 200 to 21,226 bp were scored with all the selected RAPD primers. Based on the matrix of genetic similarity, cluster analysis was performed. The similarity coefficients thus generated were used for constructing dendrogram using the UPGMA (unweighted pair-group method with arithmetic average) and the SHAN (sequential hierarchical agglomerative nested clustering) option in NTSYS-pc software package (17). The three dimensional principle coordinate analysis (PCA) was conducted with the same program using EIGEN module. This multivariate approach was chosen to complement the cluster analysis information. Genetic similarities between micropropagated and mother plants were used to calculate the Jaccard's similarity coefficient (18).

Reintroduction of micropropagated plants

After confirming the genetic stability, the tissue cultured plants were maintained in greenhouse for a period of six months. Nearly, five hundred micropropagated plants were reintroduced in rainy season (June - August) into their original habitat near Mani region (Udupi district, Karnataka, India) with the help of local people. After one year, the survival rate of reintroduced plants was recorded. The growth conditions were 22 ± 2 ^{0}C with 70% relative humidity, in normal day light conditions.

Results

Micropropagation

Previously standardized micropropagation protocol was used to establish the large scale propagation of *in vitro* plants (4) for the assessment of genetic fidelity. The highest frequency of nodal explants responding (83.3%) was observed on MS medium supplemented with 2 mg/l BA and 0.3% AC. For subculture, incorporation of NAA (1 mg/l) in combination with BA (0.5 mg/l) gave a maximum of 9.7±1.2 shoots/explant. High percentage of rooting (91.6) with a maximum root length of 3.8cm was observed at 30 min exposure of multiple shoots in 10 mg/l indole-3-butyric acid (IBA) solution. Rooted plantlets were acclimatized in growthchamber (Sanyo, Moriguchi-city, Osaka, Japan) under temperature of 25±2 ^{0}C, 80% relative humidity, irradiance of 50 µmol m^{-2} s^{-1} with 16 h of photoperiod for 4 weeks. Then plantlets were hardened in greenhouse conditions for a period of six months before transferring to their habitat.

Genetic stability by using RAPD primers

We screened thirty eight RAPD primers for this analysis but only thirty two primers were useful in reproducing the banding patterns. Therefore, remaining six primers were discarded as they were producing ambiguous and non reproducible amplification profiles. Other 32 primers produced a total of 245 clear and reproducible bands of which, 227 bands were monomorphic with 92.67% and remaining 18 bands were polymorphic with 7.34%. Indeed, all these 32 primers generated identical banding patterns in two independent amplifications that were performed for all the samples. The number of bands per each primer varied from 2 to 12 with an average of 7.65 bands per primer. A total of 5390 fragments (numbers of plantlets analyzed ∞ number of bands in all the primers) were generated showing homogeneous RAPD banding patterns. The selected RAPD primers, their annealing temperature, total number of bands scored, their base pairs size, monomorphic bands and polymorphic bands for each primer are summarized in table 1. RAPD amplifications were observed for monomorphic pattern in primer OPE-20 (Fig. 1a) and polymorphic in primer OPC-05 (Fig.1b).

The dendrogram constructed on the basis of Jaccard's similarity matrix, followed by UPGMA based clustering analysis (Fig. 2a) showed that the genotypes were grouped into single cluster with the donor mother plant, which comprises of twenty tissue cultured plants, while plant 'Om1' fell apart from this clustering. The coefficient of similarity in the dendrogram generated by the RAPD data among the regenerated plants ranged from 0.953 to 0.997 with a mean of 0.976 indicating the genetic similarity of 97%. The linear relationship among 21 micropropagated plants and donor plant are shown in PCA (Fig. 2b). In the three-dimensional plot of PCA where *in vitro* plants 'Om2 to Om21' fell within donor plant showing genetic similarity but plantlet 'Om1' lies apart from all showing significant changes in PCA. The PCA and dendrogram both results showed that the micropropagated plants exhibited distinct genetic similarity with mother plant. All groups showed genetic stability among each other and comes under the donor group showing monomorphism. Most of the micropropagated plants reintroduced into the Mani region (Karnataka, India) showed better establishment without any morphological variation, with 70% of survival rate.

Discussion

Plant tissue culture has been successfully used for large scale propagation of number of plant species including many medicinal plants (19). The genetic integrity of micropropagated plants can be determined with the use of various techniques. The choice of a molecular marker technique depends on its reproducibility and simplicity. In this study, RAPD technique was employed to assess the true to type clonal fidelity of *O. missionis* plantlets. In order to confirm genetic integrity, the DNA profile of 21 regenerated plants was compared with the DNA profile of the mother plant. Many workers suggested the role of *in vitro* propagation of rare and endangered plants as an effective means for conservation but there are limited reports on reintroduction of micropropagated plants in wild (20) or to *ex situ* conservations. Screening the tissue culture derived plants at an early stage or during hardening and prior to reintroduction using molecular markers will assist in reintroducing true-to-type plants (21) and protecting their genetic integrity (22).

