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Biological Fractals and Morphological Change in Bacterial Colonies

Kranti Zakde[1]* Pradnya Ingle[2] and Surekha Munde[2]

[1] Department of Applied Science, Jawaharlal Nehru Engineering College, Aurangabad

[2] Department of Physics, Milind College of Science, Aurangabad

[2] Department of Physics, PES College of Engineering, Aurangabad

***Corresponding Author's E-mail:** kzakde@mgmu.ac.in, **Mob.:** +91-8983436885

A B S T R A C T

The collection of patterns and shapes in nature has long been a source of joy and wonder to laymen and scientists alike. Discovering how such patterns emerge spontaneously from an orderless and homogeneous environment has been a challenge to researchers in the natural sciences throughout the ages. Many phenomena display the emergence of patterns during diffusive growth, ranging from the growth of snowflakes to solidification of metals, from the formation of a coral reef to cell differentiation during embryonic development. Alan Turing understood that patterns would evolve in systems driven out of equilibrium, where competition and interplay between various tendencies exists. The observations shows that the diffusion field drives the system towards decorated (on many length scales) irregular fractal shapes. This paper describes cooperative patterning during growth of bacterial colonies a standard modeling approach was developed by combining a detailed study of the cellular behavior and dynamics during colonial development and invoking concepts derived from the study of pattern formation in nonliving systems.

Keywords : *fractal, fractal shapes, diffusion, bacterial colonies etc.*

1. Introduction

Pattern formation is thought to be more complex in biological systems than in physical and / or chemical systems. Biological phenomena usually occur through the underlying complexities Complex biological factors and environmental (physical-chemical) conditions.

However, aside from the morphogenesis of individual organisms, population formation of simple biological objects can sometimes be completely dominated by physical conditions. The formation of bacterial colonies which are one of the simplest biological objects are studied.

Common bacteria such as Esaheriahia (E.) aoli are unicellular organisms. A very small number of bacteria (parent cells). Once it is placed on the surface of a suitable medium such as solid agar containing sufficient nutrients and incubated for some time, repeat growth and cell division several times. Eventually the progeny bacteria become large in number and usually swarm on solid medium to form visible colonies. Colonies vary in size, shape and color depending on the bacterial species. Its appearance also changes with the variation of environmental conditions. Examination

of the growth of bacterial colonies is a treasure house for the study of pattern formation. For example, Matsuyama et al. recently showed that the bacterium Serratia marescens undergoes fractal morphogenesis in the process of spreading growth on agar surfaces.

2 Growing conditions: Considering hostile conditions of low level of nutrients, a hard surface, or both, under such conditions, not unlike certain ecosystems in natural environments, complex colonial patterns are observed. Drawing on the correspondence with diffusive patterning in nonliving systems [4, 5, & 6] the above observations can be understood as follows:

(1) The cellular reproduction rate, that determines the growth rate of the colony, is limited by the level of nutrients available for the cells.
(2) The latter reproduction is limited by the diffusion of nutrients towards the colony (for low nutrient substrate).

Hence, the colonial growth should be similar to diffusion limited growth in nonliving systems, such as solidification from a supersaturated solution, growth in a Hele-Shaw cell, electrochemical deposition, etc [7, 8]

Studies on pattern formation in abiotic systems demonstrated that different shapes are observed for the same system as the control parameters are varied (e.g. under cooling, supersaturation). Although a number of morphologies are possible under a particular set of conditions, only one is generally selected. The commonly accepted morphology selection principle state that the particular morphology selected is the fastest growing one [9, 10]. Hence the observed patterns can be organized in a morphology diagram similar to a phase diagram (of liquid, solid, gas).

There is a relatively sharp transition from one shape to the other, as the control parameters are varied and different morphologies are selected. It has been demonstrated that the concept of morphology diagram can also be applied to the growth of bacterial colonies [11, 12, 13], i.e. the patterns exhibited by a given strain can be organized as a variety of regimes (each for a characteristic pattern) on a graph of nutrients and hardness of agar [l4,l5]

3 Types of bacterial colonies observed:

With general modeling, we were able to conclude that three kinds of chemo tactic response were involved in the self-organization. The analysis of patterning behavior of the models suggests bacterial processes and structural development. Bacterial self-organization phenomena.

