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SKINETIC OF PRODUCTION OF EXOCELLULAR CATALASE BY *Aspergillus phoenicis K30* MUTANT ON DATE FLOUR EXTRACT AND FUNGUS MORPHOLOGY UNDER PARTIAL OXIDATIVE STRESS

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ABSTRACT

The specific productivity of extracellular catalase by *A. phoenicis K30* on DFSE at 5408.16 U/g cell dry weights at 48 h of fermentation. This production situated after the endocellular one. Involve that, the best total activity of enzyme (53.0 U/ml) was produced during exponential and stationary phases of fungus growth. The amount of the extracellular catalase activity was reflected by an increase in density and spherical form of pellets. To that effect, in the external layer, which forms the active zone of pellets, the ramified hyphal morphology has been showed to have an important influence on the formation of a range of extracellular catalase by our strain. The kinetic of fungus growth has revealed that, the stationary phase, in which, the amount of extracellular catalase was produced was remarkably short, followed by a rapid phase of decline. This is due, to our understanding, to the accumulation of mycelium on metal mechanism in the tank of bioreactor. As consequence, the width of submerged fermentation phase was considerably decreased, involved the oxidative stress, which affected directly the morphology of mycelium in submerged phase, characterised by the cell autolysis and lessening in the exocellular catalase activity.
Keywords: *Aspergillus phoenicis*, mutant strain, exocellular catalase, fungus morphology, bioreactor, oxidative stress, date flour.

INTRODUCTION

Filamentous fungi are attractive host organisms for the production of commercially useful proteins. This is mainly, due to their ability to secrete large amounts of proteins into their environment. The fungi *Aspergillus niger* group is one of the most important organisms for the industrial production of many enzymes (Berka *et al* ,1992 ; Verdos *et al* ,1995).

Catalases (H_2O_2 oxidoreductase, EC 1.11.1.6) which catalyse the decomposition of hydrogen peroxide to oxygen and water and physiologically they act as regulators of H_2O_2 levels in organelles (Ventaeshwaren *et al* ,1999) is the class of enzymes widely produced by this kind of micro-organisms, but, to our knowledge no information, about the production of this enzyme by *Aspergillus phoenicis* has been published. However, this species

taxonomically, belongs to the *Aspergillus niger* group (Belgian Coordinated Collections). Catalases are used in several industrial applications such as food or textile processing to remove hydrogen peroxide (Akertek and Tarhan,1995).

Catalases are naturally located in cytoplasm and peroxisoms and not usually excreted from the cell. Industrially, the extracellular liberation of enzyme is more advantageous than the extracting the intracellular one. However, there is little information regarding the regulation of catalase synthesis in *A. niger* mutants and the influence of physiological conditions on the excretion of this enzyme into the medium using a synthetic medium (Petruccioli *et al* ,1995;Gromada,1997).

Therefore, efforts have been undertaken to improve the production of extracellular catalase by mutants of *Aspergillus phoenicis K30* on date flour soluble extract submerged medium using a mutagenesis techniques (Kacem-chaouche *et al*,2004).

Production of extracellular catalases by filamentous fungi and their mutants in natural media such as date flour has not, to our knowledge, been investigated. The use of this date flour product as a fermentation medium presents an economical potential for countries producing it such as Algeria.

The Algerian phenicicole estimated today at more than 13 millions palm trees with an immense annual date production of around 420000 tons. Although this large date production is of different varieties, a large proportion of it is of the dry type which presents a down grade quality (Anonymous,2003). The dry date pulp contains about 80 % of the essential sugars such as sucrose, fructose and glucose (results not published). In addition to this considerable amount of sugars, it was proved as a good source of some important minerals. Therefore, the present study was undertaken to investigate the possibility of producing an extracellular catalase by *Aspergillus phoenicis K30* mutants in date flour soluble extract medium in different conditions, using a down grade quality of dry date.

MATERIALS AND METHODS

Strain:

The *A. phoenicis* wild-type strain was isolated from foodstuff, in the east of Algeria [4]. It was maintained as a spore suspension in 30 % glycerol physiological water at –20 °C. This strain, which produces a small amount of an exocellular catalase in solid medium and in shaken submerged culture, was used in mutagenesis experiments. The induction and selection of mutants of *Aspergillus phoenicis* which produces an amount of exocellular catalase has already been described (Kacem-chaouche *et al*,2004).

