METHODOLOGY:

For the proposed research work the investigator has framed work into different parts.

1) Isolation of organism (Aspergillus niger):
   
a) Isolation of Rhizosphere Soil From 54 places and 23 species of trees of Nandurbar District:

   There will be wide screening schedule for the isolation of potent strains in this work soil properties are considered as major functional aspect. With this regard entire Nandurbar district is divided in equal block in squares and that geographical zone is divided in to 54 squares. The further consideration is with collection of rhizosphere soil of forest trees. With this regard dominant tree species belongs to Nandurbar forests are considered for rhizosphere soil collection. The above approach is new approach and never discussed in any research communication, people have collected rhizosphere soil samples, but the way of collection is not scientific which represents somehow the proper representation of soil. With this respect one study the soil parameters in randomized block design. There will be variation in different soils throughout the district. In addition to natural soil type’s soil from representative agricultural fields also considered for collection.

   The natural source of Aspergillus niger is soil. For the isolation of high citric acid yielding strains of A.niger, for this propose soil samples from 54 place of different trees rhizosphere were collected.

   The present study is based on the isolation of rhizosphere soil for collection of high potential strain of Aspergillus niger. Common trees found in selected equal 54 blocks of entire Nandurbar district. For this propose there is selected 54 villages and 23 trees species found commonly in that region. The details are given bellows;

   First name is for village name and second name is for tree species. The plant species are identified by using “Flora of Dhule and Nandurbar district” written by, Patil D. A., 2003.

1) Roshmal khurd – Garuga pinnata Roxb.
2) Genda – Terminalia bellirica (Gaertn.) Roxb.
3) Savaryadigar – Acacia chundra Willd.
4) Khadki – Butea monosperma Lamk.
5) Pimpalkhunta – Ficus recemoso L.
6) Bhagdari – *Madhuka longifolia* (Koem.)
7) Khuntamauli – *Mangifera indica* L.
8) Dhadgaon – *Dalbergia sissoo* Roxb.
9) Jugani – *Bauchanania lanza* Spreng.
10) Lighapani – *Bauhinia variegata* L.
11) Hunakhab – *Spondias pinnata* (L.f.) Kurz.
12) Sakaliumbber – *Bombax ceiba* L.
13) Asali – *Ficus religiosa* L.
14) Bijari – *Tarminalia crenulata* Roth.
15) Kakarda – *Syzygium cumini* L.
16) Lakadkat – *Tamarindus indica* L.
17) Morkhi – *Boswellia serrata* Roth.
18) Valmba – *Tectona grandis* L.
19) Kankadamal – *Tamarindus indica* L.
20) Amoni – *Tamarindus indica* L.
22) Borad – *Azadirachta indica* A. Juss.
23) Pimpri – *Tamarindus indica* L.
25) Korai – *Tectona grandis* L.
26) Akkalkuwa – *Tectona grandis* L.
27) Daswad – *Azadirachta indica* A. Juss.
28) Dhurkheda – *Tamarindus indica* L.
29) Shahada – *Azadirachta indica* A. Juss.
30) Vadchhil – *Azadirachta indica* A. Juss.
31) Malgaon – *Bauhinia racemosa* Lamk.
32) Vedawad – *Azadirachta indica* A. Juss.
33) Lahan Shahada – *Azadirachta indica* A. Juss.
34) Vikharan – *Acacia nilotica* (L.) Willd.
35) Sasde – *Bauhinia racemosa* Lamk.
36) Katali – *Adansonia digitata* L.
37) Kakadde Khurd – *Tamarindus indica* L.  
38) Umaj - *Azadirachta indica* A. Juss.  
39) Pacharbari – *Bauhinia racemosa* Lamk.  
40) Nandurbar – *Azadirachta indica* A. Juss.  
41) Tishi – *Acacia nilotica* (L.) Willd.  
42) Karli – *Acacia nilotica* (L.) Willd.  
43) Nawagaon – *Adansonia digitata* L.  
44) Dudhare - *Azadirachta indica* A. Juss.  
45) Malwan – *Adansonia digitata* L.  
46) Waghade – *Adansonia digitata* L.  
47) Gangapur – *Acacia nilotica* (L.) Willd.  
48) Balwad – *Azadirachta indica* A. Juss.  
49) Vaidane – *Azadirachta indica* A. Juss.  
50) Bedkipana – *Acacia nilotica* (L.) Willd.  
51) Chichpala – *Adansonia digitata* L.  
52) Bandharpada – *Bauhinia racemosa* Lamk.  
54) Kamad – *Bauhinia racemosa* Lamk.