(i) Simple branching: As the food level is lowered, the colonial patterns shift from compact to tip splitting, reminiscent of the viscous fingering observed in the Hele-Shaw system, to ”bushy’ fractal patterns (figure 4.1). At even lower peptone levels, the colonies revert to more organized structures of fine radial branches. Branching patterns were observed in other strains of lubricating bacteria, like Serratia marcescens and Salmonella anatum (Matsuyama & Matsushita 1991; Matsuyama et al. 1992) and in Bacillus subtilis (Fujikawa & Matsushita 1991; Matsuyama ct al . 1993;Matsushita & Fujikawa 1990; Mendelson & Salni 1996)[16]

Fig. 1 Bacterial colony growth with simple branching.

Figure 1 refers to an additional concept introduced to describe the Patterning of bacterial colonies. This

is further analyzed for determination of fractal dimension.

(ii) Chiral branching: Bacteria display marrons chiral properties, at the branch-tips exhibit minute twists, all with the same handedness. Still, the observation of a pattern of strongly twisted, chiral branches, all of them with the same handedness, was unexpected (Fig.2). First, swimming bacteria generated it. In general, transitions will occur into the morphotype whose colonies can expand faster and organize in a more complex pattem at the given growth conditions. Optical microscope observations indicate that, in the case of chiral branching, the cells are much longer and the random walk tums into a quasi-one dimensional random walk.

(iii) Vortex branching : More than 50 years ago, observations of collective migration of B. circulans on hard surfaces showed turbulent-like bacterial flow with eddy (vortex) formation, merging and splitting of vortices, rotating 'bagels', and more (Smith & Clark 1938; Wolf 1968). All of these phenomena are also observed in the self-organization of new P. vortex strain when growing on a very hard surface [17]. The self-generation of vortices provides high bacterial density to facilitate movement on such surfaces,

(a) The fine radial branching growth results from domination of the chemorepellent response;
(b) The transition to the 'bushy' fractal branching growth corresponds to the turning on of the food chemotaxis above a threshold level;
(c) The internal observed patterns of the tip-splitting branches are a chemoattractive response.

The sharp transitions between the various regimes imply that at each regime, a characteristic biological feature dominates the growth. Bacterial cells are also contained in the trails left behind the leading vortices. Some are immobile, while others move, swirling with complex dynamics. The migrating groups of cells are reminiscent of the "worm" motion of slime mold or schools of multicellular organisms. The whole intricate dynamics is confined to the trail of the leading vortex, and neither a single cell nor a group of moving cells can pass out of the boundary of the trail. Only vortices formed in the trails oan break out of the trail and create a new branch.

4 Experimental Method:

The bacterial colonies were maintained on standard tryptose blood agar base (TBAB) plates made from difco medium. For growing colonies of the morphologies described above, a softer version was made, which contained only 0.6% agar, instead of 1.5%. The method for making this media [18] is to dissolve 10 grams (g) of tryptose, 3 g of beef extract, and 5 g of NaCl in 1.0 liters of distilled water. Six grams of agar was added. To sterilize, the mixture was autoclaved at 121° C for 20 minutes. The solution was cooled to 48° C and maintained at this temperature for one hour. Then, the plates were poured and the agar was allowed to solidify at 23° C in a 50% relative humidity chamber. For clearer resolution on a microscope, this media could be melted and dropped onto a cover slip. Then, to keep the media from drying out, the cover slips were placed on a specimen slide, and this was set on a bent glass rod, in a petri dish with a small amount of water. These plates were stored in an incubator at 24° C. For viewing, the cover slip would be taken off the slide and placed under the microscope. This ensures a minimum amount of glass and agar between the microscope and the bacteria. Cultures were inoculated using sterile toothpicks.