Date Flour Soluble Extract (DFSE)

To prepare the date flour soluble extract (DFSE), which forms the basal medium in this study, the pulp of a downgraded quality of dates (var. MECH-DEGLA) of *Phoenix dactylifera* (Produced in the south of Algeria), was dried for 48 h at 45° C. The dates were crushed into a powder and

suspended in distilled water. After about 3 h of agitation, the suspension was centrifuged for 1h at 4700 rpm. The supernatant was completed to 1000 ml to obtain the final concentration of the DFSE per litre.

Culture medium:

The culture medium was prepared as follow (per litre) : 40 g date flour (prepared as described above) ; 10 g corn steep ; 2 g $Ca(NO_3)_2\ 4H_2O$; 0.5 g KH_2PO_4 ; 0.5 g $MgSO_4\ 7H_2O$; 0.5 g KCl ; 0.001 g $FeSO_4\ 7H_2O$.

Fermentation processes:

Cultures were carried out in a 2-L stirred-thank bioreactor (Braun Biotech International GgmbH) filled with a working volume of 1.5 L culture medium sterilised at 121 °C for 30 min and inoculated with a final concentration of 1×10^6 spores/mL. The conidia were harvested from 7-days old culture on PDA medium. The bioreactor was equipped with a mechanical foam-breaking system (TEGO antifoam KS 911, Goldschmidt, Essen, Germany). The conditions of fermentation were pH 6, temperature 30° C, aeration 1 v/v/m, and agitation 200 rpm (using the 2 X six Rushton turbine impellers). All fermentation parameters were controlled by a Biolafitte regulation unit. The samples were taken every 6 h. The fungal mycelium was separated from the culture fluid by filtration on Whatman paper No.3 The filtrate was used for the determination of exocellular catalase activity and the mycelium was used for endocellular enzyme activity, fungi morphology and dry weight determination. The cultivations were carried out twice under the same conditions to verify the reproducibility of results.

Microscopy and morphological measurements:

To determine the morphology of tips in hyphal tree and its relation to extracellular catalase production, the mycelia were harvested and fixed using a solution containing: 13 mL of 40 % formaldehyde and 5 mL glacial acetic acid, added to 200 mL of 50 % (V/V) ethanol, and stained with lactophenol cotton blue(Packer and Thomas,1990). In different cases, the cell morphology was analysed with Zeiss Axioscop 2 MOt, fluorescence Microscope (AxiocamhRc,hallbergmoos, Germany). The microscopic analysis has also permitted the observation of pellet formation and determination of spore concentrations.

Analytical procedure:

The number of inoculum was determined by microscopy using a Burker counting chamber (0.100 X 0.0025 mm^2). The exo-and endocellular catalase activity was measured respectively in culture filtrates and cell free extract as described previously (Caridis *et al*,1991). The cell free extract was prepared by breaking down intermittently the washed mycelium, in 0.1 M phosphate buffer (pH 7.5) in ice-bath, using the ultra sonic vibrations (Sonnifier Main Components Model 250/450, VWR, Louven, Belgium) operating at 60 constant out put for 15 sec. The action was done in triplicate.

One unit (U) of catalase activity was defined as the amount of enzyme catalysing the decomposition of one µmol of H_2O_2 min^{-1} at 25° C and pH 7.5.

To determine the cell dry weight, the mycelia were harvested by filtration on Whatman paper No.3, and washed twice with distilled water, followed by drying to constant weight at 105° C.

RESULTS

Cell growth and exocellular catalase production:

Figure 1, represents the results of catalase production in 2-L bioreactor. The exocellular catalase began to be extracted into the medium after 18 h of growth and the best value of exocellular catalase activity (43.5 U/ml) was obtained at 48 h of culture. After this time, the activity of exocellular enzyme dropped progressively. However, the endocellular catalase was produced at 12 h of cultivation and the most important value (10 U/ml) was obtained at 36 h of fermentation. The endocellular catalase was produced during the exponential phase, in contrast to the exocellular catalase which was considerably, produced during stationary phase. So, the endocellular catalase was produced before exocellular one. Moreover, the best activity of total catalase occurred only during stationary phase of growth. As it can be seen in the (fig.1), the concentration of biomass began to increase around 12 h post-inoculation period and the maximum value (9.9 g/L) was obtained at 42 h of fermentation. From the results of biomass estimation, three phases of growth distinguished a development of fungus (fig.1). The kinetic revealed that at a stationary phase the biomass was doubled in comparison with the beginning of exponential phase (fig.1). However, the stationary phase was short in comparison with the others phases and the decline phase was dramatically rapid. Moreover, the figure 2 showed that the exocellular catalase production was correlated with the biomass evolution. The linear regression calculation has revealed that the index of global correlation R equal to 0.9531. Nevertheless, the correlation index, during lag phase was 0.9659 and that of stationary phase was 0.9068.

