

Protein and DNA Isolation from *Aspergillus Niger* as well as Ghost Cells Formation

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Abstract

Recently, a protocol for ghost cells preparations was introduced. It was given the name sponge-like protocol: Prokaryotes, eucaryotes and virus were turned to ghost cells using such protocol. In this study, with slight modifications, *Aspergillus niger* ghost cells were prepared using the same protocol. Both the Minimum Inhibitory Concentration (MIC) and the minimum growth concentration (MGC) values for H₂O₂, NaOH, NaHCO₃ and SDS against *A. niger* were determined. Five different randomization experiments were conducted instead of the full Plackett-Burman design. During the ghost preparation steps, the released Protein and DNA were measured spectrophotometrically at 280nm and 260nm, respectively. The quality of the prepared ghost cells were evaluated during the preparation steps using light microscope. Transmission electron microscope was used for evaluating the final steps. Protein and DNA electrophoresis were conducted to evaluate the quality of the released protein and DNA after each randomization experiment. The data obtained prove correct evacuation of the fungal cells from their cytoplasmic content during the successive steps. The study not only introduces a protocol for preparing ghost cells from *Aspergillus niger* but also enables the isolation of both of protein and DNA. The idea, the concept and the tools used in this study could establish a more sensitive method for protein and DNA isolation using any of four utilized chemical compounds. This proposes the same concept of enzyme-induced cell lysis which is based on minimizing the effect of used chemicals or enzymes. The study recommended extending the benefit of the sponge-like protocol from being a protocol for ghost cells preparation to DNA and protein isolation technique using the same concept.

Keywords: *Aspergillus niger*; ghost cells; DNA isolation; Protein isolation

Introduction

Aspergillus niger is one of the most commonly found fungi either in in/outdoor environments. It causes black mould diseases in foods as vegetables, grapes, apricots, onions, peanuts, etc. Some reports prove that it is responsible for producing some dangerous mycotoxins. *A. niger* is less likely to cause human

disease than some other *Aspergillus sp.* One of the most famous diseases it causes to human is the Aspergillosis; a lung disease. It is one of the otomycosis causing fungi, resulting in ear pain, temporary hearing loss, and head pain etc. In severe cases, it causes damage to ear canal and tympanic membrane. In fact, most people breath in *Aspergillus* spores including *A. niger* every day. In most cases, it causes nothing for healthy individuals if entered in a little quantity [1, 2]. However, it turns to be pathogenic in cases such as immunocompromized patients, illness, lung disease, and if inhaled in large amount. One could conclude that weak or compromised immune system is the most claimed causative agent for *A. niger* pathogenesis as well as exposure to large amount of spores or continuous source that enable their accumulation in the body particularly the lung. *A. niger* spores could be found everywhere but one of the most dangerous sources is the dry grinded seeds where the spore will not be germinated and could by one or another way reach to our bodies as spore. Spores are more dangerous than vegetative cells except in case of mycotoxin-producing strains. *A. niger* is not harmful in all cases, it is also beneficial and can be used in different biotechnological applications such as in the citric acid production [3].

Evacuating microbes from their cytoplasmic content is a natural phenomenon [4]. Pores could be introduced to the microbial cells as a result of different mechanisms [4-8]. Such as the evacuation of the gram-negative bacteria by the bacteriophage infections [4]. The bacteriophage E lysis gene is used for evacuating the cells and turning them to ghosts by controlling its expression using heat sensitive promoter [4, 5, 8-11]. Recently, the Sponge Like protocol was introduced [12-15]. Its main concept is using active chemical compounds that could introduce pores in the microbes and degrade the DNA at concentrations which did not change the surface antigens or the 3D structure [13]. This enables evacuating gram-negative and gram-positive bacteria, eukaryotes and viruses [4, 12-14, 16-27].

In this study, *A. niger* representing the fungi was prepared as ghosts. In addition, the idea for using the Sponge Like protocol for releasing both of DNA and protein for various applications was established. Using such protocol based on using chemical compounds or enzymes, not only the ghost cells are produced but also both of DNA and protein are isolated.