If you want to preserve your bacterial colony, you can stain it. Staining the colony kills the bacteria to prevent further growth, and makes the colony easier to look at both with the naked eye and under a microscope. The ingredients required for preparation of agar in the way it's needed for the favorable pattern to grow are as follows:

Stirring rod, beaker, graduated cylinder, 0.1% Coomassie Brilliant Blue R stain (available from Sigma), methano1, acetic acid (or vinegar), distilled water, Staining Solution, Rinsing Solution.

4.1 Preparation of agar:

An agar plate is a sterile Petri dish that contains agar plus nutrients (media), used to culture microorganisms. Selective growth compounds may also be added to the media, such as antibiotics. For growing bacteria under various nutritional conditions and for preparing agar plates to grow the bacteria let us consider the types of agar used for the same. Like other growth media, the formulations of agar used in plates may be classified as either defined or undefined; defined medium being synthesized from the individual chemicals as required by the organism so that the exact molecular composition is known, while undefined medium is made up of natural products such as yeast extract, where the precise composition is unknown[19]. Several types of agar are used with various nutrients but the main types are:

(a) Blood agar plate (BAP): Contains mammalian blood (usually sheep), typically at a concentration of Wl0%. BAP are an enriched, differential media used to isolate fastidious organisms

(b) Chocolate agar (CHOC): A type of blood agar plate in which the blood cells have been lysed by heating the cells to 56 °C; used for growing fastidious (fussy) respiratory bacteria. (No chocolate is actually contained in the plate; it is named for the coloration only)

(c) Mannitol Salt Agar (MSA): MSA is also a selective and differential media. Mannitol is the differential part, it indicates organisms that ferment mannitol.

(d) Nutrient agar: Safe to use in school and college science labomtories because it does not selectively grow pathogenic bacteria.

Method of preparation: To prepare 1 liter of staining solution (be sure to follow the exact order of these steps):

1. The first step is take a 1000 ml beaker and add 400 ml distilled water to it.
2. Then add 500 ml methanol to the distilled water taken in the beaker.
3. Add 100 ml acetic acid to it. If acetic acid is not available then vinegar can also be used instead of it as a substitute.
4. 1 gram of 0.1% Coomassie Brilliant R stain powder is then added to the third step solution. The entire solution of all contents is mixed thoroughly until the solution becomes a uniform blue.The ingredients can be scaled proportionally for smaller amounts of staining solution. Use the base proportions of (4:5: l) distilled water:methanol:acetic acid.

The rinse solution can be made by following Steps 1-3 for making the staining solution. Be sure not to add the stain powder to your rinse solution.

1. Pour staining solution onto plate. Solution should just cover the surface of the nutrient agar. Let the staining solution stand on the nutrient agar for approximately 45 seconds. This may vary depending on the size and color of the colony that you are staining.
2. Pour solution off the nutrient agar. Pour on rinse solution. Agitate rinse solution for approximately ten seconds. Let the rinse solution stand on the nutrient agar for approximately 50 more seconds.
3. Pour off the rinse solution. If any rinse solution remains, you can invert the plate onto a paper napkin until it is dry.

4.2 Analyzing bacterial colonies:

Various bacterial strains (e.g. strains belonging to the genera Bacillus, Paenibacillus, Serratia and Salmonella) exhibit colonial branching patterns during growth on poor semi-solid substrates. These patterns reflect the bacterial cooperative self-organization. Bacteria grown on agar surfaces can grow into a variety of colony shapes, ranging from a basic circle to an intricate fractal-like pattern. The diversity is caused by the motility of the bacteria, which is the result of the affects of agar concentration and nutrient concentration, as well as numerous other factors.

The obtained bacterial colonies are analyzed using a program called box counting for determination of fractal Dimensions. The selected pictures of bacterial colony growths were saved as black and white bitmap

files for implementing it to a Turbo basic programme that converts the picture file into a matrix of pixels that are occupied and unoccupied. The program then displays a log-log plot of the box size versus number of boxes. Fractal Dimension then is found from the slope of the best-fit straight line.