Mycelial morphology :

The microscopically analysis of mycelial morphology during 2 L-bioreactor cultivation revealed that at 6 h of fermentation the spore stage was prevailing (fig.3 A). The appearance of pellet form was observed around 20 h post-inoculation. However, at this time the pellets had a loose flocculent form, so the pellet formation was just at an early stage (fig.4 A). The observation of external layer hairy of this pellet, showed some septa and absence of ramification form in the apical elongations of hyphae (fig.3 B). After 20 h of growth, the flocculent mycelium became progressively more compact and dense (spherical pellets) accompanied with an increasing in size of pellet. In addition, the septa and ramifications in the apical elongations of hyphae were clearly apparent (fig. 4 B, seen also fig.3 C, D). This morphology increased progressively to attain much branched hyphae with more septum after 48 h of fermentation (fig.3 E). At the end of the fermentation, the pellets had a

relatively big size and a low density with increased size in the inner layer. In comparison with biomass kinetic and activity of enzyme, the exponential and stationary phases were represented by dense and compacts pellets. Moreover, the decline phase was represented by a relatively big size of pellets with low density accompanied by autolysis of cells in external layer. In addition, both of endo-and exocellular catalase was produced before the decline phase. In this fermentation, the best production of exocellular catalase by *A. phoenicis K30* mutant was directly correlated with ramified hyphae morphology in external layer of a dense and compact pellet. The stationary phase was short and the decline one was dramatically rapid. During this period, the accumulation of mycelium on metal mechanism of bioreactor was noticed. Consequently, the width of aerated layer submerged culture was decreased (fig. 5).

DISCUSSION

As described above (Kacem-chaouche *et al*,2004), the production of exocellular catalase by the strain *A. phoenicis K30* mutant on DFSE in shake-flask cultures was demonstrated. In this study the scale-up was investigated using the same medium. In 2-L bioreactor cultures, the increased production of exocellular catalase was preserved. This result proved that the selected strain is performing in large scale. This performance was also verified in 20 L bioreactor (Kacem-chaouche *et al*,2004). The specific productivity of extracellular catalase in this culture attained 5408.16 U/g cell dry weights at 48 h of fermentation. However, as noted before (Kacem-chaouche *et al*,2004)., the important production of enzyme was obtained later (72-96 h). This value turns to be less considerable in comparison with 500 ml shake-flasks and 20L-bioreactor cultures, but it was important regarding the short time of fermentation in which it was produced.

The inoculum size employed in this culture has allowed a pellet growth form of mycelium. The structural analysis of pellets during a performing step (until 48 h post-inoculation) revealed a compact and a dense morphology with a small hollow core at the end of this period. This morphology let us conclude that, the pellet was constituted in three layers; the inner layer (core) consists of a hollow zone with some dispersed hyphae, the external thin layer appeared more dense, constituted of a compact ramified mycelium, and the intermediate biggest layer which consists of a strong decrease in mycelial density. During the decline phase, the pellet morphology showed an increased size in the inner layer and a microscope observation of the mycelium in this region has revealed that the autolysis of mycelium was in advanced step. Thereafter, the global size of the pellets did not increase further. It is interesting to note that the increase in the cell dry weight with the course of culture time is not reflected in an increase of pellets size, but in an increase of pellets density. These observations are in agreement with others studies, which revealed that the medium employed maximum growth was soon followed by exponential decline due to autolysis. If however, conditions are such that pellet growth occurs, a logarithmic plot of