Materials and Methods

Fungal strain

A. niger strain used in this study was kindly identified and obtained from Al-Azhar University Mycology Center (Cairo, Egypt).

Cultivation Conditions

The *A. niger* strain was cultivated in one litre flask contains 500 mL Sabouraud's dextrose broth at 28°C for 7 days at 150 rpm.

Determination of the MIC and MGC Values for NaOH, SDS, NaHCO₃ And H₂O₂

Standard broth microdilution susceptibility assay for determining the MIC values for each of NaOH, SDS, NaHCO₃ and H₂O₂ was conducted [13, 14]. The MIC value for each compound

was calculated as well as the concentration which allows first fungal growth which is abbreviated as MGC (the concentration showing first growth after the MIC).

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Randomization experiment

Five experiments were conducted to map the best preparation conditions either during the cells cultivation or during the ghost cells preparation. The different variables were four chemical compounds representing SDS, H₂O₂, NaHCO₃, and NaOH, and three physical parameters represented as cultivation temperature, shaking rate during treatment, and the cultivation time. The seven variables were randomized according to the design in Table 1.

Table 1: The randomization experiments for ghost cells preparation including 7 variables in +1 or -1.

Experiment Number	NaOH	SDS	NaHCO ₃	H ₂ O ₂	Shaking rate during treatment	Cultivation time	Cultivation temperature
1	+1	-1	+1	+1	+1	-1	-1
2	+1	+1	+1	-1	-1	-1	-1
3	-1	+1	-1	+1	-1	-1	+1
4	+1	-1	-1	+1	-1	+1	-1
5	+1	-1	-1	-1	+1	-1	+1

Each variable of the four used chemical compounds was represented at two levels (high and low), which are denoted by +1 (MIC) and -1 (MGC) as in Table 1. The +1 in shaking conditions refers to shaking at 150 rpm during the different treatments while -1 refers to static incubation during treatment, +1 in growth time represents cultivation for 10 days while -1 represents cultivation for 5 days, +1 in growth temperature refers to cultivation at 28°C while -1 represents cultivation at 25°C.

The biomass of the different cultivation conditions was collected and washed gently by 0.5% saline and recentrifuged at 6000 rpm for 10 min. The supernatant was then discarded. 5X stock for each of NaOH, SDS, NaHCO₃ and 2X stock for H₂O₂ were prepared from both +1 (MIC) and -1 (MGC), which were determined as mentioned above.

All the experiments were conducted in three steps. The first step contains NaOH, SDS, NaHCO₃ where 1 ml from each was added to 1 ml water and finally 1 ml of the fungal suspension (0.5 gm of the fungus mat/ml) was added to get a final concentration of each of NaOH, SDS, NaHCO₃ equal to 1X. The second step contains H₂O₂. The H₂O₂ was used as 2X and one ml of the fungal suspension was added to reach final concentration of H₂O₂ equal to 1X.

After each treatment step, the supernatant was collected by centrifuging the fungus at 6000 rpm. After that, the fungal pellet was washed using 1X Phosphate Buffered Saline (PBS) (alternatively common saline solution can be used). In the third step, the cell pellets were washed using 60% Ethanol and left at room temperature. After each of the above washing steps and centrifugation the supernatant was preserved to determine the amount of the released protein and DNA.

Fungal Cells Evaluation Using Light Microscope

Fungal sample from each randomization experiment was examined by light microscope. The quality of the cells from each experiment has been determined based on the cellular structure as being either intact or deformed and then the overall fungal ghost quality is given as %.

Determination of the DNA Concentration

The concentration of DNA in the supernatant after each step for each randomization experiment was determined by measuring the absorbance at 260 nm. Quartz cuvette was used. An extinction 260 = 1 corresponds to 50 µg dsDNA mL⁻¹ [28].

Determination of the Protein Concentration

Concentration of released protein from each step in each randomization experiment (the different supernatants) was determined using the spectrophotometer at 280 nm. Quartz cuvette was used. The protein concentration was derived from Bovine Serum Albumin (BSA) standard curve (Figure 1) [15].