The visual assessment of over hundred plants derived from the axillary bud culture did not reveal any morphological variation in the micropropagated plants of *Eucalyptus tereticornis* and *E. camaldulensis* (23) and *Tylophora indica* (24). The identification of variability in micropropagated plants derived

Table 1 Number of amplification products generated with RAPD primers in the analysis of *O. missionis* plants.
(A.T-Annealing Temperature, P.B-Polymorphic Bands, M.B-Monomorphic Bands)

Primers	Sequence (5' to 3')	Size range (bp)	A T^0C	Number of bands		
				P B	M B	Total
OPA-04	AATCGGGCTG	1584-564	40.0	0	4	4
OPA-11	CAATCGCCGT	2027-560	40.0	0	8	8
OPA-14	TCTGTGCTGG	3100-235	35.9	0	11	11
OPA-20	GTTGCGATCC	1904-200	35.0	2	5	7
OPB-01	GTTTCGCTCC	4268-564	41.5	2	9	11
OPB-04	GGACTGGAGT	1375-564	39.6	0	4	4
OPC-01	TTCGAGCCAG	1584-564	35.0	2	2	4
OPC-02	GTGAGGCGTC	2100-564	33.0	1	6	7
OPC-04	CCGCATCTAC	1904-200	33.0	0	8	8
OPC-05	GATGACCGCC	2100-200	40.0	3	6	9
OPC-07	GTCCCGACGA	1584-265	40.0	1	6	7
OPC-08	TGGACCGGTG	1750-564	40.0	4	6	10
OPC-11	AAAGCTGCGG	1845-560	40.5	0	8	8
OPC-15	GACGGATCAG	947-564	31.5	0	2	2
OPD-05	TGAGCGGACA	2027-200	36.9	0	10	10
OPD-06	ACCTGAACGG	1904-245	39.5	0	5	5
OPD-07	TTGGCACGGG	1450-200	45.0	1	5	6
OPD-11	AGCGCCATTG	2576-200	41.2	0	8	8
OPD-16	AGGGCGTAAG	3530-564	34.0	0	11	11
OPE-03	CCAGATGCAC	3450-564	36.9	0	9	9
OPE-05	TCAGGGAGGT	947-200	31.5	0	3	3
OPE-06	AAGACCCCTC	2027-200	39.6	0	11	11
OPE-19	ACGGCGTATG	2027-831	39.6	0	9	9
OPE-20	AACGGTGACC	1400-564	32.7	0	5	5
OPM-16	GTAACCAGCC	2027-250	45.0	1	9	10
OPM-20	AGGTCTTGGG	1467-200	40.0	0	7	7
OPN-03	GGTACTCCCC	1375-200	40.0	0	7	7
OPN-04	GACCGACCCA	2027-564	40.0	0	9	9
OPN-06	GAGACGCACA	1904-200	33.0	0	7	7
OPN-09	TGCCGGCTTG	3650-245	45.0	0	12	12
OPN-10	ACAACTGGGG	1850-564	33.0	0	7	7
OPN-12	CACAGACACC	1845-270	45.0	1	8	9
Total 32				**18**	**227**	**245**

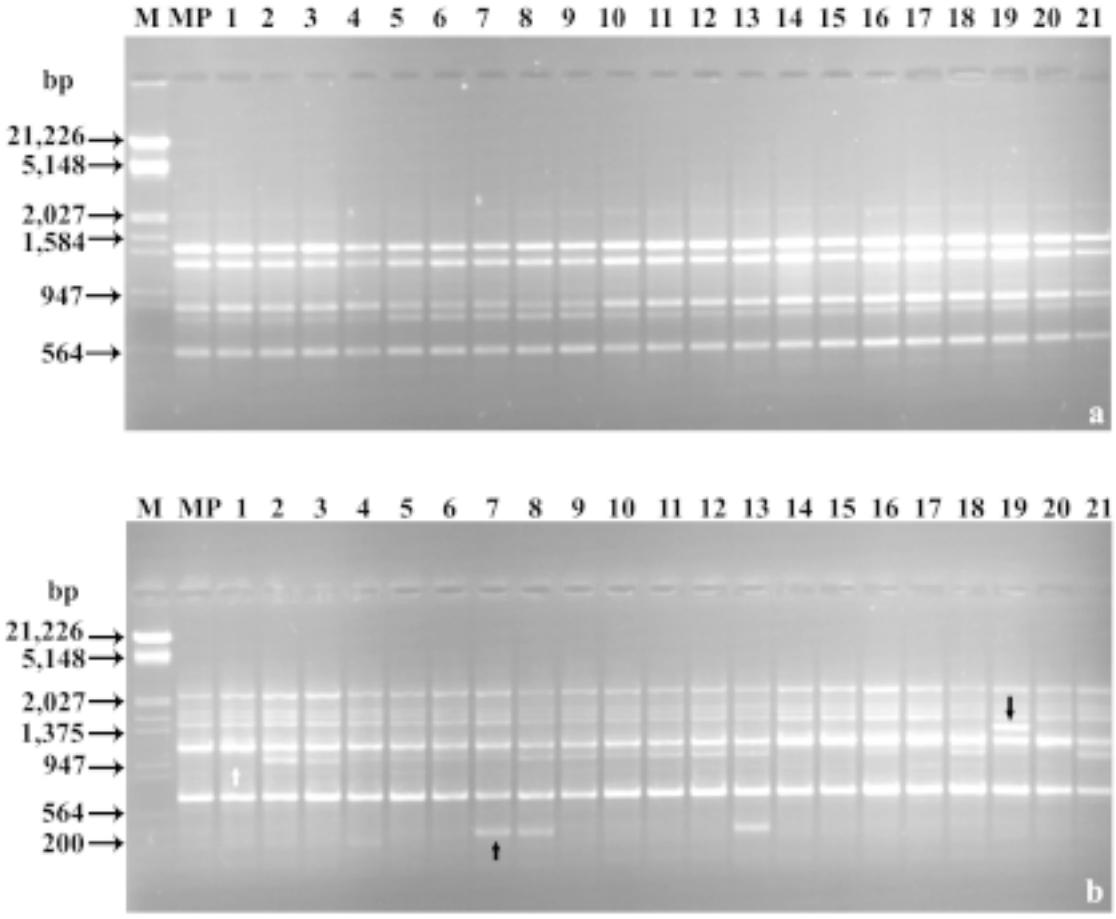

Figure 1 RAPD amplification in *O. missionis* plantlets with primers
a) OPE-20 Monomorphic gel
b) OPC-05 Polymorphic gel (two types of band variations i.e gain of bands at 1375 and 200 size bp, marked by black arrows and loss of band at 947 size bp, labeled by white arrow),
Lanes- M: Molecular marker λ DNA / *Eco*R I - *Hind* III Double digest,
MP: Mother plant, 1-21: Micropropagated plants.