**b) Serial dilution method:**

Fifty four *Aspergillus niger* culture were isolate from fifty four soil sample of selected places of Nandurbar district of Maharashtra by serial dilution method. The soil samples were collected in sterile polythene bags. One gram of soil sample was dissolved in 100 ml of sterilized distilled water. The soil suspension was further diluted to $10^4$ - $10^6$ times. One milliliter of this diluted suspension was transferred to individual petri plates containing PDA medium.

In this way, 54 different samples were collected. Each sample was diluted in serial dilution so as to get 1/100, 1/1000 and 1/10000 dilution of each sample. These dilutions (0.1 ml) of each sample were spread separately on sterile Potato-Dextrose agar medium containing streptomycin and Penicillin to avoid growth of bacteria.
Method for Potato-Dextrose agar (PDA) medium:

For the development of one litter medium there is required following **Composition of PDA Medium with pH is 4.5:**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Composition</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Potato</td>
<td>200 g</td>
</tr>
<tr>
<td>2</td>
<td>Dextrose</td>
<td>20 g</td>
</tr>
<tr>
<td>3</td>
<td>Agar-agar</td>
<td>15 g</td>
</tr>
<tr>
<td>4</td>
<td>Streptomycin</td>
<td>5 g</td>
</tr>
<tr>
<td>5</td>
<td>Penicillin</td>
<td>5 g</td>
</tr>
<tr>
<td>6</td>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The medium was prepared by peeling the 200 g potato in small pieces and boiled with 500 ml distilled water for 30 minutes and filtered the preparation by muslin cloth and remove the supernatant and take the liquid in measuring slender for adjustment of 1000 ml with addition of distilled water, then after fill the preparation in 1000 ml conical flask, then after dissolve 20 g Dextrose and add 5 g Streptomycin and 5 g Penicillin for avoid bacterial growth. The pH was maintained at 4.5. The agar was then dissolved by heating the medium for about 15 minutes and cooled the medium at room temperature, the after ploughed the conical flask containing medium with cotton plough. Then medium is autoclave for 15 minutes at 15 lbs. pressure.

The prepared medium of PDA of 10 ml are filled in petri plate in laminar air flow, then after One ml of the serial diluted suspension was transferred to individual petri plates containing PDA medium.

Plates were incubated at 28°C 48 hours. Isolated cultures were observed and they were purified by sub-culturing on Potato-Dextrose agar (PDA).

c) **Classification, Identification and Maintenance of the fungal cultures:**

The young *Aspergillus niger* colonies were transferred to potato dextrose agar (PDA) slants. The cultural and morphological characteristics of *A. niger* isolate were observed.
The PDA slants were then inoculated by transferring a minute amount of *A. niger* conidia from the petri plates and incubating at 30\(^0\)C (4-6 days) for maximum sporulation. Culture was kept in refrigerator at 4\(^0\)C for further study.

**d) Screening isolated cultures for citric acid production:**

To study the *Aspergillus niger* strain producing organic acid from carbon substrates can be detected by incorporation of pH indicators bromocresol dye in Potato Dextrose agar medium and growing fungal strain on it. A color change of medium from blue to yellow in the vicinity of colony indicates organic acid production.

**Dye Method:**

The *Aspergillus niger* cultures were screened qualitatively in petri plates containing PDA medium with Bromocresol green as an indicator. The medium was prepared by peeling the 200 g potato in small pieces and boiled with 500 ml distilled water for 30 minutes and filtered the preparation by muslin cloth and remove the suparnant and take the liquid in measuring slender for adjustment of 1000 ml with addition of distilled water, then after fill the preparation in 1000 ml conical flask, then after dissolve 20 g Dextrose and add 5 g Streptomycin and 5 g Penicillin for avoid bacterial growth and addition of 1.5 g Bromocresol green in medium. The pH was maintained at 4.5. The agar was then dissolved by heating the medium for about 15 minutes and cooled the medium at room temperature, the after ploughed the conical flask containing medium with cotton plough. Then medium is autoclave for 15 minutes at 15 lbs. pressure.

