Digital Image Analysis of Actinomycetes Colonies as a Potential Aid for Rapid Taxonomic Identification

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High frequency isolation of actinomycetes poses a challenge for the taxonomists hence simple and rapid identification methods are required. Our work to catalogue biodiversity of actinomycetes of Goa yielded several distinct morphotypes. After their tentative identification, the feasibility to distinguish these using digital image analyses (DIA) was explored. Digital images of wild colony morphotypes were processed using public domain SCION image analysis software. DIA revealed some intricate digital characters. A combination of these with standard morphological and microscopic characters could be potentially useful for preparing a digital identification key of the actinomycetes strains with potential application in rapid taxonomic identification.

INTRODUCTION

Actinomycetes are prokaryotes having high GC content in their DNA, with various metabolic possibilities (Goshi *et al*.,2002) and are responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents and enzymes which are lead molecules of pharmaceutical interest (Kin, 2006., Shantikumar *et al*., 2006).

Identification and classification are difficult aspects of actinomycetes research in traditional systems. Although identification is possible with the help of traditional morphological criteria and by performing several biochemical tests (Locci *et al*., 1989) colony isolation is often a frustrating task and the identification involves the time consuming examination of

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morphological characters. In view of these difficulties, advanced methods for identification of actinomycetes using computer software Actinobase, have been developed for genus-level identification based on archived image files linked to descriptions of International Streptomyces Project (ISP) and other sources (Surajit. *et al*.,2006) Besides this, 'The Atlas of Actinomycetes' (http://www.jmc.riken.go.jp/saj/DigitalAtlas) , which is the world's first comprehensive reference guide to genus level identification contains 440 photomicrographs of 190 strains corresponding to 55 genera which includes reviews of taxonomy and structural descriptions of compounds produced (Miyadoh & Hotta, 2002).

Affordable computers and electronic imaging devices have led to the widespread availability of low cost digital image processing systems for microscopy which allow rapid quantification of many parameters which could only be described qualitatively. Digital image processing, involves the formation and manipulation of images by computer, such as image processing, image analysis and computer graphics. Applications have been reported in the fields of medicine, food hygiene, environmental microbiology (in the soil and aquatic environments) and biotechnology (fermentation), using a great variety of optical systems, cameras and image processing hardware and software (Wilkinson & Schut, 1998).

Merger of the techniques of imaging and flow cytometry as a single instrumental application has been tested in the field of plankton ecology where digital images are extremely useful in identification and automated detection of algal species toxic to human beings (Micheal & Charles, 1998). Work has been done on automated morphometry of yeast cells which could improve understanding of cell physiology (Pons & Vivier, 1998). Digital micromorphometry could be used to examine excreta specimens from patients regularly and thereby monitoring their gut flora (Meijer & Wilkinson, 1998). Digital image processing has been used for analysis of basidiospore characters and aided definition of species and species groups. This

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method has been also used by Cox & Thomas (1992), Jones *et al.*, (1992), for classifying and measuring fungal pellets grown in liquid culture and for spore counts of white rot fungi respectively. Hilber & Schuepp (1992), used digital image analysis for measuring lengths of fungal germ tubes (Mitchell *et al*., 1997).

Morphological studies of filamentous fungi and actinomycetes by image analysis have been carried out (Spohr *et al*., 1998, Treskatio *et al*., 2000). Staining procedure with image analysis has been used to quantify the percentage viability of *Streptomyces clavuligerus* (Sebastine *et al*., 1999). Image analysis has also been used to identify recombinant/ nonrecombinant colonies of *E.coli* (Spohr *et al*., 1998).

In addition to microscopes, the agar plate is the most familiar icon of microbiology. Image analysis has been used to monitor the development of macrocolonies of *Bacillus cereus* and *Salmonella typhimurium.* More specialized systems already allow automatic selection and robotic picking of colonies based on differences in colony morphology. Using image analysis, enzymatic activity of bacteria and its antimicrobial activity could be determined (Peters *et al*., 1998).

Automated classification of organisms using digital images is another application (Micheal *et al.,* 1998). Ogawa *et al.,* 2005 showed the use of simple multicolour digital image analysis for identification of bacteria (example *E.coli* 0157:H7) and to assess their metabolic activity.