from the same donor mother plant as in *Populus deltoides* (11) and *Piper longum* (25) using RAPD and in few other cases however provides evidences for the existence of variants. Thus, suggesting visual phenotypic evaluation may not be sufficient for characterization of *in vitro* plants. Screening for DNA variations among several millions of base-pair could be more problematic and exhaustive than scoring for a few morphological variations. The RAPD technique was found useful in examining genetic fidelity of tissue culture-clones. With the use of RAPD markers, clonal fidelity of tissue cultured plants has been determined in micropropagated species

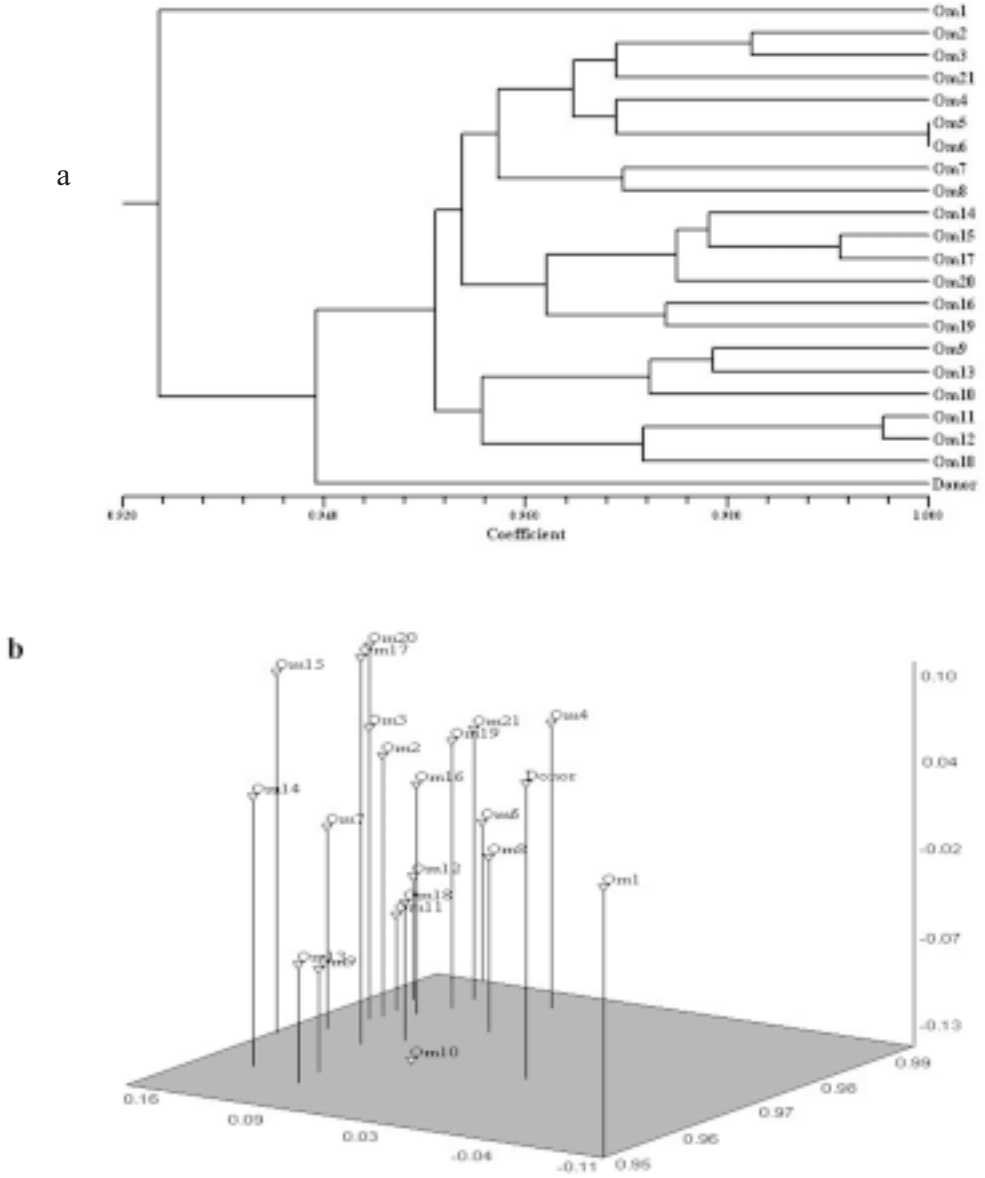

Figure 2 a) Dendrogram illustrating coefficient similarities among 21 regenerated plants (Om1 to Om21) and the single donor plant of *O. missionis* by UPGMA analysis (NTSys-pc) calculated from 245 bands generated with 32 selected RAPD primers. b) The three-dimensional plot of principle coordinate analysis (PCA) where *in vitro* plants Om2 to Om21 are all within donor plant showing genetic similarity and plantlet Om1 lies apart from all showing significant changes in both dendrogram and PCA.

of *Drosera anglica* and *D. binata* (26), *Cedrus* (27), *Swertia chirata,* (28), *Macadamia tetraphylla* (29), sugarcane (30) and *Mucuna pruiens* (31).