biomass against time shows that exponential growth soon comes to an end and a less rapid increase in biomass follows (Doohyn and Chang,1999;El-Enshasyh *et al.*,1999) The production of exocellular catalase was demonstrated and the kinetic of this production revealed that, this excretion of enzyme was situated after the production of the endocellular catalase. This situation may be explained by the accumulation of the enzyme as endocellular catalase in the first step, followed by the excretion of the enzyme as exocellular catalase in the next step. There have been many studies on the excretion of protein into the medium by filamentous fungius *A. niger* (Pluschkell *et al*,1996;Wosten *et al* 1991). The excretion of catalase during exponential and stationary phases of growth was probably caused by a specific process and not by cell lysis. This hypothesis was demonstrated for glucose oxidase (Pluschkell *et al*,1996), who revealed the presence of a signal peptide confirming that the glucose oxidase (GOD) is actively secreted in the culture medium. In contrast, there are only a few reports on the influence of environmental conditions on the excretion of catalase into the culture medium (Nishikawa *et al*,1993;Wongwicharn *et al*,1999).

Around 48 h of fermentation, the accumulation of mycelium on bioreactor mechanism was observed which, provoked the diminution of space liquid phase. This state has created two distinct phases in the tank of bioreactor; the first phase was solid due to the adherent hyphae to metal mechanism which were not counted in biomass measurements. The second phase was submerged medium which contained a mycelium as pellets. In this last phase, the initial rate of aeration was increased and as a consequence, the level of oxygen was also increased. The reduction in biomass concentration and reduction in enzyme activity may have been due to oxidative stress (Wongwicharn *et al*,1999). To confirm this hypothesis, in an other study, we have tested a level of aeration equal to 1.5 v/v/m in same bioreactor and conditions of culture. We have concluded that a high level of oxygen enrichment provoked a dispersion of mycelium, production of pigment, formation of adherent hyphae, and early autolysis of cell was detected. The literature consulted reveals that, mechanical agitation (stirring) helps to distribute dissolved air uniformly throughout the working volume. It also reduces the width of static layers of liquid that surround cells and through which oxygen must diffuse to reach the cell. Agitation facilitates also the passage of metabolic products from the cells to the medium. In addition, agitation will hinder the formation of cell clumps and mycelial aggregates which grow less well than free cells and individual hyphal filaments. But excessively high oxygen concentrations may prove toxic (Nykanen *et al.*,Agger *et al.*,1998) Beside, a further increase in impeller speed from 400-900 rev.min^{-1} caused a dramatic reduction in enzyme activity (Amanuallah *et al.*,1999) To conclude, the exocellular catalase production was related neither to the fungal biomass nor to the size of pellet. However, this production may be directly related to the external layer of the dense pellet region. It has been shown that the ramified morphological form of the hyphae has an important influence on the formation of a range of metabolites by the filamentous fungi (Wongwicharn *et al*,1999).

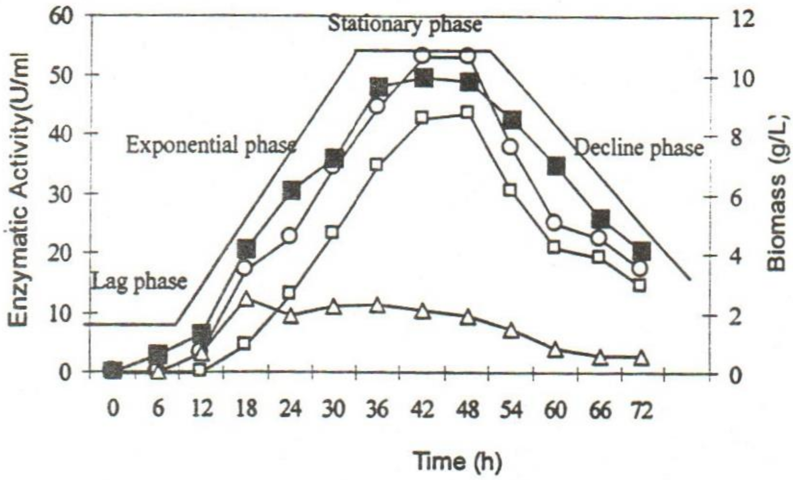

Figure 1: Catalase production during growth of *A. phoenicis K30* mutant on DFSE 2-L bioreactor, (-Δ-) endocellular catalase activity, (-□-) exocellular catalase activity (-○-) total catalase activity and (-■-) biomass.