**Composition of PDA Medium with Bromocresol green and pH, 4.5:**

<table>
<thead>
<tr>
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<td>5 g</td>
</tr>
<tr>
<td>6</td>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>7</td>
<td>Bromocresol green</td>
<td>1.5 g</td>
</tr>
</tbody>
</table>
The prepared medium of PDA of 10 ml are filled in petri plate in laminar air flow and at room temperature allowed to cool small quantity of the conidial spot was given aseptically to each of these petri plates. The petri plates incubated at 30°C for 24 hours. Yellow zones due to citric acid formation were formed. On the basis of larger citric acid zone compared with control, the best strains of *A. niger* were picked and transferred to the PAD slants.

The cultures were incubated for 4-6 days at 30°C until maximum sporulation. Five *A. niger* isolates were selected from fifty four *A. niger* for further screening.

**First screening:** Done in 54 rhizosphere soil sample. It gives higher potential power of strain of *A. niger* for citric acid production, in this manner 5 strain are selected from 54 rhizosphere soil sample strain of *A. niger* which are collected from soil sample of village Bhagdari, Khuntamovli, Leghapani, Morakhi and Valmba.

**2nd screening:** The prepared medium of PDA of 10 ml are filled in petri plate in laminar air flow and at room temperature allowed to cool small quantity of the conidial spot five *Asperillus niger* was given aseptically or controlled environment to each of these petri- plates. The petri-plates incubated for 12 hours at 30°C. Yellow zones due to citric acid formation were formed. On the basis of larger citric acid zone compared with control, the best strains of *A. niger* were picked and transferred for culture to the PAD slants this strains are isolated from soil sample of Leghapani, Bhagdari and Valmba village from trees of *Bauhinia variegate* L., *Madhuka longifolia* (Koem.) and *Tectona grandis* L. respectively.

The cultures were incubated for 4-6 days at 30°C until maximum sporulation. Three *A. niger* isolates were selected from five strains of *A. niger* for further submerged fermentation.

**Abbreviation for these three strains:**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Place</th>
<th>Name of trees</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leghapani</td>
<td><em>Bauhinia variegate</em> L.</td>
<td><em>Asperillus niger</em>LBV10</td>
</tr>
<tr>
<td>2</td>
<td>Bhagdari</td>
<td><em>Madhuka longifolia</em> (Koem.)</td>
<td><em>Asperillus niger</em>BML6</td>
</tr>
<tr>
<td>3</td>
<td>Valmba</td>
<td><em>Tectona grandis</em> L.</td>
<td><em>Asperillus niger</em>VTG18</td>
</tr>
</tbody>
</table>
e) Preparation of conidial inoculation:

Conidial inoculation was used in the present study. Conidia from 4-6 days slant culture were used for the inoculation. Ten milliliters of sterilized distilled water was added with slant having profuse conidial growth on its surface. An inoculum needle was used to break the conidial clamps. The tubes were shaken vigorously to obtain a homogenous mixture of the spore suspension. The homogenous mixture of the conidial suspension was added in distilled water and makes a final 100 ml. The soil suspension was further diluted to $10^4 - 10^6$ times. One milliliter of this diluted suspension was transferred to submerged fermentation medium for estimation of citric acid.

f) Designing of media for fermentation of citric acid production:

Production and growth of microorganism or fungi are strongly affected by the medium composition such as concentrations of carbon, nitrogen, phosphorous, and potassium. Thus, citric acid productivity by *Aspergillus niger* can be improved by optimizing the medium composition. In this respect there was select for Carbon source which are flower of *Madhuka longifolia*, Flower of *Bauhinia variegate*, pithy pulp of fruit of *Adansonia digitata* and flower of *Bombax ceaba*.

Botanical description:

1) *Madhuca longifolia*:

*Madhuca longifolia* is one of the most important of Indian forest trees, not because it may possess valuable timber - and it is hardly ever cut for this purpose - but because of its delicious and nutritive flowers. It is a tree of abundant growth and, to the people of Central India; it provides their most important article of food as the flowers can be stored almost indefinitely. It is large and deciduous with a thick, grey bark, vertically cracked and wrinkled. Most of the leaves fall from February to April, and during that time the musky-scented flowers appear. They hang in close bunches of a dozen or so from the end of the gnarled, grey branchlets. Actually the word ‘hang’ is incorrect because, when a bunch is inverted, the flower stalks are sufficiently rigid to maintain their position. These stalks are green or pink and furry, about 5 cm. long. The plum-coloured calyx is also furry and divides into four or five lobes; within them lies the globular corolla, thick, juicy and creamy white. Through small eyelet holes at the top, the yellow anthers
can be seen. The stamens are very short and adhere to the inner surface of the corolla; the pistil is a long, protruding green tongue. It is at night that the tree blooms and at dawn each short-lived flower falls to the ground. A couple of months after the flowering period the fruit opens. They are fleshy, green berries, quite large and containing from one to four shiny, brown seeds.