At present there is acute shortage of microbial taxonomists in India and very few people are exposed to industrial research and practicing molecular biology of actinomycetes (MTCC, 1998) owing to constraints experienced in isolation and identification. The present work is a part of an ongoing study to survey, explore, isolate, identify and catalogue actinomycetes diversity from different habitats in the state of Goa. It involved high frequency isolation of actinomycetes on different media. However, rapid and specific taxonomic diagnosis was not

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possible and therefore an attempt was made to see the feasibility of digital image analysis (DIA) of actinomycetes colonies.

MATERIALS AND METHODS

Bacterial strains and isolation

Five soil samples (20-30g each, fresh weight) were collected at a depth of 10-20 cms in clean polythene bags from 5 x 5 m quadrat below the canopy of a 400 years old sacred *Ficus benghalensis* L. tree from the lateritic plateau, Taleigao, Goa (lat. $15^0$27'-36·77'N and long. $73^0$49'-59·44'E). The samples were designated as FB1, FB2…FB5, respectively, brought to the laboratory, mixed homogeneously and sieved through 250 μm brass sieve. The sieved fraction was used for isolation purposes within 24 h of collection. A synthetic opaque medium, Arginine Vitamin Agar (AVA) composed of a) Basal media(g/l)**:** Glycerol 0.8 ml; Glucose, 1 g; K_2HPO_4, 0.3 g**;** L-arginine, 0.3 g; NaOH, 0.3 g; $MgSO_4.7H_2O$, 0.2 g; Agar, 18 g and b)Trace element solution (1ml added to the basal media) composed of $Fe_3SO_4.7H_2O$,50 mg**;** $CuSO_4.5H_2O$,, 50 mg**;** $MgSO_4.7H_2O$, 50 mg**;** $ZnSO_4.7H_2O$, 50 mg; Distilled water, 50 ml**;** pH 6.8 was used for isolation purpose (Nonomura & Ohara, 1969).

To eliminate unwanted microbial contaminants, the AVA medium was supplemented with nalidixic acid (0.0176 mg/ml), neomycin and polymyxin b sulphate (0.01 mg/ml) as antibacterials and nystatin (0.004 mg/ml), actidione (0.000192 mg/ml), terbinafine (0.00192 mg/ml) as antifungals.

One gram of the sieved soil sample was kept overnight for drying at 70^0C in a stockli dehydrator. For plating, one ml suspension each from 10^{-2} and 10^{-3} dilutions was evenly spread on the surface of AVA medium in triplicates which were incubated at 28^0C for 7-28 days and examined daily under Zeiss Stermi1000 stereomicroscope for detecting colonies

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with characteristic of actinomycetes morphology. Individual colonies were marked in random order on the reverse of the plates and designated as ACT-1, ACT-2….ACT-n.

Digital imaging actinomycetes colonies

Multiple images (700KB-1.1MB) of these 14 days old actinomycetes colonies were captured using Sony Cybershot digital camera 4.2 MP DSL-LI, mounted on a vibration free tripod. For this purpose the AVA plates with lids removed were kept at a distance of 15-20 cm from the camera lens and the colony surface was illuminated with incident polychromatic light from a 60 Watt tungsten filament lamp so as to obtain maximum contrast (Peters *et al*., 1998). The morphotype number corresponding to the digital image was recorded. From the AVA isolation plates distinct morphotypes were selected for transfer to the maintenance media. Oat Meal Agar Medium (HIMEDIA Laboratories Pvt. Ltd. Mumbai, India.) containing Oat Meal Agar (OMA), 72.5 g/l; pH 7.2 was used for maintaining the actinomycetes pure isolates on slants and thereafter in 10% (v/v) sterile glycerol at 16-20^{0}C to ensure long term viability of the strains.

RESULTS AND DISCUSSION

Altogether 200 actinomycetes isolates were obtained from soil sample and were thereafter identified morphologically to the generic level by comparing the morphology of spore bearing hyphae with entire spore chain as described in Bergey's manual (Locci, 1989) along with colony characteristics, morphology of substrate, aerial hyphae, morphology of spores and pigment produced; by using air dried smears stained in 1% w/v crystal violet under oil immersion lens of Olympus BX41 microscope. The substrate, aerial mycelium and pigment colour were visually recorded by using a standard colour chart. Out of these, five morphotypes belonging to five different genera, ACT-16 (*Actinopolyspora* sp.), ACT-33 (*Actinomadura* sp.), ACT-43 (*Nocardia* sp.), ACT-46 (*Micromonospora* sp.) and ACT-48

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(*Streptomyces* sp.) respectively were selected for digital image analysis. The colony morphology of these strains is given in Table1.

