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COMPARISON OF MORPHOLOGICAL, CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS OF AN ANTIBIOTIC PRODUCING *Streptomyces kanamyceticus* K2J and its Mutant, M_6.

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ABSTRACT

The morphological, cultural and physiological characteristics of an antifungal antibiotic producing strain *S. kanamyceticus* K2J and its non-producing mutant M_6 were studied by growing the cultures in maltose sodium nitrate mineral broth (MSB), nutrient agar (NA), glucose asparagines agar (GSA), glycerol nitrate agar (GNA), potato dextrose agar (PDA), yeast peptone agar (YPA), starch agar (SA), gelatin medium (GM), beef extract peptone nitrate broth (BPNB), yeast extract tyrosine NaCl agar (YTNA) and litmus milk (LM). The non-antibiotic producing mutant M_6 shows fragmentation of mycelium into shorter elements, while absent in the antibiotic producing strain K2J in maltose sodium nitrate mineral broth. The colour of the pigment produced by the two strains in NA, GSA, GNA, PDA, YPA, SA, GM and YTNA are also different. In most of the media used, antibiotic producing strain produces spores while the non-producing strain is asporogenous. *S. kanamyceticus* K2J cannot utilize dulcitol as carbon source and utilizes fructose, arabinose and galactose very poorly as carbon source whereas the M_6 mutant shows some amount of growth in these carbon sources. M_6 utilizes valine, proline, asparagines, leucine and histidine whereas K2J utilizes these amino acids very well.

Key words: mutant, antibiotic, mycelium, asporogenous.

INTRODUCTION

An antibiotic that is to be produced commercially must first be produced successfully in large-scale fermentors. But rarely do antibiotic producing strains just isolated from nature produce the desired antibiotic at sufficiently high concentration so that commercial production can begin immediately (Brock and Madigan, 2000). One of the main tasks of the industrial microbiologist is to isolate new high-yielding strains. Strain selection involves mutagenesis of the initial culture, plating of mutant types and testing of these mutants for antibiotic production. In most cases, mutants produce less antibiotic than the parent, so that only rarely would a higher yielding mutant strain be obtained. Nevertheless, the empirical techniques of random screening for the selection of improved strain have proved to be extremely effective in increasing the yields of antibiotics (Stanbury, P. F., 1984). It is of interest to know whether these low antibiotic producing strains and the non-yielding strains differ with the parent strain in morphological and biochemical characteristics. Nomi (1963) described the cultural, morphological and physiological characteristics of high and low streptomycin producing strains of *S. griseus*. He reported that fragmentation of the mycelium into shorter elements might be

inversely proportional to streptomycin production and the yellow soluble pigment produced by high potency strains. Szabo' et al. (1961) compared three non–producing streptomycin mutants with the parent strain and found that the former had a shorter life cycle in submerged culture than the latter. Bhadra and Majumdar (1972) also compared the morphological, cultural and physiological characteristics of neomycin producing and non-producing strains of *S. fradiae* and showed that the cultures behaved differently in regard to carbon and nitrogen utilization. It was, therefore, considered of interest to study the relation between the morphological, cultural and physiological characteristics of the organism *S. kanamyceticus* and its antibiotic producing power. In the present investigation, a study has been made on the comparison of properties of antibiotic producing strain *S. kanamyceticus* K2J and its non-producing mutant M_6.

MATERIALS AND METHODS

2.1 Induced mutation and selection of a non-antibiotic producing mutant M_6.

The culture *S. kanamyceticus* K2J showing significant antifungal activity against *Aspergillus niger, Aspergillus paraciticus* and *Aspergillus flavus* was used in the present study. It was grown for 7 days at 27°C on agar slants of the medium consisting of 1.5% maltose, 0.51% $NaNO_3$, 0.1% yeast extract, 0.05% $MgSO_4.7H_2O$, 0.004% $CaCl_2.2H_2O$, 0.005% $FeSO_4.7H_2O$ and 0.00005% $ZnSO_4.7H_2O$ (pH 7.2) and was sub cultured at monthly intervals (Bhadra and Majumdar, 1972). The chemical mutagens used were ethylene mine and aflatoxin and induction of mutation was done as described by Nomi (1963).