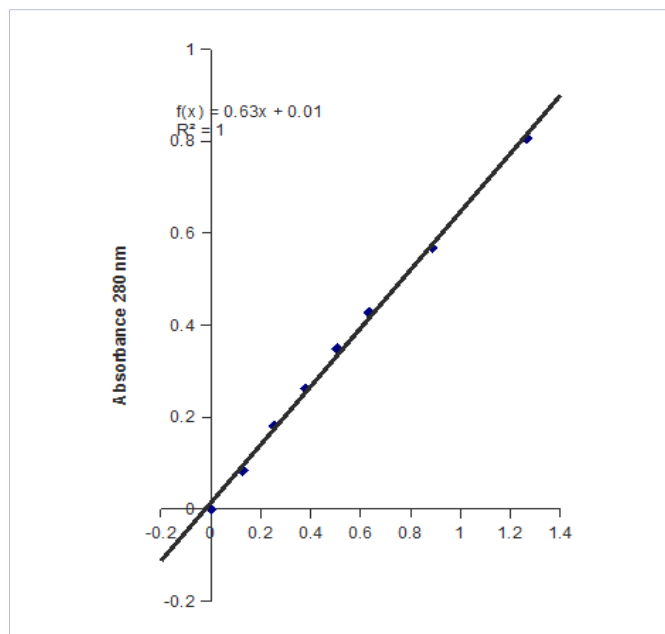


Figure 1: Bovine serum albumin protein standard curve

Determination of the Fungal Viability

The various fungal ghost preparations were investigated for the possibility of the presence of any viable cells by subjecting them to growth on Sabouraud's dextrose agar plates at 28°C for 7 days.

Transmission Electron Microscope for Examination of *A. niger* Gs

Transmission electron microscope (JEOL TEM 100 CX) was used for the examination of *A. niger* Gs.

Agarose Gel Electrophoresis

DNA in the supernatant for each randomization experiment was examined using agarose gel electrophoresis. 1% agarose gel containing 0.5 µg/mL ethidium bromide was run in a horizontal gel electrophoresis unit (Mini-Sub DNA cell, BioRad). The running buffer was TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). Electrophoresis was carried out at 100 V for 1 h on an Amersham-Pharmacia Biotech (Uppsala, Sweden) power supplier unit ECPS3000/150. The stained bands were visualized with UV light (309 nm) using a transilluminator, and the gel was recorded as digital image using a gel documentation system (UVI-Tech).

Polyacrylamide Gel Electrophoresis

Protein in the supernatant for each randomization experiment

was examined on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). About 50 µl of each sample were heated for 2-10 min at 100°C after the addition of 5 µl of 5× loading buffer. Spin down for 1 second to remove debris and subjected to SDS-PAGE with a 0.5-mm-thick gel in Invitrogen device Novex Minicell. Prestained marker heated at 95°C was used to calibrate protein mobility.

Results and Discussion

The production of microbial ghost cells is an aim for scientists as well as the isolation of DNA and protein. There are a large number of protocols for isolating and purifying DNA and protein. Each protocol is designed to match certain criteria and uses certain compounds in defined steps. Adding new idea concerning isolation of both DNA and protein will add new possibility and choices for different situations. The production of ghost cells is achieved by removing the cytoplasm from the microbial cells. The process is very sensitive but easy to conduct after determining both MIC and MGC. From the first step in the Sponge-like protocol, both DNA and protein were given special interest as parameters could be easily monitored spectrophotometrically and indicate microbial cells loss of them during cell cytoplasm loss. *A. niger* was used for first time to evaluate the Sponge-like protocol as a tool which is able to produce microbial ghosts and also could be adjusted to isolate DNA and protein.

As described earlier in the first protocol, the main feature that distinguishes this protocol from others was the determination of both MIC and MGC. An early work was done by introducing modifications in the plasmid alkaline lysis protocol via omitting the last step and adding the phenol extract step to turn it from plasmid isolation to DNA isolation protocol [29]. Such non-enzymatic DNA isolation protocol was used with different microbes and proves efficiency [29]. However, that study revealed variation in the prepared DNA quality as a fixed protocol was applied to different microbes that showed different responses to the used chemical compounds.