The captured images of these five morphotypes were imported and converted to 24 bitmapped images using SCION image processing software (USA) beta, freeware version 4.0.2 (an image processing and analysis program for the IBM PC) to get distinct image panels for each morphotype with respective DIA output-original image, find edge function (FEF), surface pixel plot density (SPPD), histogram profile (HP) and pixel profile plot (PPP). These panels are shown in Figure 1. The FEF output clearly delineates the colony margin and captures the location, size and shapes of exudate droplets and aids in pinpointing exudate variability whereas SPPD output gives characteristic cross sectional view of the smooth, rough or undulating colony topography which is likely to be strain specific. HP output displays different profiles indicating the distribution of gray values within the selection which appears to be strain specific. PPP output gives a 2D plot profile with fixed Y scale values and shows distinct cross sectional differences in colony landscape. SPPD, HP and PPP outputs considered together give distinct differences in colonies.

A tentative digital identification key of the actinomycetes which we consider to have potential application in rapid taxonomic identification was prepared as given below with the help of colony attributes obtained from useful DIA output (Table 2).

1. SPPD and PPP output shows undulating topography with a sharp relief …….. *Actinopolyspora* sp., (Fig1.1 b, d); *Actinomadura* sp., (Fig 1.2 b, d); *Nocardia* sp., (Fig 1.3 b, d); *Micromonospora* sp., (Fig 1.4 b, d); *Streptomyces* sp., (Fig 1.5 b, d).

2. HP gives symmetrical or asymmetrical profile:

a. symmetrical profile…… *Streptomyces* sp. (Fig 1.5c)

b. asymmetrical profile……..*Actinopolyspora* sp.(Fig1.1c); *Actinomadura*

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sp.(Fig 1.2c); *Nocardia* sp.(Fig 1.3c); *Micromonospora* sp. (Fig 1.4c).

3. FEF output distinctively shows Exudate Droplets (ED):

4. ED with very low, low or moderate density:

a. very small (0.1mm), spherical, which are confined to a colony sector and distributed more towards the edges without much clustering…….. *Nocardia* sp. (Fig 1.3a)

b. small (0.1-0.2mm), oval, distributed more towards the edges with moderately uniform clustering…….....*Actinopolyspora* sp. (Fig 1.1a)

c. small to large (0.1-0.4mm), spherical, distributed randomly towards the edges with dense clustering…….. *Actinomadura* sp. (Fig 1.2a)

5. ED very small with high density:

a. ellipsoidal, distributed centrally with dense clustering ……*Micromonospora* sp. (Fig 1.4a)

b. oval, distributed uniformly with very dense clustering towards the edges ……..*Streptomyces* sp. (Fig 1.5a)

Scion Image software has been previously used by (Miguélez *et al*.**,** 1999) to study the ultrastructural changes in hyphae of *Streptomyces antibioticus* which undergoes cellular degeneration. It has not escaped our notice that possible variables such as type of media, dilution factor, incubation temperature, growth conditions, incident light conditions, density of the colonies, may have to be considered to make this technique reproducible and reliable. The digital keys can be expanded by including more strains and digital image database of known strains of actinomycetes could be created and used as a reference for rapid

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matchmaking and taxonomic identification. Tropical habitats harbor rich diversity of actinomycetes, however their identification poses problems. Newer technologies like 16S rRNA studies which make possible the recognition of microbial strains up to genus level using sequence signatures are now available. However, these are expensive for large collections with little funding. Therefore, digital keys with image database of the actinomycetes would be useful under the demanding tropical conditions for making an educated guess about the identity of the large number of wild type Actinomycetes isolates. Further work is in progress to expand the digital key.

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Table1. Colony morphology of actinomycetes strains

Isolate no.	Colony type			Colony texture	Aerial mycelium growth			Aerial mycelium colour	Substrate mycelium colour	Diffusible pigment colour
	F	C	R	Hard	S	M	A			
ACT-16	+	-	-	Hard	-	-	+	Grey	Light orange	Pale orange
ACT-33	+	-	-	Hard	-	+	-	Dark green	Brownish green	Blackish brown
ACT-43	+	-	-	Hard	-	-	+	White	Dark brown	Dark brown
ACT-46	-	+	+	Hard	+	-	-	Grey	Grey	Grey
ACT-48	+	-	-	Hard	-	-	+	Grey	Brownish green	Brownish green

Abbreviations: F, flat; C, cerebroid; R, raised; S, sparse; M, moderate; A, abundant; +, present; -, absent.