2.1.1 **Ethylene mine treatment**:

The K2J strain was first grown on maltose- sodium nitrate mineral agar slants for 7 days at 27°C for the development of spores. A uniform spore suspension was prepared in 25 ml sterile water by shaking with a few glass beads for 3 hr. on a rotary shaker at 220 r.p.m. It was then filtered through sterile cotton. The filtrate was diluted with sterile water to make a suspension containing $2x10^9$ spores/ml. One ml. spore suspension was added to 9 ml of ethylene mine solution of different dilutions 1:1000, 1:2000 and 1:5000 from a stock solution of 1:10 (1 ml ethylene mine in 9 ml water). At intervals of 1 hr., 0.1 ml of the sample from each concentration was diluted to 100 ml with sterile normal saline solution and were plated on maltose-sodium nitrate-yeas extract-mineral agar medium and kept at 27°C for 7-8 days. Mutants were isolated as survived colonies (about 50% survival rate) and were transferred to maltose-sodium nitrate- yeast extract –mineral agar and incubated at 27°C for 7 days.

2.1.2 **Aflatoxin treatment**:

The strain K2J was first grown in 30 ml of Lepage medium (composition: 2% glucose, 1% yeast extract, 0.5% NaCl, 0.025% $MgSO_4.7H_2O$, 0.0001% $FeSO_4.7H_2O$; pH 7.0) in an Erlenmeyer flask placed on a rotary shaker (220 r.p.m) for 48 hours at 27°C. One ml of this 48 hr. old culture suspension was used to inoculate 50 ml Lepage medium taken in a 250 ml Erlenmeyer flask and aflatoxin was added to the medium at different concentration range of 0.5-5.0µg/ml and incubated at 27°C for 5 days in a rotary shaker (220 r.p.m).. Duplicate flasks were used for each concentration of aflatoxin. One ml sample was collected every 24 hr. and plated, after dilution, on a maltose-sodium nitrate – yeast extract-mineral agar media and incubated at 27°C for 7 days. Mutants were

isolated as survived colonies (about 50% survival rate) and were maintained on maltose- sodium nitrate-yeast extract –mineral agar slants.

2.1.3 Determination of antibiotic production

Antibiotic production was determined by fermentation process using shake flask method with a medium consisting of 1.5% maltose, 0.1% peptone, 0.05% $MgSO_4.7H_2O$, 0.004% $CaCl_2.2H_2O$, 0.005% $FeSO_4.7H_2O$ and 0.00005% $ZnSO_4.7H_2O$ (pH 7.5) (Nomi, 1963).
Inoculums were prepared by growing the isolates in 10 ml Lepage medium in a 50 ml Erlenmeyer flask on a rotary shaker for 48 hrs. incubated at 27°C.
To 20 ml of the medium (above) in a 100 ml Erlenmeyer flask was inoculated with 0.4 ml of 48 hours old culture suspension of each isolate and incubated for 7 days at 27°C on a rotary shaker (220 r. p. m). At the end of fermentation, the broths were filtered and the antibiotic potency of the filtrates was determined by a modified cup plate method using *A. niger* as the test organism (Garrod *et al.*, 1981). All the strains were maintained on maltose sodium nitrate yeast extract mineral agar slants.
Treatment with aflatoxin produced 9 non-producing isolates. They were sub cultured successively three times in maltose- sodium nitrate- yeast extract- mineral agar media to confirm their antibiotic non-production abilities. Out of these, a non-producing mutant strain M_6 was selected for the comparison studies.

2.2 Morphological characteristics of S. kanamyceticus K2J and its mutant M_6

The morphological characteristics of *S. kanamyceticus* K2J and *S. kanamyceticus* M_6, were studied by growing the cultures in a synthetic liquid medium consisting of 1.5% maltose, 0.51% $NaNO_3$, 0.05% $MgSO_4.7H_2O$, 0.004% $CaCl_2.2H_2O$, 0.005% $FeSO_4.7H_2O$ and 0.00005% $ZnSO_4.7H_2O$ (pH 7.5).
To 20 ml of the synthetic liquid medium in a 100 ml flask was inoculated with 0.2 ml of spore suspensions (harvested from 7 days old cultures in maltose-sodium nitrate mineral agar slants) of K2J and M6 strains and incubated under stationary conditions for 7 days at 27°C. Morphological characteristics studies of the cultures at different ages (0- 7days) were observed under the microscope (magnification - X1000).

2.3 Cultural and physiological characteristics of S. kanamyceticus K2J and its mutant, M6

The cultural and physiological characteristics of the two strains were studied after
growing the strains for 10 days at 27°C on a variety of media such as nutrient agar, glucose-asparagine agar, glycerol nitrate agar, potato dextrose agar, yeast peptone agar, starch agar, gelatin medium etc comprising both complex and synthetic media.

2.4 Utilization of carbohydrates

The utilization of carbohydrates by the two strains was studied in the basal medium consisting of 0.51% $NaNO_3$, 0.05% $MgSO_4.7H_2O$, 0.004% $CaCl_2.2H_2O$, 0.005% $FeSO_4.7H_2O$ and 0.00005% $ZnSO_4.7H_2O$ (pH 7.5).