After improving the Sponge-Like protocol and proving its efficacy [15], it becomes clear that different microbes have different responses to the used chemicals and thus they show different MIC and MGC values. One of the benefits of this protocol that it can be used for strain differentiation rather than ghost cells preparation. Additionally, enzymes and proteins are able to introduce pores in the microbes when used at their MIC and MGC values [4, 22]. The white egg lysozyme was used to induce an emergence protocol for ghost cells preparation that could be used globally [22].

Our scientific group are working extensively to establish the Sponge-like protocol and to investigate the possibility of its use in DNA and protein preparation. This study is the first trial to prepare fungal ghost cells and investigate the possibility of DNA and protein isolation from fungal cells via Sponge-Like protocol. *A. niger* was selected as a representative fungus. The MIC and MGC values of different used chemicals were determined. NaOH, SDS, H₂O₂, and NaHCO₃ showed MIC values of 0.01g/mL, 0.0001g/mL, 0.3mL/mL, and 0.01g/mL, respectively, while gave

MGC values of 0.001 g/mL, 0.00001 g/mL, 0.15mL/mL, and 0.001 g/mL, respectively.

After determining MIC and MGC for such compounds against *A. niger*, five randomization experiments were conducted. Results of these randomization experiments are summarized in Table 2. Data from experiments number 2 and 3 in step one show high release for the protein due to the presence of SDS as +1 in both. In experiment number 3, the culture cultivation temperature was 28°C which might be responsible for the slight increase in the released protein than in experiment number 2.

In step two (H_2O_2) in experiment number 3, results still show high protein release and the same was in step 3. Experiments 4 and 5 in step one, there are no treatment differences but the amount of the released protein in experiment 4 is higher than experiment 5. The apparent explanation that in experiment 4, the cells are grown for a longer time (10 days) and for that they contain more protein. In experiments 4 and 5 in the second step still experiment number 4 has higher release to the protein for the same reason in addition to the high amount of the H_2O_2 might be responsible for the increase in the released protein. In experiment number one SDS was -1 for that it shows lower release to the protein than in experiments number 2 and 3, however and due to the presence of shaking, its release is continuous in step 2 and 3. Considering the effect of the shaking rate as in experiments number 1 and 5 where the SDS was -1, apparently it induces the release of the protein from cells in the successive steps. H_2O_2 apparently not highly effective in the protein release as it was in its +1 at experiments number 1, 3 and 4 but only experiment 3 shows the highest released protein due to the presence of the +1 SDS. Simultaneously, the effect of SDS on the released amount of DNA is clear in step 1 as experiments number 2 and 3 show the highest DNA release. The presence of NaOH and $NaHCO_3$ in +1, decreases the amount of the released DNA because they might cause degradation for it, that can be observed in step

1 at experiment 2 and 3. H_2O_2 in experiments number 2 and 5 shows the lower release which might be due to the degradation of the DNA where NaOH is present in its +1 which might have a synergistic effect with H_2O_2 to degrade DNA and in the absence of NaOH in experiment 3. The amount of the released DNA is more than the experiments number 2 and 5. So in the same experiment (No_3) step 2 where H_2O_2 is -1 shows decrease in the amount of the DNA than step 1 which proves that H_2O_2 is a DNA-degrader. The Ethanol step still show variable release to each of the protein and the DNA according to the pores formed and the protein and the DNA still existe.

In experiments 1 and 5 where the shaking was +1 it might have negative effect on the cells quality. That might be explained by the fact that cells upon their losing to their cytoplasmic content also loss their rigidity.

The different experiments show different ghost cells quality as presented in Table 2. One should put in his consideration that the representative images are taken after the third step for each experiment which represent the final preparation. The quality was given as a percentage and calculated based on 20% for the appearance of clear nuclei in the hypha, 20% for the correct 3D structure of the hypha, 20% for the absence of the spores in the tested sample, 20% for the absence of the sporangia, and 20% for the general appearance of the sample.