4.3 Fractal characterization of Bacterial colonies:

We conducted several experiments for the growth of bacterial colonies under different conditions of medium, nutrients etc. It is observed that when the conditions are favourable the growth is uniform and homogeneous with uniform growth of boundary. Such a growth of bacterial colony under conditions i.e. at lower peptone levels, the colonies revert to more organized structures of fine radial branches as shown in Fig. 2. The figure clearly shows that the growth is uniform in all directions (isotropic) and does not show selective growth in certain preferred direction or retarded growth in certain regions. This is in contrast to Fig. 3 that shows relatively irregular growth and the effect is more prominent in Fig. 4 where the pattern has altogether different

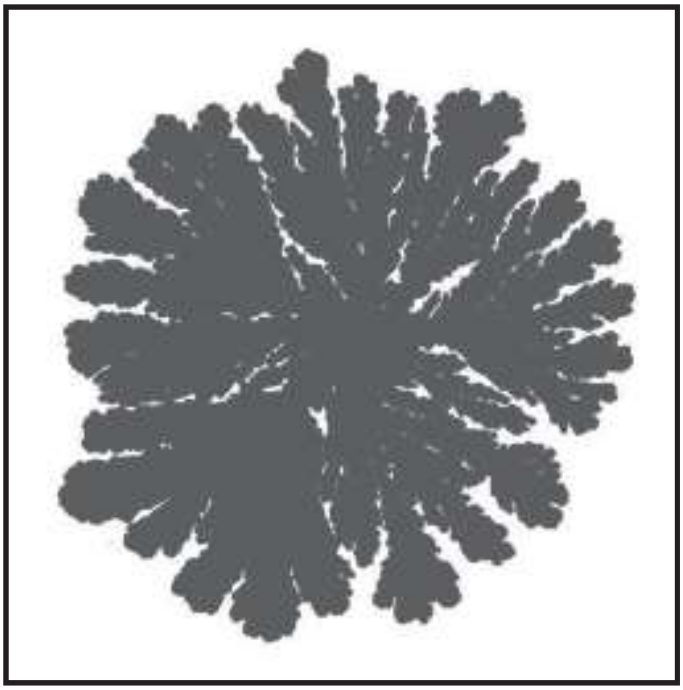

Fig. 2 BMP image of Ideal colony with dense branching.