To the basal medium sterile carbon source at 1% concentration was added. To 10 ml of medium in a 50 ml flask was inoculated with 0.2 ml cell suspension and incubated at 27°C on rotary shaker. Growths of the strains were observed.

2.5 Utilization of amino acids

The procedure and medium used for this study is same as in section 2.4 above, except sodium nitrate ($NaNO_3$) was omitted from the medium, and was supplemented with 1% maltose. Amino acids at a concentration equivalent to 84 mg N per 100 ml were added. Cellular growth was observed on the 7^{th} day of incubation.

RESULTS

Induced mutation and selection of a non-antibiotic producing mutant M_6.

The results of treatment of *S. kanamyceticus* K2J with ethylene mine and aflatoxin are as shown in Table 1.

Table 1: Production of antibiotic by mutants of *S. kanamyceticus* K2J

Mutagens	No. of isolates tested	*Antibiotic production (µg/ml)			
		A	B	C	D
Ethylene mine	65	28	-	37	-
Aflatoxin	45	15	2	19	9

*A- similar to parent (102µg/ml); B- more than parent; C- less than parent (20-65µg/ml); D- No production (no measurable zone)

Morphological characteristics of K2J *and* M_6.

The type of growth, the nature of vegetative growth and that of aerial mycelium formation are listed in Table 2 and Figures 1a and 1b.

Table 2: Morphological characteristics of K2J and M_6 in Maltose-$NaNO_3$-mineral broth

Morphological characteristics	K2J	M_6
Growth	Surface growth; many long and highly branched vegetative mycelia of diameter 0.7μ to 0.8μ	Submerged growth; relatively few fragmented short mycelia of diameter 1.6μ to 2.0μ.
Aerial mycelium	Abundant and highly branched	Poor and simply branched
Sporulation	White spore formation; sporophores Flexible and hooked; no true spirals.	No spore formation; Fragmented mycelia in the broth.

Cultural and morphological characteristics of K2J *and* M_6.

The cultural and physiological characteristics were observed for 10 days on different synthetic and complex media. The results are as shown in Table 3.

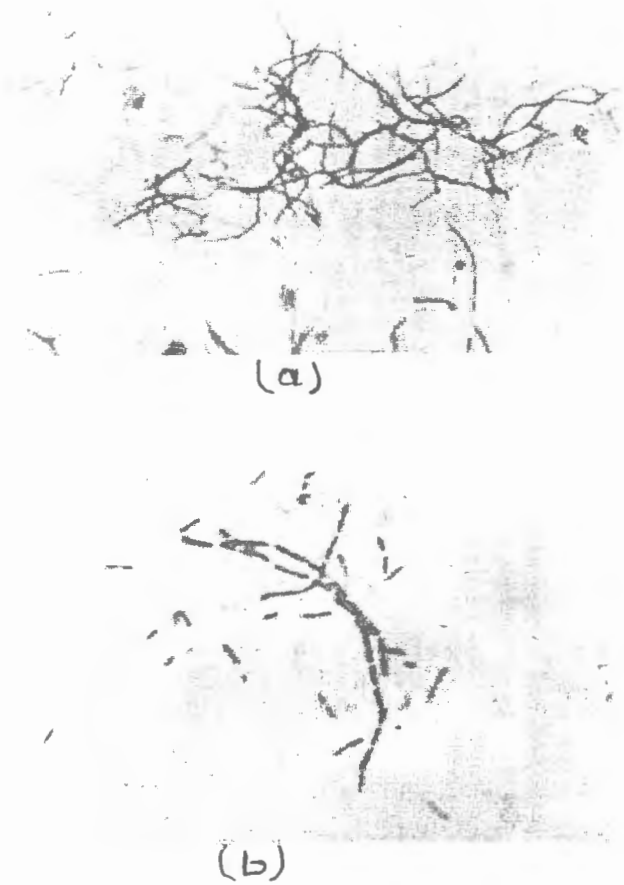

Fig. 1 a) Microphotograph of vegetative Mycelium of S. *kanamycetitus* k2J.

b) Microphotograph of vegetative Mycelium of Mutant M6.