Figure 2 summarizes the results of light microscope examination to prepared fungal ghost cells after each experiment. The wild type shows clear sporangia with well-differentiated spores and hyphae. The prepared fungal ghost cells in the five experiments lost the ability to produce the sporangia including the spores. Hyphae elongation was evident. The loss of the nuclei was observed clearly but in variable degrees. The samples were shaken during ghost cells preparation showed a sort of hyphal intertwining and shrinkage (Figure 2 images d and h).

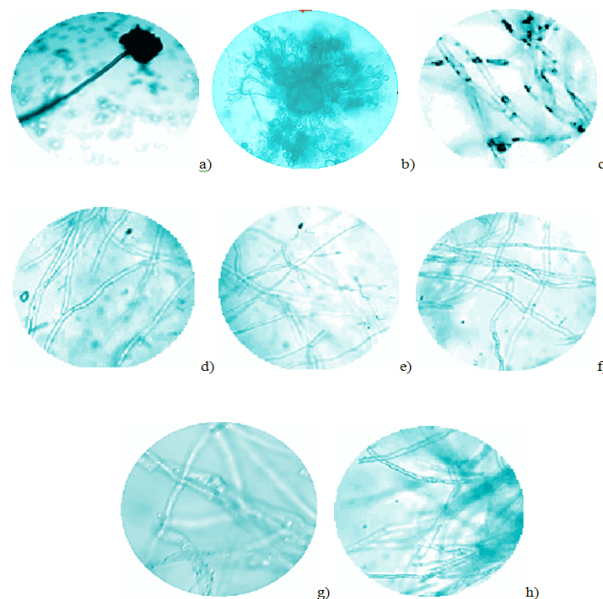


Figure 2: a) *A. niger* wild type untreated b) *A. niger* sporangia untreated c) *A. niger* hyphae untreated d) ghost cells from experiment 1 e) ghost cells from experiment 2 f) ghost cells from experiment 3 g) ghost cells from experiment 4 h) ghost cells from experiment 5

No growth was obtained on Sabouraud’s dextrose agar plates after ghost cells cultivation from any of the five experiments which prove the loss of the cytoplasmic content during the process and the death of the fungus. However, the hyphae elongation was not definitely known to occur after which of the three steps of ghost cells preparation.

In conclusion, this study succeeded in fungal cell evacuation and ghost cells preparation releasing both DNA and protein content.

The examination by transmission electron microscope results in a clear prove for the evacuation of the analyzed samples. By comparing experiment 1 with experiment 3 as in Figure 3, the image 3a from experiment number 1 magnification of the section of *A. niger* cells at 1200x represents less loss of *A. niger* cytoplasm while the image 3b in experiment number 3 magnification of the section of *A. niger* cells at 1200x the sample lost most of

its cytoplasmic content. This does not agree with the results in Table 1 where experiment 1 gives 70% quality while experiment 3 gives 60% quality. Even if they are both near in their quality % however, electron microscope gives better judgement. Image 3b shows better loss for the cytoplasmic content which proves the power of the Sponge-Like protocol in evacuating the microbes. Comparing image 3c which represents sample from experiment number 1 magnified at 10000x with image 3d which represents sample from experiment number 5 magnified at 10000x, the result agrees with that in Table 2 where the quality of the experiment 1 was 70% and of experiment 5 was 55%. The transmission electron microscope proves that the different preparation based on each experiment give different results, however, *A. niger* cells were turned to ghost cells. Some cells did not lose their cytoplasmic content however, they become nonviable as proved by the viability test. Those samples which show existence of the cytoplasm need more washing processes.