The 21 randomly tagged plants were found phenotypically normal and essentially identical with their mother plant at hardening stage which partly suggest the minimal or absence of somaclonal variations. A total of 32 primers resulted in 245 distinct and reproducible bands showing homogeneous RAPD patterns. Band intensity of each gel confirmed their monomorphic nature of 92.67% and with low level of genetic variation of 7.34% in RAPD primers among the plantlets analyzed. Similarly, an average of about 97% genetic fidelity was maintained among the 18 micropropagated populations raised from shoot tip explants of sugarcane (32), 97.4% homology and 7.6% polymorphism were observed in 15 micropropagated plants of *Syzygium travancoricum* (22) and 7.74% polymorphism and 92.25% genetic similarity were seen in *in vitro* plants of *Gypsophila paniculata* (33). Whereas, high level of polymorphic variations were observed in regenerants of *Codonopsis lanceolata*, a medicinal plant, with 24.9% of polymorphism, 23.2% polymorphism were seen in micropropagated apple root stock plants (12) and 32% in micropropagated plants of *Robinia pseudoacacia* (10). By using ISSR primers, low polymorphism of 3.92% among the twenty one *in vitro* grown *Dictyospermum ovalifolium* plantlets was reported (34).

In *O. missionis* plantlets, a total of 5390 bands were generated using 32 RAPD primers. Similarly, a total of 6520 reproducible bands were obtained in *Quercus serrata* plantlets (35), 1056 bands from micropropagated plants of *Syzygium travancoricum* (22), 1856 and 2320 scorable bands in *Drosera binata* and *Drosera anglica* respectively (26) and 925 bands in ten tissue culture clones of *Mucuna pruriens* (31). In *O. missionis*, the number of bands of each primer varied from 2 to 12, with an average of 7.65 bands per primer. On similar findings, bands varied from 1 to 7, with an average of 3.02 bands per primer in *Quercus serrata* (35), 2 to 8 bands, with an average of 4.6 per primer in micropropagated plants of *Syzygium travancoricum* (22) and in *Hagenia abyssinica* regenerated plantlets, number of bands per primer ranged from 4 to 11 with an average of 7.2 bands per primer (5). In conclusion, a simple, efficient and high fidelity protocol for comparative assessment of genetic stability in micropropagated plantlets and mother plant of *O. missionis* has been established. The present study indicates that the molecular analysis of plants at the hardening stage provides an early evaluation of plants for clonal fidelity. The result presented demonstrate that the RAPD technique proved to be effective in generating reproducible results useful in assessment of genetic fidelity of micropropagated plants in *O. missionis*. Since *O. missionis* is threatened medicinal plant, production of genetically true to type plants is important for the germplasm conservation.

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Screening for pharmaceutically important exopolysaccharide producing *Streptococci* and partial optimization for EPS production

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Abstract

Capsular exopolysaccharide, hyaluronic acid produced by *Streptococcus equi* subsp. *zooepidemicus,* carries high importance in pharmaceutical as well as biomedical field. In this direction, isolation and screening for exopolysaccharide producing streptococcus from nasopharynx of horses from Maharashtra and its neighbouring place was carried out. Out of 70 samples, none was observed to be *Streptococcus equi* subsp. *zooepidemicus* while, exopolysaccharide producing BM2 was isolated which showed close association with *Streptococcus dysgalactiae* with potential to produce hyaluronic acid. BM2 was compared with *Streptococcus equi* subsp. *zooepidemicus* (ATCC 43079), *Streptococcus equi* subsp. *zooepidemicus* (MTCC 3523) which produced exopolysaccharide 0.052, 0.050 and 0.097 g/l respectively. *Streptococcus equi* subsp. *zooepidemicus* (MTCC 3523) gave higher productivity; hence primary optimization studies were carried out to achieve maximum its growth and exopolysaccharide production.

Keywords : Exopolysaccharide, Horses, Mastitis, Streptococci, *Streptococcus dysgalactiae*

Introduction

Polymeric materials are gaining importance due to their extensive applications in pharmaceutical and medical sectors especially in diverse bio-medical fields like tissue engineering, implantation of medical devices and artificial organs, prostheses, ophthalmology, dentistry, bone repair *etc* (10). Among the polymers, Hyaluronic acid (HA) is a high-value biopolymer with a wide variety of medical, cosmetic and pharmaceutical applications (14). Commercially, HA is produced by the fermentation process using microbes like *S. equi* subsp. *zooepidemicus.* Being chemically identical to mammalian HA, streptococcal HA is non-immunogenic and hence regarded as a viable substitute for that from other sources. *S. equi* subsp. *zooepidemicus* is pathogenic Gram-positive bacteria which produce HA to protect against phagocytosis during infection. It is a catalase-negative, facultative anaerobe but is also aerotolerant microorganism (6). *S. equi* subsp. *zooepidemicus* belongs to the β-hemolytic Group C *streptococci*, which is mostly an opportunistic pathogen of non-human animal species, including important domestic animals like horses, cows, pigs, sheep, and dogs. Thus it is a pathogen of veterinary concern. It may be found in the nasopharynx, on the tonsils, in the respiratory tract, and on the genital mucous membranes of healthy horses and cattle. *S. equi* subsp. *zooepidemicus* is an important cause of respiratory tract infections in foals, young horses and is associated in uterine infections in mares. It is also a well-known cause

of mastitis in cows, mares and is the most frequently isolated opportunistic pathogen of horses (16). β-haemolytic and Lancefield's group C streptococci can be differentiated on the basis of fermentation of sugars like sorbitol and trehalose. *S. equi* subsp. *zooepidemicus* generally ferments sorbitol, but not trehalose (9, 15). The other streptococci like *S. pyogenes, S. faecalis, S. dysgalactiae, S. equi* and *S. equisimilis* belonging to Lancefield group A and C can also be used to produce an exopolysaccharide (EPS); especially hyaluronic acid (11). In this study, search for *S. equi* subsp. *zooepidemicus* was carried out in some part of Indian region and the EPS production by strain *S. equi* subsp. *zooepidemicus* (MTCC 3523) was analysed.