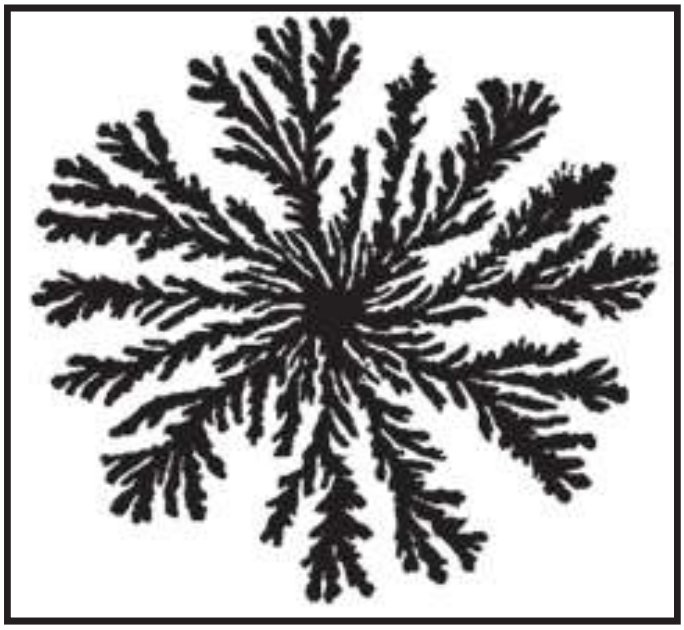

Fig. 3 BMP image of growth of Bacterial colony with ramified boundaries

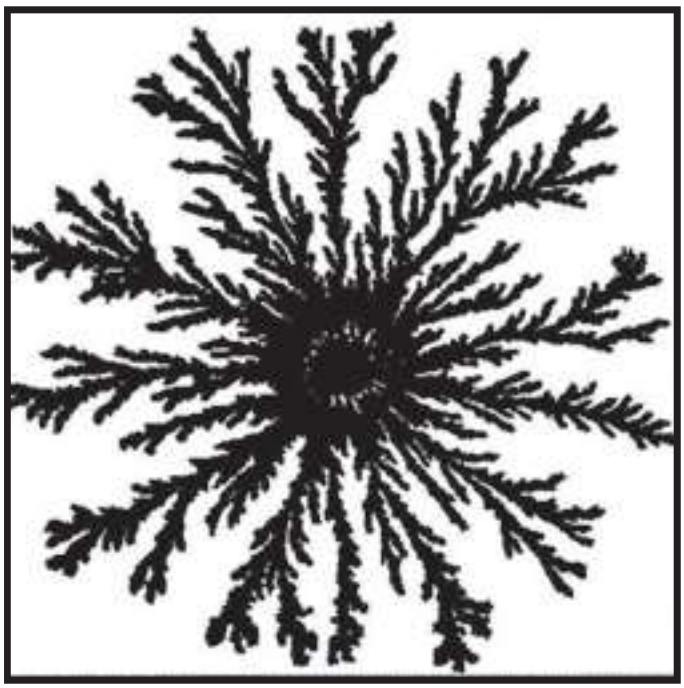

Fig. 4 BMP image of Bacterial colony resembling that of a DLA pattern

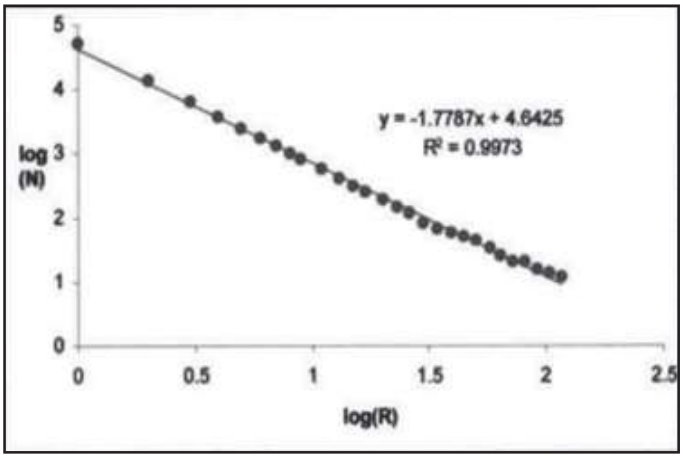

Fig. 5 Plot for box counting method for Fig. 2

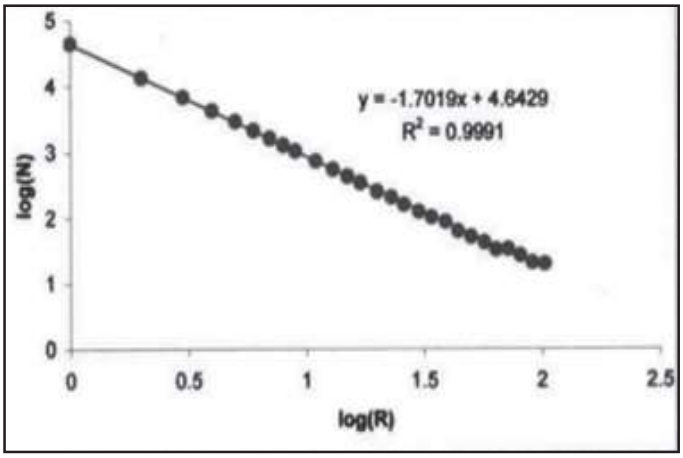

Fig. 6 Plot for box counting method for Fig. 3