**Medicinal uses:**

Medically the tree is very valuable. The bark is used to cure leprosy and to heal wounds; the flowers are prepared to relieve coughs, biliousness and heart-trouble while the fruit is given in cases of consumption and blood diseases.

**Natural Habitat:**

*Madhuca longifolia* is a frost resisting tree of the dry tropics and sub-tropics, common in deciduous forests and dry sal plain forests. The tree is usually found scattered in pastures and cultivated fields in central India.

**Products:**

Food: The sweet, fleshy corolla is eaten fresh or dried, powdered and cooked with flour. The fruit contains valuable oil that is sometimes used for cooking by the locals. Outer fruit coat is eaten as a vegetable and the fleshy cotyledons are dried and ground into a meal. Ripe fruits are used for fermenting liquor. Fodder: Leaves, flowers and fruits are lopped for goats and sheep. Seed cake is also fed to cattle. Timber: The heartwood is reddish brown, strong, hard and durable.

2) **Bauhinia variegata:**

*Bauhinia variegata* is a small to medium-sized deciduous tree with a short bole and spreading crown, attaining a height of up to 15 m and diameter of 50 cm. In dry forests, the size is much smaller. The bark is light brownish grey, smooth to slightly fissured and scaly. Inner bark is pinkish, fibrous and bitter. The twigs are slender, zigzag; when young, light green, slightly hairy, and angled, becoming brownish grey. Leaves have minute stipules 1-2 mm, early caducous; petiole puberulous to glabrous, 3-4 cm; lamina broadly ovate to circular, often broader than long, 6-16 cm diameter; 11-13 nerved; tips of lobes broadly rounded, base cordate; upper surface glabrous, lower glucose but glabrous when fully grown. Flower clusters (racemes) are
unbranched at ends of twigs. The few flowers have short, stout stalks and a stalk like, green, narrow basal tube (hypanthium). The light green, fairly hairy calyx forms a pointed 5-angled bud and splits open on 1 side, remaining attached; petals 5, slightly unequal, wavy margined and narrowed to the base; 5 curved stamens; very slender, stalked, curved pistil, with narrow, green, 1-celled ovary, style and dot like stigma. Pods dehiscent, strap-shaped, obliquely striate, 20-30 by 2-25 cm; long, hard, flat with 10-15 seeds in each; seeds brown, flat, nearly circular with coriaceous testa.

3) **Adansonia digitata:**

*Adansonia digitata* is a large, round canopied tree with a swollen trunk, about 10-25 m in height, often with a bole of 3-10 m (giant individuals attain a girth of up to 28 m); bark is soft, smooth, fibrous, reddish-brown, greyish-brown or purplish-grey; bark of leaf-bearing branches is normally ashy on the last node; a green layer below the outer, waxy layer of the bark, presumably to assist in photosynthesis when the tree has shed its leaves. The thick, fibrous bark is remarkably fire resistant, and even if the interior is completely burnt out, the tree continues to live. Regrowth after fire results in a thickened, uneven integument that gives the tree its gnarled appearance resembling an elephant’s skin but that serves as added protection against fire. Mature thick and extensive lateral roots anchor the tree on the ground and end in clusters of potato like tubers; the thick, strong, prominent taproot at 6 months is 3 times the length of the seedling; roots grow fast but never penetrate far beyond a depth of 2 m, which explains why in old age they are often found toppled when the branches increase in weight. Leaves alternate, digitately 3- to 9-foliate; leaflets oblong to ovate, 5-15 x 3-7 cm, lower leaflets being the smallest and terminal leaflet the largest; leaflets dark green, with short, soft hairs; lateral veins looping; apex and base tapering; margin entire; petiolules absent or almost so; petiole up to 12 cm long. Flowers a waxy white, up to 20 cm in diameter, axillary, solitary, pendulous, bisexual; all floral parts in 5s; calyx deeply lobed, with white, silky hairs inside; large, crinkly, spreading petals; many stamens on a large central column that is shed with the petals; ovary superior, 5-10 chambered; petals bruise easily and become brown; flowers have an unpleasant scent. Fruit ovoid, 12 cm or more in length, with a hard, woody shell, covered with yellowish-grey velvety hairs, indehiscent; seeds smooth, embedded in a whitish powdery pulp, have little or no endosperm.
4) **Bombax ceiba:**