Materials and Methods

Sampling: Sampling was done from Raver a rural place in northern side of Maharashtra state, eco-sensitive hilly area of Matheran in Maharashtra state and Ratlam in Madhya Pradesh (India) using sterile transportation swabs with Amies charcoal media (HiMedia, India). Sampling was carried out by nasopharyngeal swabs from 18 horses at Raver, 8 horses and 25 different ponies at Matheran and 21 horses from Ratlam. At Raver, sampling was done twice, in April and November which was summer (~ 40°C) and winter (~ 28°C). While sampling at Matheran was done in late winter in February (~ 18°C) while sampling in Ratlam was done in summer *i.e.*, in April (~45°C). Milk samples of 1 cow and 3 different buffaloes suffering from bovine mastitis were also obtained from Yawal a rural place close to Raver. Samples were transported from place of collection to laboratory by keeping it in cool boxes.

Isolation and screening of bacterial strains

Isolation was carried out by streak plate method using β-Streptococcus selective agar medium (HiMedia, India) containing (g/l): Peptone 1.0, Meat extract 0.6, Yeast extract 0.5, L-lysine 0.02, Sodium chloride 6.0, Disodium hydrogen phosphate 2 and agar 15 with final pH 7.3. After autoclaving, medium was cooled at 45-50°C and sterile defibrinated sheep blood was added at the concentration 7-10%. Isolates were cultivated on slants of streptococcus agar containing (g/l): Glucose 20, Pancreatic digest of casein 20, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.1, agar 15 and final pH was adjusted at 6.8 at 25°C. Cultures which showed β-haemolysis were isolated and further characterised by Gram staining and biochemical characters like catalase test, sorbitol and trehalose utilization were sequentially carried out only when preceding test was found affirmative. Sugar fermentation test was carried out using phenol red broth containing sorbitol or trehalose (10 g/l). Simultaneously standard strains *S. equi* subsp. *zooepidemicus* (ATCC 43079) and *S. equi* subsp. *zooepidemicus* (MTCC 3523) were obtained from Microbiologics, USA and Microbial Type Culture Collection & Gene Bank (MTCC), India, respectively.

Biochemical characterization and genomic analysis of isolate

Identification of EPS producer isolate BM2 was carried out by biochemical characterization using HiStrep biochemical test kit (HiMedia, India) which subsequently was confirmed by 16S rRNA gene sequence analysis and phylogenetic studies. Universal primers 16F27N (5'-CCAGAGTTTGATCMTGGC TCAG-3') and 16R1525XP (5'-TTCTGCAGT CTAGAAGGAGGTGTWTCCAGCC-3') (5) were used for the amplification of 16S rRNA gene of the BM2.

The sequence of BM2 has been deposited at National Centre for Biotechnology Information (GenBank), http://www.ncbi.nih.gov under the accession number FJ238093.

Production and estimation of EPS

Inoculum: The inoculum was prepared in a 250-ml shake flask with 50 ml of Todd Hewitt Broth comprising (g/l): Brain heart infusion 500, Peptic digest of animal tissue 20, Dextrose 2, Sodium chloride 2, Disodium phosphate 0.40, Sodium carbonate 2.50, pH 7.8 and incubated at 37°C for 12 h.

Cultivation conditions: The shake flask experiments were performed in 500 ml Erlenmeyer flask with a working volume of 100 ml, and the agitation was maintained at 120 rpm. The fermentation medium contained (g/l): Casein enzyme hydrolysate 20, NaCl 2, $MgSO_4 \cdot 7H_2O$ 1.5, K_2HPO_4 2.5 and pH adjusted to 7.0. Glucose solution 5 g/l was autoclaved separately and added to it after cooling. This medium was inoculated with 1% (v/v) inoculum. Culture flasks were incubated at 37°C for 24 h.

Cell biomass: Samples were centrifuged at 8000xg for 5min. The weight of cell biomass was measured after repeated washing of the cell pellets with distilled water and drying at 60 °C for 24h.

Analysis of EPS: Fermented broth was diluted with an equal volume of 0.1% w/v SDS and incubated at room temperature for 10min to free capsular EPS which was filtered through 0.45µm membrane as per method of Blank *et. al* (4). Filtrate was subjected to determine mucopolysaccharides by a turbidimetric assay (8). Briefly, 200 µl of the filtrate was mixed with 200 µl of 0.1M acetic acid (pH 6) and 400µl of 2.5% w/v cetyltrimethyl-ammoniun bromide (CTAB) in 0.5M NaOH. The mixture was incubated for 20min at room temperature and A_{595} was recorded. A standard curve was established using a 0.18 g/l EPS stock solution prepared using standard HA of microbial origin (Focuschem, China).

Growth curve: Growth pattern of selected streptococcal strain was determined by incubating at 37°C, 120rpm for 30h in above mentioned fermentation medium. Samples were drawn at the interval of 2 h to determine cell growth and EPS produced. Cell growth was monitored turbidimetrically by spectrophotometer at 595nm. Culture samples were diluted in water to an absorbance of less than 1 before measurement which was later multiplied by the dilution factor.

Partial optimization of medium ingredients for EPS production

Carbon source: Six carbohydrates *viz.* glucose, sucrose, starch, galactose, fructose and lactose were evaluated. Fermentation medium contained (g/l); Carbon source 5, casein enzyme hydrolysate 20, NaCl 2, $MgSO_4.7H_2O$ 1.5 and K_2HPO_4 2.5. Selected carbon source taken was starting from 0.5% to 2.5% with addition up of 0.5% each time to find out optimum concentration.

Nitrogen source: Five different organic nitrogen sources *viz.* tryptone, peptone, soyapeptone, yeast extract and meat extract were evaluated. Fermentation medium was similar as above except the nitrogen source. Thus, the medium composition was (g/l); nitrogen source 20, NaCl 2, $MgSO_4.7H_2O$ 1.5 and K_2HPO_4 2.5. Glucose (1.5%) was used as a carbon source. Concentration of selected nitrogen source was optimized by assessing its concentrations starting from 1.5% to 5% with addition of 0.5% each time to find out optimum concentration.