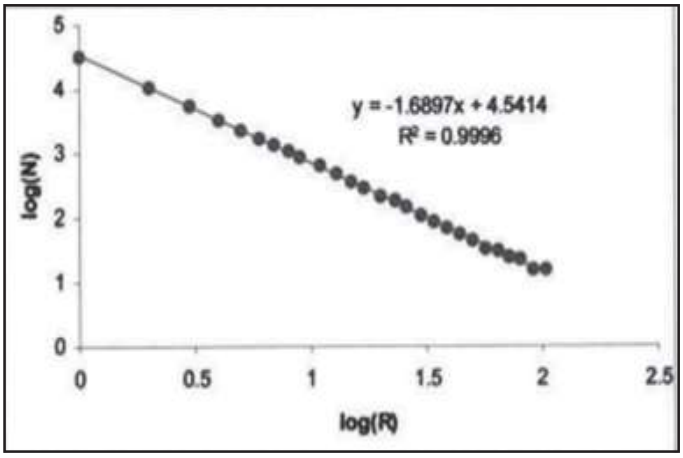

Fig. 7 Plot for box counting method for Fig. 4

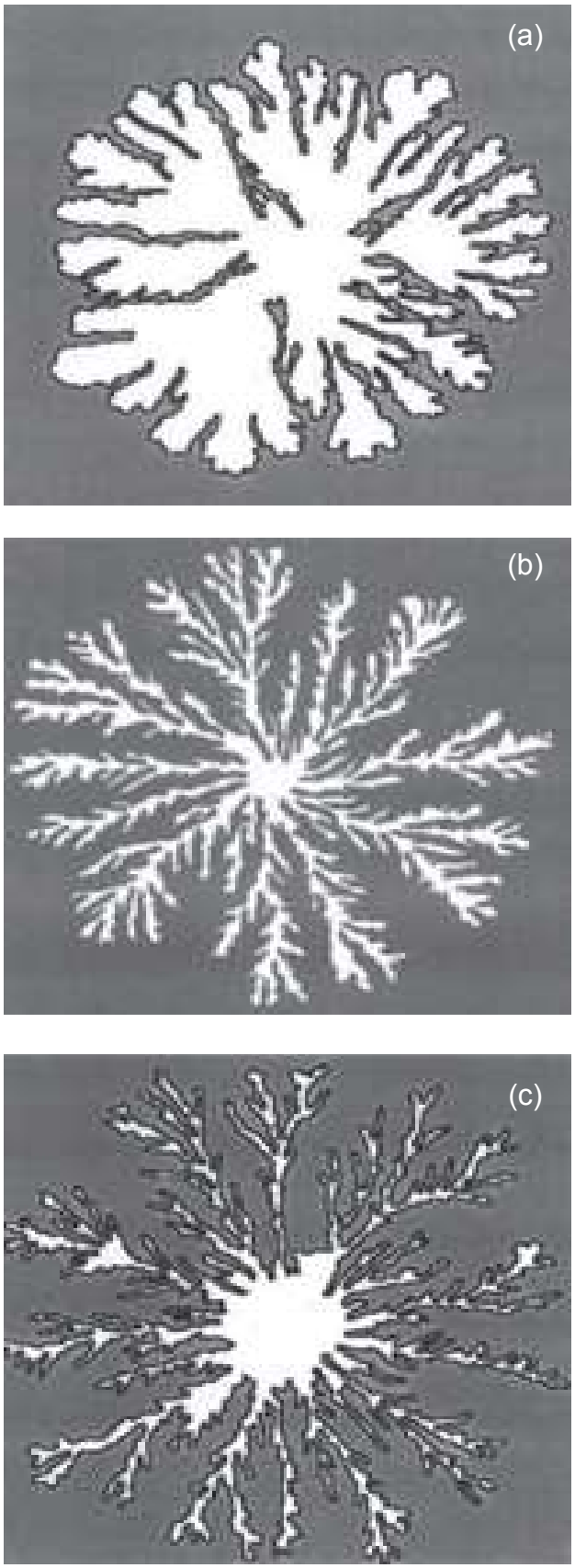

Fig. 8 (a,b,c) Figures converted for Richardson's plot method

morphology with ramified boundary as compared to Fig. 2. growths in Fig. 3 and 4 are examples of the extreme conditions where the growth appears to be dendritic with a morphology resembling that of a DLA.