Deciduous trees; to 45 m high; bole straight, buttress 1-2 m high, armed with conical prickles; bark 20-30 mm thick, grey mottled with white, longitudinal fissures shallow; blaze pink, marked with triangular rays; branches horizontal and more or less whorled; branchlets prickly. Leaves digitately-compound, alternate, stipulate; stipules small, lateral; rachis 12-25 cm, stout, swollen at base, glabrous; leaflets 5-7, whorled; petiolule 14-25 mm long, stout, glabrous; lamina 10-20 x 2-6 cm, elliptic, elliptic-ovate or elliptic-obovate; base attenuate or cuneate; apex caudate-acuminate; margin entire, glabrous, chartaceous; lateral nerves 8-14 pairs, parallel, slightly ascending, prominent, secondary laterals also seen; intercostae reticulate. Flowers bisexual, dark crimson, 6-7 cm across, solitary or 2-5 together; pedicels 1-2 cm long, thick; calyx campanulate, irregularly lobed, lobes 3-4 x 3 cm, coriaceous, glabrous to sparsely puberulous outside, silky inside, falling of with corolla and stamens; petals 5, 8.5-18 x 3.5-5 cm, obovate to elliptic-obovate, recurved, fleshy, tomentellous outside, imbricate; stamens 65-80, 3-7.5 cm long in 5 bundles; staminal tube short; filaments flat, angular, connate only at the base of the bundles; anthers reniform; ovary conical, tomentose, 5-celled; ovules many; style exceeding the stamens; stigma 5-fid, lobes spreading. Fruit a capsule, 8-10 x 3 cm, downy tomentose, cylindrical, cuneate on both ends, blackish and glabrous at maturity, the columella brownish; seeds numerous, pyriform, smooth, dark brown, embedded in white cotton.

g) **Fermentation technique:**

The selected carbon sources which are flower of *Madhuka longifolia*, flower of *Bauhinia variegata*, pithy pulp of fruit of *Adansonia digitata* and flower of *Bombax ceaba* (100, 150, 200 and 250 g). Were cut in small spices and crushed in mixture machine with 50 ml distilled water. After make final volume 1000 ml with distilled water, then after 100 ml this solution add in separate 250 ml Erlenmeyer flasks. After add KH$_2$PO$_4$ (50,75,100 and 125 mg), NaNO$_3$ (300,350,400 and 450 mg), MgSO$_4$.7H$_2$O (15, 20, 25, and 30 mg), and adjust 4.5 initial pH with HCl and 0.1 NaOH. The flask was autoclaved after flask with cotton plugged at 121°C for 15 minutes. Per medium inoculated by 1.0 ml (6.0 X 106) of *A. niger* (selected strains Asperillus niger LBV10, Asperilllus niger BML6 and Asperilllus niger VTG18) conidial suspension of *A. niger* after cooling at room temperature and incubated in static incubator for 8 days at 28°C.
Later the fermentation, addition of distilled water (1:4 W/V) for the dilution of medium. The filtrates was used for citric acid detection after medium were filtered.

**h) To found the study of effect of media components:**

To found the study of effect of media components such as percentage of carbon sources, phosphate, nitrogen, magnesium source and effect pH, methanol and initial pH with H₃PO₄ in growth medium on citric acid fermentation all the components, except one to be studied, are kept constant with respect to control medium and one component concentration is changed in particular range. These broths were then tested for citric acid production by selected strains of *A. niger*.

**i) To found the study of effect of physical parameters in growth medium:**

To found the study of effect of physical parameters such as electric current and UV treatment on strain.

**j) Estimation of citric acid production:**

After fermentation medium was diluted with distilled water (1:4 W/V). The medium was then filtered and filtrate was used for the subsequent analysis.

Citric acid was determined titrimetrically by using 0.1 NaOH and phenolphthalein as indicator and calculated as % according to the formula:

\[
\text{% citric acid} = \frac{\text{Normality} \times \text{volume of 0.1 M NaOH} \times \text{Equivalent weight of citric acid} \times \text{dilution factor}}{\text{Weight of sample (g)} \times 10}
\]

(Kareem, S. O.et al. 2010)