Table 3: Cultural and physiological characteristics of K2J and M_6

*Medium	**characteristics	K2J	M_6
NA	VM	cream	colorless
	AM	,,	white
	RS	,,	nil
	DP	nil	colorless
GSA	VM	yellow	colorless
	AM	,,	white
	RS	,,	nil
	DP	brown	colorless
GNA	VM	lemon	pink
	AM	white	,,
	RS	yellow	,,
	DP	brown	,,
GNA	VM	white	white
	AM	brown	yellowish white
	RS	,,	yellow
	DP	nil	nil
YPA	VM	white	colorless
	AM	,,	,,
	RS	colorless	,,
	DP	yellow	nil
GYA	VM	white	colorless
	AM	,,	,,
	RS	colorless	nil
	DP	yellow	nil
MSB	VM	white	colorless
	AM	,,	,,
	RS	yellow	,,
	DP	,,	nil
SA		hydrolyzed	
GM		liquefied	liquefied
BPNB		nitrate reduced	nitrate reduced
YTNA		no melanin formation	no melanin formation

* NA- nutrient agar; GSA- glucose asparagines agar; GNA- glycerol nitrate agar; PDA- potato dextrose agar; YPA-yeast peptone agar; GYA- glucose yeast extract agar; MSB- maltose sodium nitrate mineral broth; SA-

starch agar; GM- gelatin medium; BPNB_ beef extract peptone nitrate broth; YTNA- yeast extract tyrosine NaCl agar.
** VM-vegetative mycelium; AM-aerial mycelium; RS-reverse side; DF-diffusible pigment.

Utilization of carbohydrates and amino acids by the two strains, S. kanamyceticus and mutant M_6..
The results showed that *S. kanamyceticus* K2J cannot utilize dulcitol as carbon source and utilizes fructose, arabinose and galactose very poorly as carbon source, while the M_6 mutant showed some amount of growth in these carbon sources. There are no significant differences in the utilization of other carbohydrates by the two strains. *S. kanamyceticus* K2J utilizes valine, proline, asparagine, leucine and histidine very poorly whereas M_6 utilizes these amino acids very well. There is no significant difference in the utilization of other amino acids like glutamic acid, serine, aspartic acid etc. by the two strains. The results are shown in Table 4.

Table 4: Utilization of carbohydrates and amino acids by K2J and M_6

Carbohydrates	K2J	M_6	amino acid	K2J	M_6
maltose	+++	++	proline	+++	+
mannitol	++	+++	asparagine	+++	+
mannose	++	+++	methionine	++	++
starch	+++	+++	glycine	++	+++
dextrin	++	+++	phenylalanine	+++	++
galactose	+++	+	lysine	+++	++
arabinose	++	±	valine	++	+
glucose	+++	++	arginine	+++	++
fructose	++	±	aspartic acid	+++	++
sucrose	+	+	leucine	++	+
raffinose	++	++	threonine	+++	++
inositol	-	-	histidine	+++	+
dulcitol	+	-	tyrosine	++	+++
glycerol	++	++	serine	++	+++
lactose	-	-	glutamic acid	++	++

+++ indicates heavy growth
++ „ medium growth
+ „ poor growth
± „ very poor growth
- „ no growth

DISCUSSION

Virtually any characteristic of an organism can be changed by mutation. In industries, there are a variety of chemical, physical or biological agents that are used to get the mutant of an organism with suitable characteristics. A selection method, based on a temperature sensitive autotypic phenotype, has been used to genetically improve a second fermentation *Saccharomyces cerevisiae* yeast strain

by UV mutagenesis. The mutations carried by the resulting strains affected cell morphology, growth kinetics, sporulation and the release of nitrogenous compounds in an accelerated autolysis model. Their fermentation power was not severely impaired (Gonzales *et al.*, 2003). It is well known that antibiotic producing ability of a microorganism varies greatly for different strains of a given species. This ability may also be lost under different cultural conditions or mutation. A number of investigators have made systematic studies on the relationship between the morphological, cultural and physiological characteristics of different strains of a microbial species and their antibiotic producing capacities. In course of this study on the development of high yielding mutants with chemical mutagens, ethylene mine and aflatoxin, a non-antibiotic producing mutant M_6 was isolated and its morphological, cultural and physiological properties were compared with those of the antibiotic producing strain K2J. It is observed that the antibiotic producing strain K2J differs from the non-producing isolate M_6 with respect to morphological and some cultural and physiological characteristics. Thus, there is fragmentation of mycelium of M_6 into shorter elements while K2J does not show any such fragmentation of mycelium. Again, K2J shows good sporulation whereas M_6 does not produce spores. The strains K2J and M_6 also differ in their ability to produce pigments in different media and colour of the pigments produced by the two strains is also different. These findings agree with that of Nomi (1963) who reported on the variation in properties of different strains of *S. griseus* and their streptomycin producing capacity. He suggested that fragmentation of the mycelium into shorter elements is inversely proportional to streptomycin production and the difference in the ability of different potency strains of *S. griseus* to produce pigments. There are some differences in the ability of two strains to utilize some carbohydrates such as dulcitol, fructose, arabinose and galactose and some amino acids as valine, proline, asparagines, leucine and histidine. The present study has shown a possible correlation between antibiotic producing ability of the organism and its morphological and physiological properties relating to spore formation.

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