One can analyze Fig. 2, 3 and 4, with a view to characterize the morphologies on the basis of fractal dimension using box-counting technique. The photographs of the three growth patterns were scanned, processed and converted to gray scale and then to two colour bitmaps using suitable threshold to obtain the image (Fig. 8). These images were then analyzed using computer programmes in turbobasic to implement the box counting. Fig. 5, 6 and 7 show the results of box counting for the three cases of Fig. 2, 3 and 4 respectively.

The number of boxes of side 'L' required to cover the pattern is recorded as 'N'. Log (N) is plotted against log (R) and the fractal dimension is obtained from the slope of the resulting straight line. It is clearly seen that for all the plots, all the points lie along a straight line. The points plotted are actual data points from box counting and the line joining the points is the least square fit applied to these points, the goodness of the fit is seen from R^2 values that are close to 1. This indicates that for all the three cases, scaling law applies, of course with a different power law exponent. For the case where there is limited structure to the shape (Fig 2), the fractal dimension is low D = 1.78 indicating that the growth morphology has limited details and structure.

The fractal dimension is higher as the pattern is dense and larger area is occupied by the pattern. The values of fractal dimension D obtained for Figs. 3, 4 are 1.7 and 1.69 indicating that there are more structural details to the growth patterns. As the patterns are very much branched the fractal dimensions are on higher side approaching to that of pure DLA. When compared with Fig. 2, the area occupied by the pattern is appreciably low and the pattern has more open structure still the fractal dimension is around 1.7. This strengthens the idea that the growth is under diffusion like conditions and the patterns resemble DLA.

Table 1 Fractal dimensions using box-counting method:

Pattern in fig No.	Plot in fig. No.	Fractal Dimension
2	5	1.778
3	6	1.702
4	7	1.690

For characterization of shapes in terms of structure and texture, Richardson's plot is a powerful technique. For the implementation of Richardson's plot technique the Figures were processed to detect the boundary and the boundaries were analyzed for implementation of Richardson's plot. This was done using the picture files of the boundary of the patterns with the help of programme written in turbo basic. This programme measures the length of the boundary using different ruler length (k) and keeps a record of the count (N). The results of Richardson's plot exploration for patterns of Fig. 2, 3 and 4 are presented in Fig. 9, 10 and 11 respectively. These are plots of log (N) versus log (k), where k is the ruler size and N is the number of rulers required to cover the entire boundary. The irregularity in shape or the structure to the boundary on longer length scale is different from texture to the pattern at shorter length scale.

It is seen from Fig. 9 and the corresponding fractal dimension that the scale invariance is seen over appreciable range. The x-axis is in terms of k (the Ferets diameter). It has also been observed that the pattern has structure over longer length scale, however at lower length scales the details are limited, thus those points are not included. At larger length scale the points fairly lie along a straight line as is seen from Fig. 9. For very large values of k the points are more scattered.

Fig. 3 and 4 are much different in appearance as compared to Fig. 2 and the corresponding fractal dimensions are also different and are on the higher side. Fractal dimensions for Fig. 3 and 4 are 1.6532 and 1.6644 respectively. The plots of log (N) versus log (k) for Fig. 3 and 4 are shown in Figs. 10, 11, scale invariance is seen over appreciable range of length scale (X). The higher fractal dimension, particularly

over the longer length scale indicates presence of fractal structure to the shapes. The power law exponent for the shorter length scales was not found to be much different indicating that same exponent applies. Thus the structures of the shapes of Figs. 3, 4 have a fractal dimension of 1.653 and 1.664 respectively.