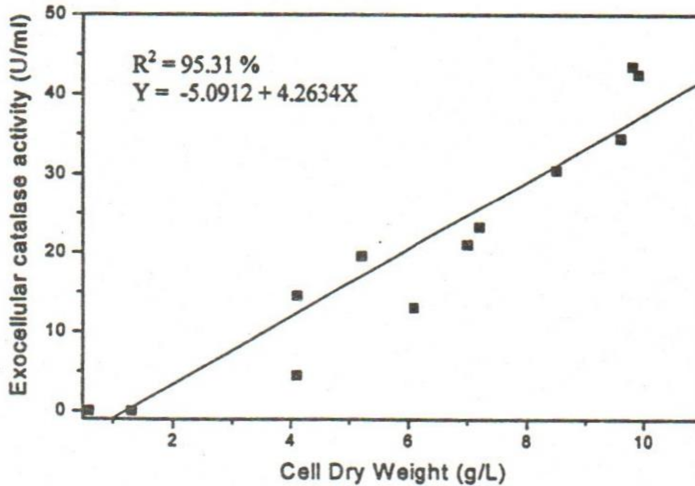

Figure 2: Correlation index of exocellular catalase activity and cell dry weight of *A. phoenicis K30* mutant on DFSE 2-L bioreactor.

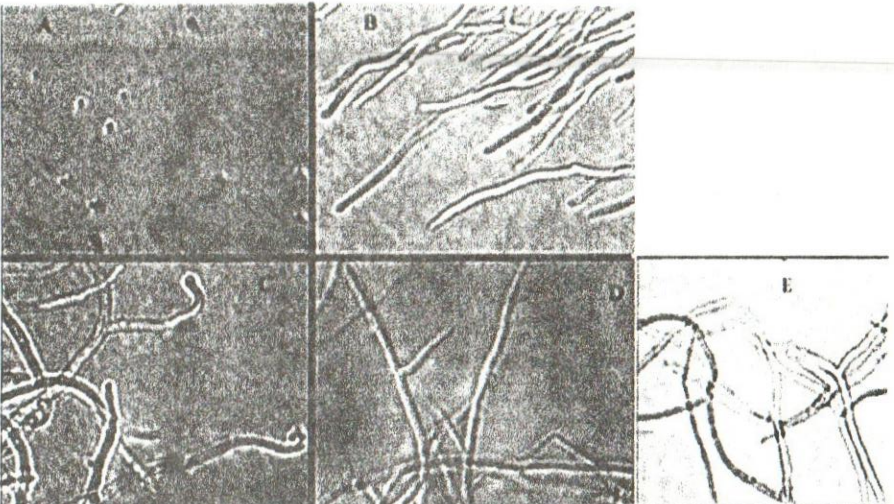

Figure 3: Simple microscopic analysis of mycelium morphology during batch cultivation of *A. phoenicis K30* mutant. Samples were taken at 6 h (spore stage) (A),24 h (B), 36 h (C), 48 h (D) and 72 h (E) post inoculation.

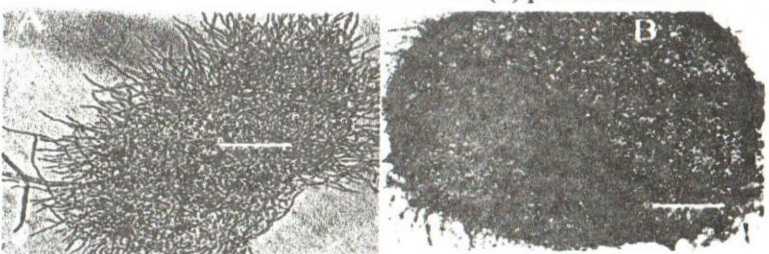

Figure 4: Microscopic observation of pellets during batch cultivation of *A. phoenicis K30* mutant Samples were taken at (A) 20 and (B) 48 h post inoculation. The bars represent 167 µm.