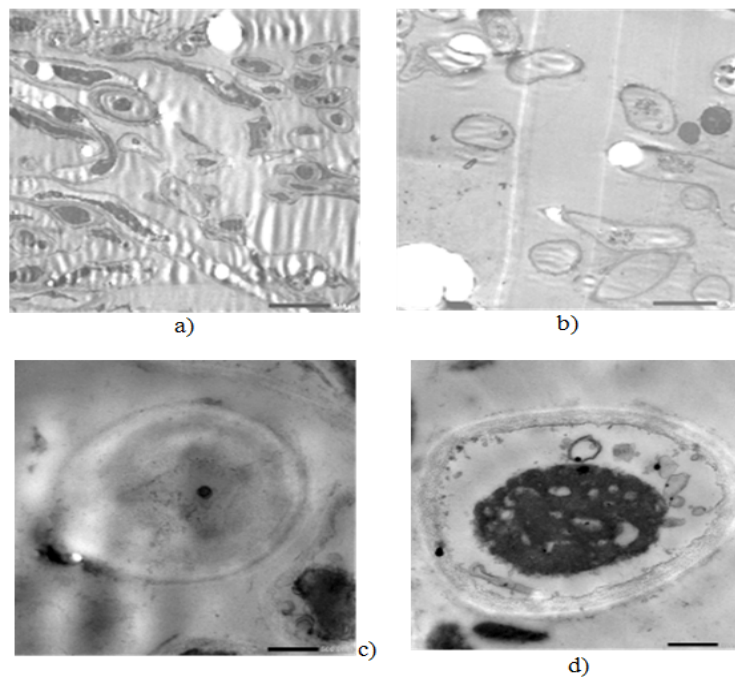


Figure 3: The results of transmission electron microscope for *A. niger* different treatments and magnifications: a) Experiment number 1 magnification of the section of *A. niger* cells at 1200x; b) Experiment number 3 magnification of the section of *A. niger* cells at 1200x; c) Experiment number 1 magnification of the section of *A. niger* cells at 10000x; d) Experiment number 5 magnification of the section of *A. niger* cells at 10000x

Table 2 : The results of randomization experiments for ghost cells preparation.

Experiment Number	NaOH/SDS/ NaHCO ₃		H ₂ O ₂		Ethanol (60%)		Cell quality %
	DNA (µg/ml)	Protein (mg/ml)	DNA (µg/ml)	Protein (mg/ml)	DNA (µg/ml)	Protein (mg/ml)	
1	4.25	0.092	17.1	0.181	2.15	0.29	70%
2	28.25	0.616	0.15	0.001	4.35	0.003	80%
3	48.5	0.752	31.45	0.416	1.9	0.138	60%
4	6.2	0.233	11.6	0.119	0.15	0.12	50%
5	3.1	0.061	0.15	0.024	0.3	0.126	55%

The SDS-PAGE analysis of the different samples supernatant prove the existence of protein in all supernatants of the five conducted experiments [Figure 4]. However, the samples represent the experiments end result which is another prove for the loss of the protein during the experiments steps. For obtaining better protein preparation, one should collect the protein of each step during each experiment and follow that with protein precipitation to concentrate the protein samples. The DNA agarose gel results which represent the end point of each of the five conducted experiments are the same [Figure 5, Figure 6]. The DNA from the samples were nearly removed. In addition, during the preparation steps more DNA in each sample is shown. The overall protocol succeeded to prepare *A. niger* as ghost cells and shows the first trial to prepare protein and DNA using the critical chemical concentration of the used compounds. One should not neglect the side effect of H_2O_2 on DNA which has been used in all of the experiments either with MIC or MGC but still effective. The data reveal more optimization is in need. The concept is clear, if MIC and MGC of the used chemical compounds are able to turn *A. niger* to ghost cells by inducing pores so the released DNA and cytoplasm can be collected. By improving such protocol, it will be a universal protocol for preparing both DNA and protein as well as ghost cells. In addition, other micro and macromolecules as well as different biological elements could be prepared.