Metal: Five different metal salts were checked, *viz.* $MgSO_4.7H_2O$ (Mg), $MnSO_4.H_2O$ (Mn), $CuSO_4.5H_2O$ (Cu), $ZnSO_4.7H_2O$ (Zn) and $FeSO_4.7H_2O$ (Fe). Fermentation medium was similar as above and each metal salt was added in separate flasks at an equimolar concentration.

Thus, the concentrations were (g/l) Soyapeptone 45, NaCl 2, metal salt 1mM and K_2HPO_4 2.5. Glucose (1.5%) as carbon source and soyapeptone (4.5%) as nitrogen source were used. Concentration of selected metal salt was optimized by assessing its different concentrations (1 – 9 mM).

Phosphate source: Four different inorganic phosphate salts were assessed *viz.* K_2HPO_4 (Dipotssium), KH_2PO_4 (Potassium), $Na_2HPO_4.2H_2O$ (Disodium) and $NaH_2PO_4.2H_2O$ (Sodium). Each phosphate source was added in medium separately in flasks at an equimolar concentration. The medium ingredient concentrations were Soyapeptone 4.5%, NaCl 0.2%, and $MgSO_4.7H_2O$ 1mM. Glucose (1.5%) was used as a carbon source. Concentration of selected inorganic phosphate salt used was 10 - 110mM for optimization.

pH: To determine the best pH for EPS production, culture was grown in the fermentation medium having six different pH values as 6, 6.5, 7, 7.5, 8, and 8.5 containing glucose 1.5%, soyapeptone 4.5%, NaCl 0.2%, $MgSO_4.7H_2O$ 1mM and K_2HPO_4 30 mM.

Results

α-haemolytic cocci were characterized by using Gram's nature, catalase test, ability to utilize sorbitol and trehalose for differentiating *S. equi* subsp. *zooepidemicus* from other strains where failure of any test lead to non performance of subsequent further tests. Out of 20 isolates from Raver, 7 were β-haemolytic and 1 was β haemolytic. But these 7 β-haemolytic isolates did not exhibit further characters of *S. equi* subsp. *zooepidemicus i.e.* cocci in shape, Gram positive, catalase negative, sorbitol fermentation reaction positive and trehalose fermentation reaction negative (data not shown). Similarly, out of 33 isolates from horses and ponies at Matheran, 23 were β-haemolytic which did not exhibit further desired characters (data not shown). Some horses at Ratlam used for local transport were also found to be free from the infection of *S. equi* subsp. *zooepidemicus* as none of the isolates out of 21 matched it (data not shown). Apart from horses, milk samples from 3 different domestic milking buffaloes and 1 cow showing mastitis symptoms were collected and further screened. Though isolates BM1, BM2, BM3 from buffaloes milk and CM1 from cow milk did not match biochemical characters of *S. equi* subsp. *zooepidemicus* (data not shown), BM2 was further studied because of its glossy and mucoid appearance on agar plates.

Characterization of isolate BM2

BM2 culture was grown on streptococcus maintenance agar medium that showed off white round shaped small glossy colony with mucoid consistency. Cells were cocci and Gram positive.

Biochemical analysis

The isolate BM2 showed Voges Proskauer's test, esculin test, L-pyrrolidonyl ß-naphthaylamine test, and o-nitrophenyl- ß-D-galactopyranoside test negative while arginine utilization was positive. Among sugars, it could utilize glucose, ribose, arabinose, sucrose and sorbitol; but not mannitol and raffinose. These results of biochemical tests of BM2 matched with *S. dysgalactiae* as per HiStrep biochemical test kit (HiMedia, India).

16S rRNA sequence of BM2

T G T A C G C T T T G G A A C T G
G A G A A C T T G A G T G C A G A A G
G G G A G A G T G G A A T T C C A
T G T G T A G C G G T G A A A T
G C G T A G A T A T A T G G A G G
AACACCGGTGGCGAAAG CGGCTCTCT
G G T C T G T A A C T G A C G C T G
A G G C T C G A A A G C G T G G G
G A G C A A A C A G G A T T A
G A T A C C C T G G T A G T C C
ACGCCGTAAACGATGAGT GCTAGGTGT
T A G G C C C T T T C C G G
GGCTTAGTGCCGGAGCTAACGC
ATTAAGCACTCCGCCTGGGGAGTACGACCGCA
AGGTTGAAACTCAAAGGAATTGACG
GGGGCCCGCACA AGCGG TGGAGC
ATGTGGT TTAATT CGAAGCAA CGCG
AAGA ACCTTAC CAGGTCTTGACATCCT
CCTGACCGGTCTAGAGATAGACTTT
C C C T T C G G G G C A G G A G T G
A C A G G T G G T G C A T G G T T G T C
G T C A G C T C G T G T C G T G A
GATGTTGGGTTAAGTCCCGCAACG
A G C G C A A C C C C T A T T G T T A
GTTGCCATCATTAA GTTGGGCACTCTA
GCGAGACTGC CGGTAATAAACCGGAGGA
AGGT GGGGATGACG TCAAAT CATC
ATGCCCCTTATGACCTG GGCTAC ACAC
GTG CTACAA TGGTTG GTACAA CGAG
TCGC AAGCCGGTGACG GCA AGCT
AATCTCTTAAAG CCAAT CTCAGT TCGGA
TTG TAGG CTGCAACT CGCCTA CATGAA
GTCGGAAT CGCTAGTA ATC GCGGA
TCAGCA CGCCG CGGTG AATACG
TTCCCGGGCCTTGTACACACCGCC
C G T C A C A C C A C G A G A G T T
TGTAACACCCGAAGTCGGTGAGG