Table 2 Fractal dimensions using Richardson's plot technique

Pattern in fig No.	Plot in fig. No.	Fractal Dimension
2	9	1.573
3	10	1.664
4	11	1.649

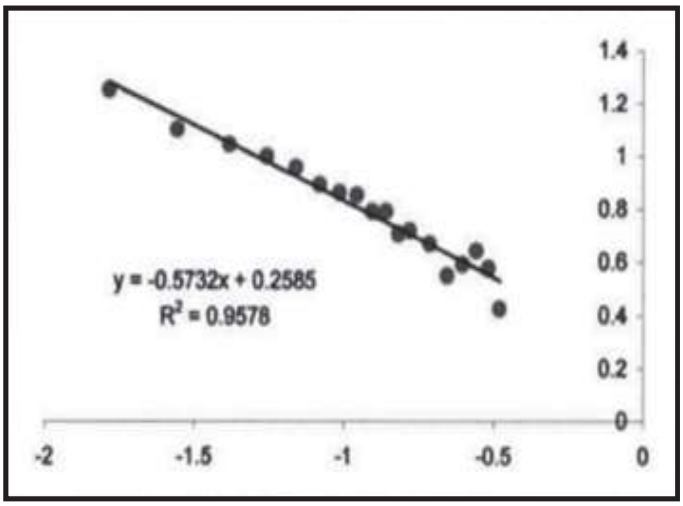

Fig 9 Richardson's plot for Fig. 2

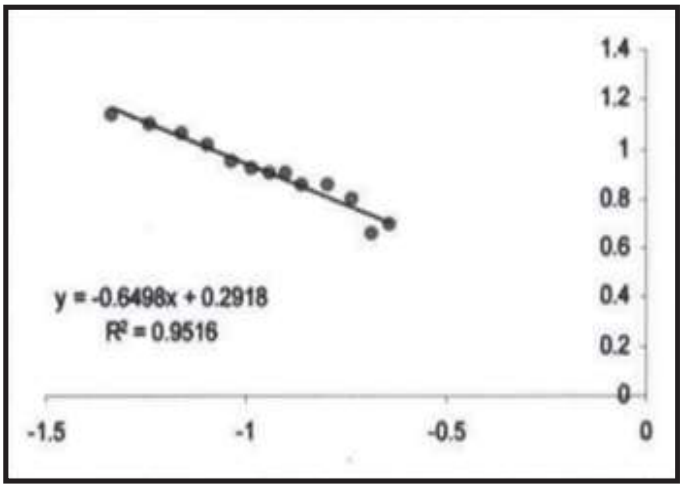

Fig 10 Richardson's plot for Fig. 3

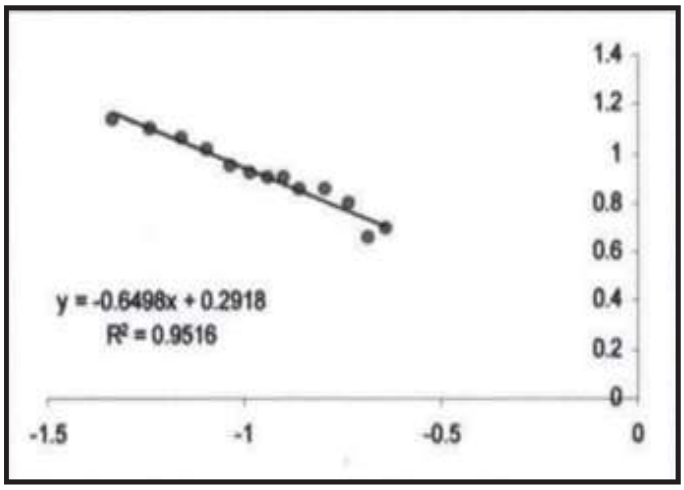

Fig 11 Richardson's plot for Fig. 4

5 Conclusions :

The observations shows that the diffusion field drives the system towards decorated (on many length scales) irregular fractal shapes.

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