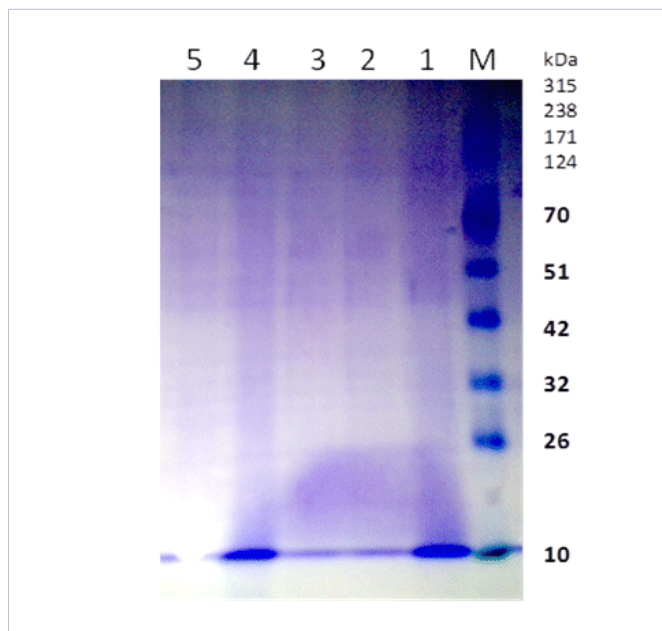


Figure 4: 12% SDS-PAGE analysis of different experiments for *A. niger* ghost cells preparation. Lanes 1-5 represent supernatant from experiments 1 to 5 respectively for *A. niger* ghost cells preparation. M, represent protein marker

Conflict of Interests

The authors have no conflict of interests to declare.

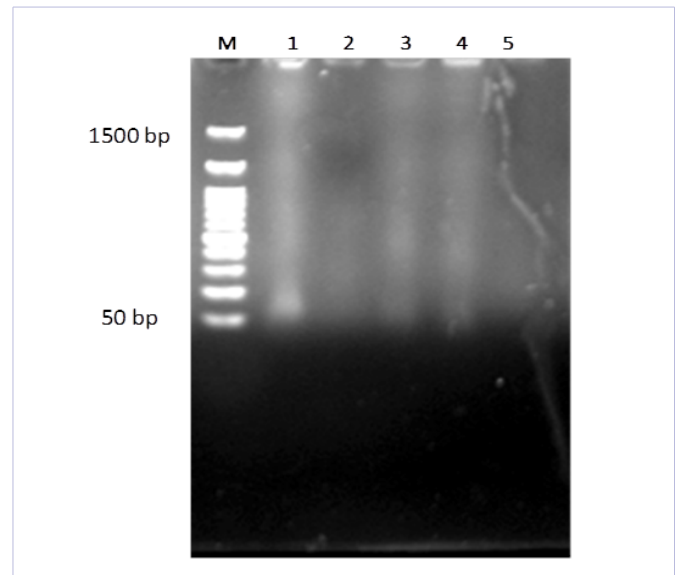


Figure 5: Agarose gel electrophoresis for *A. niger* ghost cells prepared in different experiments. DNA marker (lane M), *A. niger* during ghost preparation (lanes 1-5).

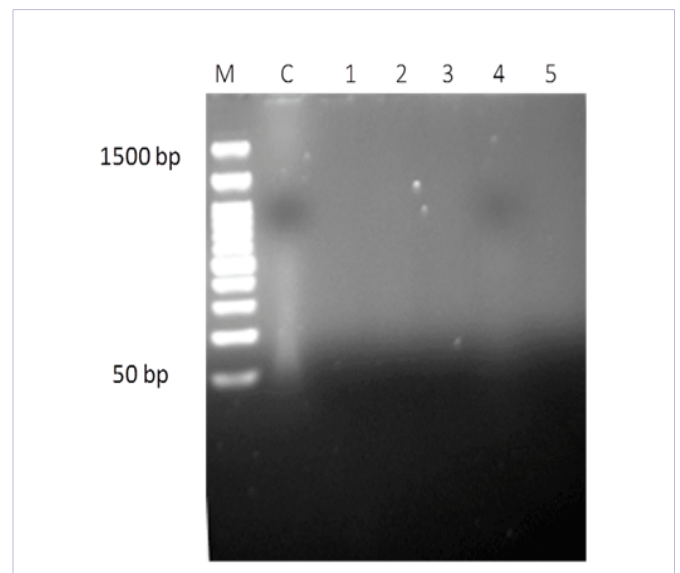


Figure 6: Agarose gel electrophoresis for *A. niger* ghost cells prepared at the end of different experiments. DNA marker (lane M); *A. niger* control (lane C); *A. niger* after ghost preparation (lanes 1-5).

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