Biochemical characterisation, 16S rRNA gene sequence analysis and phylogenetic position (Fig. 1) showed close association of BM2 (about 99% homology) with *S. dysgalactiae.*

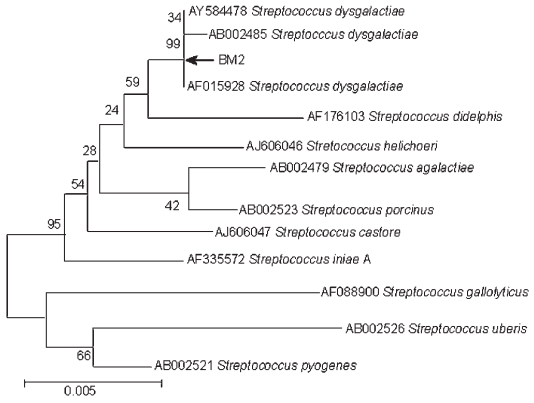
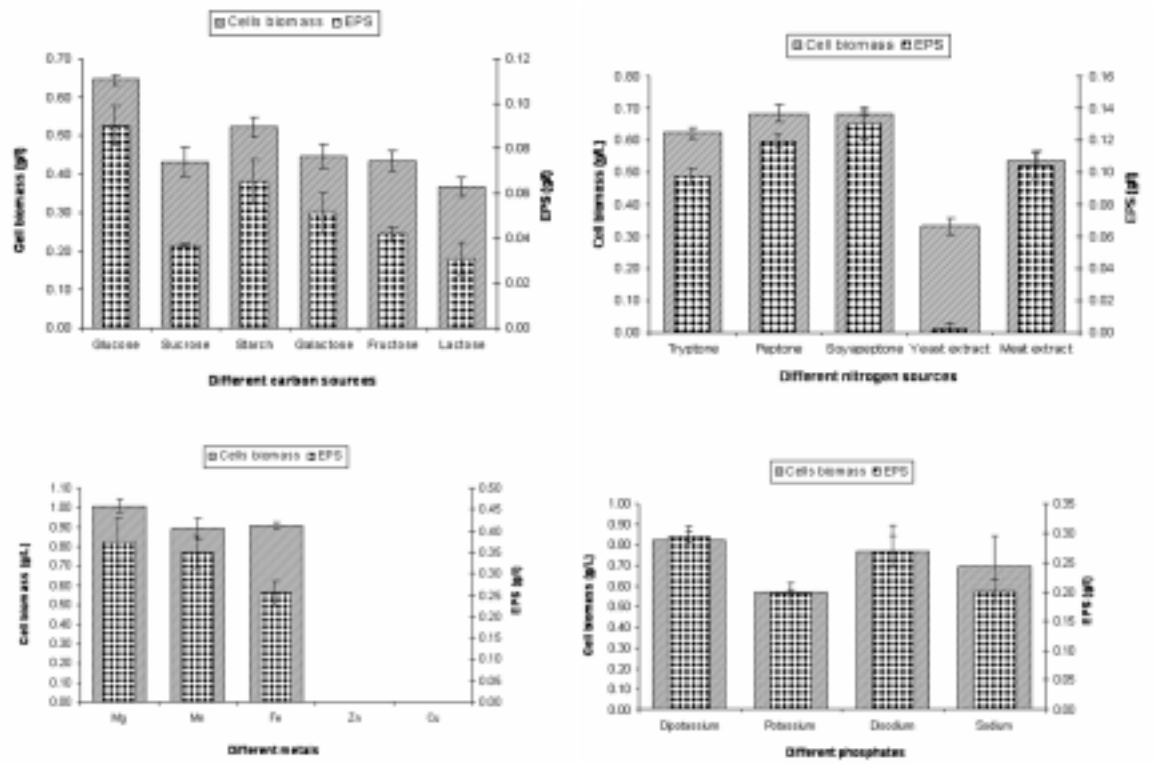


Figure 1. Phylogenetic position of BM2

The strain *S. equi* subsp. *zooepidemicus* (MTCC 3523) produced higher amount of EPS *i.e.* 0.097 ± 0.00g/l than other two strains *S. equi* subsp. *zooepidemicus* (ATCC 43079) 0.050 ± 0.01g/l and Streptococcus (BM2) 0.052 ± 0.01g/l. Hence EPS production from strain MTCC 3523 was further studied. Its growth curve revealed that logarithmic phase started from 4h and after 8h entered in to stationary phase which was observed till 30h. Similarly, capsular EPS production was observed to increase radically at 8h and stabilized with slight increase up to 30h (data not shown). The effect of the carbon sources on EPS production was studied (Fig 2a) where glucose was found to be the best carbon source with a production of 0.090g/l EPS subsequently 1.5% was the optimum glucose concentration in the fermentation medium (Table 1a). While, the best nitrogen source for EPS production was found to be soyapeptone which resulted in maximum production (0.131g/l) (Fig. 2b). Higher EPS production was obtained at the 4.5% of soyapeptone (Table 1b). Whereas, fig 2c and table 1c shows that the metal salt $MgSO_4.7H_2O$ when used at concentration 3mM enhanced the production of EPS to 0.376 g/l & 0.383 g/l respectively. While $ZnSO_4.7H_2O$ and $CuSO_4.5H_2O$ completely inhibited the growth of



(C) selection of metal for higher yield of EPS

(d) Selection of inorganic phosphate source for higher yield of EPS

Fig. 2 :

microorganism. Fig 2d and table 1d shows that use of an inorganic phosphate source K_2HPO_4 at the concentration 30mM gave higher production of EPS. Fig 3 shows that pH 7.5 is the optimum pH for higher production of EPS that yielded 0.443 g/l.