Table 2. Colony attributes used in preparation of the digital key

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Colony attributes	FEF	SPPD	HP	PPP
Shape				
symmetrical	-	-	+	-
asymmetrical	-	+	+	-
Topography				
smooth	-	+	-	+
undulating	-	+	-	+
Relief				
Sharp	-	+	-	-
not sharp	-	+	-	-
Margin				
distinct	+	-	-	-
not distinct	+	-	-	-
Exudate				
distribution	+	-	-	-
density	+	-	-	-
shape	+	-	-	-
size	+	-	-	-
clustering	+	-	-	-

Abbreviations: FEF, find edge function; SPPD, surface pixel plot density; HP, histogram profile and PPP, pixel profile plot
+, indicates usefulness of the DIA output; -, indicates not much helpful.

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Original images

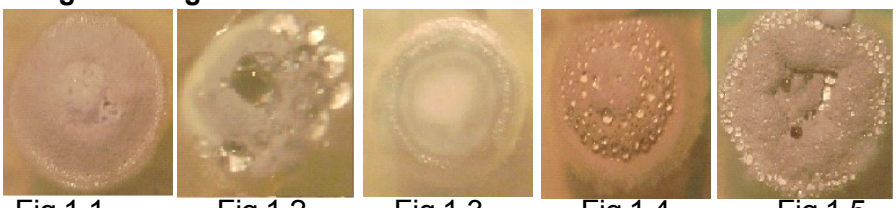

Fig 1.1 Fig 1.2 Fig 1.3 Fig 1.4 Fig 1.5

Find edge function output

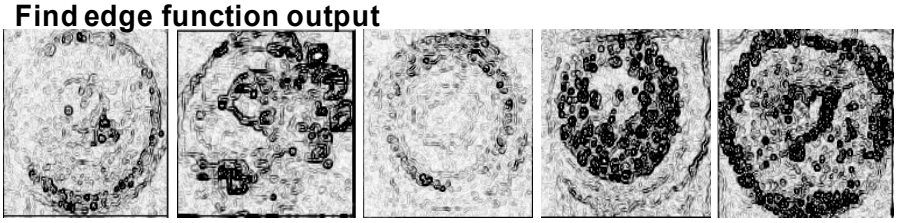

Fig 1.1a Fig 1.2a Fig 1.3a Fig 1.4a Fig 1.5a

Surface pixel plot density output

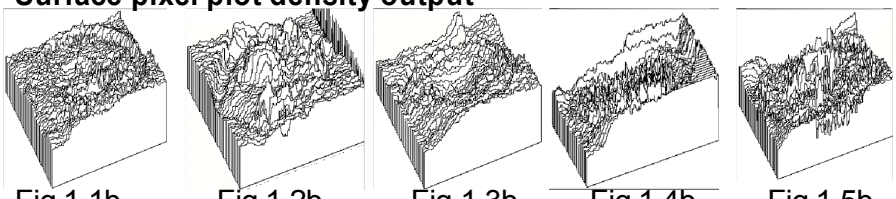

Fig 1.1b Fig 1.2b Fig 1.3b Fig 1.4b Fig 1.5b

Histogram profile output

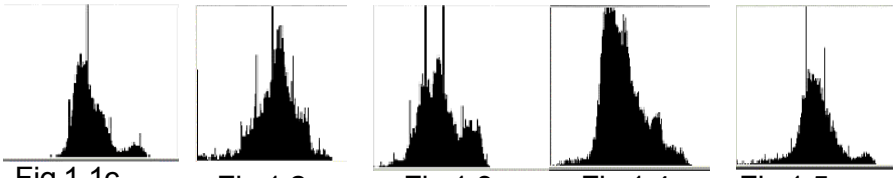

Fig 1.1c Fig 1.2c Fig 1.3c Fig 1.4c Fig 1.5c

Pixel profile plot output

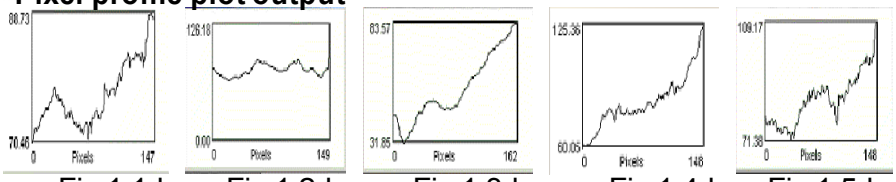

Fig 1.1d Fig 1.2d Fig 1.3d Fig 1.4d Fig 1.5d

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