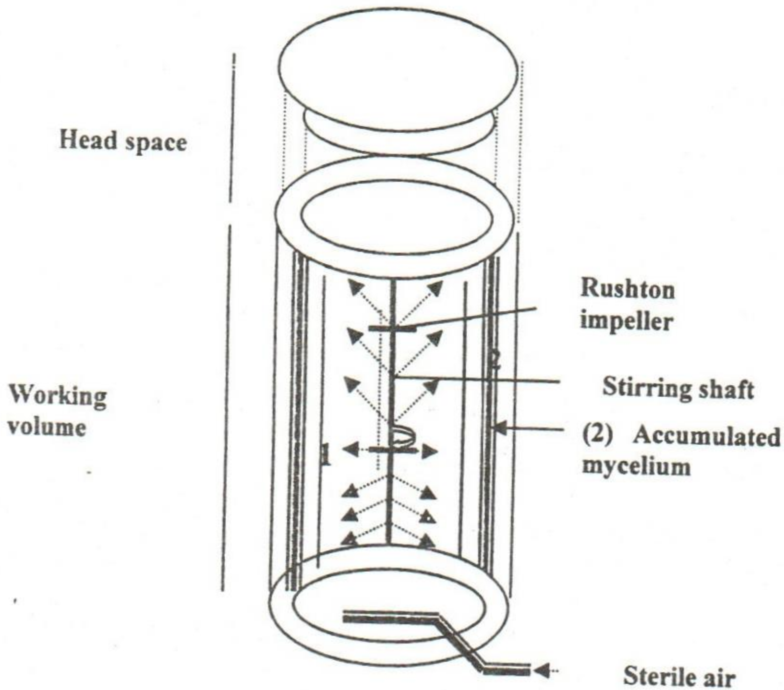

Figure 5: Diagram of a 2-L stirred bioreactor after 48 h of fermentation (1) submerged zone (hyper aerated) and (2) accumulation of mycelium on metal mechanism of bioreactor.

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الخواص الأنتاجية لأنزيم الكتاليز الخارجى المنتـج بواسـطة طفـرة مـن فطـر الأسبرجلس فونكس ك ٣٠ على مستخلص دقيق التمر والصفات الخارجية للفطـر تحت إجهاد جزئى للأكسدة .

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تم في هذه العمل دراسة إنتاج إنزيم الكاتالاز المفرز خـارج الخلايـا بواسـطة السـلالة الطـافرة *Aspergillus phoenicis K30* المنمى في مخمر ذو سعة ٢ لتر على بيئة تخمر أساسـها المسـتخلص الذائب لدقيق التمر. بلغت الإنتاجية النوعية للكاتالاز المفرز خارج الخلايا ٥٤٠٨,١٦ وحدة/ جرام مـن وزن الميسليوم الجاف بعد ٤٨ساعة من التخمر، علما أن الإنزيم يتكون داخل الخلايا. إن كمية الإنزيـم المفـرز خارج الخلايا خارج خلوي المنتجة، إرتبطت إرتباطا وثيقا بالشكل المتكور و المتماسك لميسليوم الفطر. أنتـج أكبر قدر من الكمية الإجمالية من الإنزيم خلال طوري النمو اللوغارتمي والثابت وبلغـت أقصاهـا (٥٣,٠ وحدة / مل) بعد ٤٨ ساعة من التخمر، أي عندما كانت كريات الفطر أكثر تكـورا وتماسـكا. إتضـح أن الطبقة الخارجية لكرات المسليوم هي النشطة في عملية إنتاج الإنزيم، و أظهرت الملاحظة المجهرية لمسليوم هذه المنطقة أن الشكل المتفرع منه هو الأكثر تأثيرا علىالإنتاج المتزايد للكاتلاز خارج خلوي. بتتبع حركيـة نمو الفطر خلال مراحل التخمر، تبين أن الكمية الكبيرة للإنزيم المنتجة المفرز خارج الخلايا سـجلت أثنـاء طوري النمو اللوغارتمي و الثبات، إلا أن هذا الأخير كان قصيرا و أعقبه طـور المـوت و الـذي كـان سريعا. ترجع هذه الظاهرة في إلى تراكم المسليوم على الأجزاء الحديدية داخل وعاء المخمر، وتشكل مـن جراء ذلك طورين للمسيليوم: طور شبه صلب على الأعمدة الحديدية محاذي لجدار الوعاء، و الأخر طـور سائل على شكل كريات صغيرة وهي المنتجة للإنزيم. هذا التراكم أدى إلى نقص قطـر منطقـة التخمـر، و تسبب ذلك بطريقة غير مباشرة في زيادة نسبة التهوية. هذا الإرتفاع في نسبة التهويـة أثـر سـلبا علـى مرفولوجيا المسليوم حيث بدأ ظهور التحلل الذاتي للخلايا الفطرية، مما أدى إلى نقص الكتلة الحيوية و كميـة الإنزيم معا بعد ٤٨ ساعة من وقت التخمر.