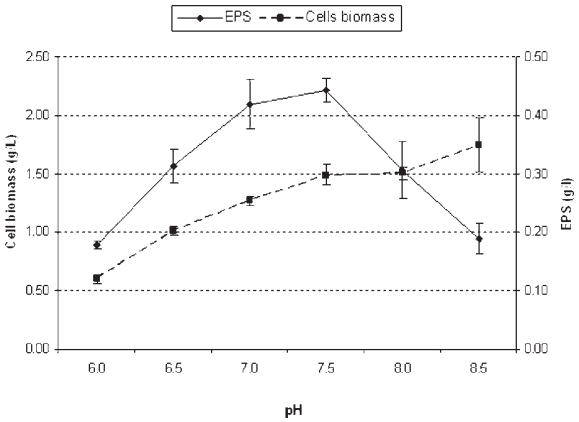


Fig.3. : Optimization of pH for higher yield of EPS

Discussion

A total of 74 samples from nasopharyngeal mucosa of horses and ponies were investigated with objective to isolate EPS producing bacteria especially *S. equi* subsp. *zooepidemicus*. Isolated cultures were 20, 33 and 21 from Raver, Matheran and Ratlam respectively where horse population was high. Though, *S. equi* subsp. *zooepidemicus* is an important pathogen of horse being associated with respiratory tract infections of foals and with uterine infections in

Table 1a. Optimization of glucose concentration for higher yield of EPS

Glucose (%)	Cells biomass (g/l)	EPS (g/l)
0.5	0.67 ±0.05	0.044 ±0.01
1.0	0.69 ±0.02	0.079 ±0.01
1.5	0.61 ±0.06	0.093 ±0.01
2.0	0.63 ±0.05	0.091 ±0.01
2.5	0.63 ±0.05	0.091 ±0.01

Table 1b. Optimization of soyapeptone concentration for higher yield of EPS

Soyapeptone (%)	Cells biomass (g/l)	EPS (g/l)
1.5	0.65 ±0.08	0.108 ±0.04
2.0	0.68 ±0.04	0.149 ±0.03
2.5	0.68 ±0.02	0.178 ±0.01
3.0	0.76 ±0.04	0.203 ±0.01
3.5	0.81 ±0.01	0.186 ±0.04
4.0	0.88 ±0.04	0.201 ±0.04
4.5	0.94 ±0.04	0.245 ±0.01
5.0	1.01 ±0.06	0.170 ±0.01

Table 1c. Optimization of $MgSO_4.7H_2O$ concentration for higher yield of EPS

$MgSO_4.7H_2O$ (mM)	Cells biomass (g/l)	EPS (g/l)
0	0.83 ±0.03	0.205 ±0.02
1	1.08 ±0.08	0.313 ±0.03
3	0.99 ±0.04	0.383 ±0.01
5	0.98 ±0.10	0.297 ±0.02
7	1.05 ±0.08	0.293 ±0.02
9	1.06 ±0.03	0.256 ±0.02

Table 1d. Optimization of K_2HPO_4 concentration for higher yield of EPS

K_2HPO_4 (mM)	Cells biomass (g/l)	EPS (g/l)
10	1.22 ±0.01	0.442 ±0.04
30	1.74 ±0.13	0.485 ±0.08
50	1.73 ±0.15	0.241 ±0.08
70	0	0
90	0	0
110	0	0

mares (1). Unexpectedly the nasopharyngeal flora of horses in these regions was found to be free from infection by this pathogen although particular animals were mucus excreting. Regions selected for isolation were having different climatic and geographical conditions, and the animals selected were also from diverse hygienic conditions. But α-haemolytic, *S. equi* subsp. *zooepidemicus* of group C causing strangles in horses was not observed which could not be answered and needs to be explored further. Perhaps mucus might be due to other microbial infection. Association of *S. equi* subsp. *zooepidemicus* with mastitis in dairy cattle like cows, goat, sheep and buffaloes is also reported (12), but instead of this organism, *S. dysgalactiae* was found in one of the milk samples of dairy animals showing symptoms of mastitis. This isolate *S. dysgalactiae* was compared with *S. equi* subsp. *zooepidemicus* ATCC 43079 and *S. equi* subsp. *zooepidemicus* MTCC 3523 strain for its EPS productivity, where MTCC 3523 proved better than others.

The specific growth rate and volumetric EPS production rate by *S. equi* subsp. *zooepidemicus* were found to be less favourable in the chemically defined media (2) and Group A as well as Group C streptococci possess fastidious nutrient requirements with respect to organic nitrogen (3, 7). Hence organic sources for carbon and nitrogen were selected to optimize the growth of selected strain *i.e. S. equi* subsp. *zooepidemicus* (MTCC 3523) in which glucose as carbon source and soyapeptone as nitrogen source showed better increase in growth of organism and production of EPS. The enzyme hyaluronic acid synthase (HAS) involved in HA synthesis in streptococcus and mammals prefer magnesium ions while *Chlorella virus* HASs prefer manganese ions to stimulate HA synthesis (17). Similarly use of magnesium sulphate showed better results than the other metal salts in EPS production by MTCC 3523 strain. Large number of ATP molecules are consumed in various enzymatic reactions involved in HA biosynthesis pathway in streptococcus, hence source of phosphate is essential to synthesize ATPs. In this study, salt dipotassium hydrogen phosphate as a source of inorganic phosphate showed better results. Earlier Johns *et. al* (13) have reported 6.7 as optimum pH for both the rate of production and yield of hyaluronic acid (HA) by *S. equi* subsp. *zooepidemicus* from glucose medium while, our results showed optimum pH for better production of EPS was 7.5.

Conclusion

The results obtained suggested that the occurrence of equine pathogen *S. equi* subsp. *zooepidemicus* could be rare in Maharashtra and its neighbouring region. These results can provide a base for further study on animal pathogens in the above said area. Secondly, the strain *S. equi* subsp. *zooepidemicus* (MTCC 3523) could be effectively used for fermentative production of HA that could be further exploited.

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