



Optimum Condition of Pullulanase Production by Liquid State and Solid State Fermentation (SSF) Method from *Bacillus Licheniformis* (BS18)

Amna Kahtan Khalaf*, Sanaa Borhan Aldeen

Department of Biology, College of science, University of Baghdad, Baghdad, Iraq

*amnabiology@yahoo.com

Abstract

Select 30 isolate from *Bacillus* to detect the ability to produce pullulanase enzyme in liquid and solid state fermentation, and use the isolate *Bacillus licheniformis* (Bs18) because the highest production of enzyme, the optimum condition for the production of enzyme by liquid state fermentation (LSF) in grown with: media contains starch + pullulan as a carbon source, peptone as a nitrogen source, inoculums size 2 ml, and incubated at 40 C° with pH 7 for 48 hrs. In addition pullulanase production by solid state fermentation (SSF) was investigated using isolated *Bacillus licheniformis* (Bs18). Optimization of process parameters were carried out ,the optimum solid substrate , Temperature , pH , incubation period , inoculation size , hydration solution and substrate to moisture ratio were found to be corn bran + rice bran (w/w) , 37°C , 6.0 , 48hr , 2ml , (0.2M) phosphate buffer , and 1:2 respectively. Calcium ions (2mM) enhanced enzyme activity by increasing its activity to 170%. Solid state fermentation had given higher production of pullulanase than liquid state fermentation (LSF).

Keywords: *Bacillus*, Pullulanase, Pullulan, Solid state fermentation (SSF), Liquid state fermentation (LSF), Optimum condition.

الطرق المثلى لانتاج انزيم Pullulanase بطريقة تخمرات المواد السائلة والصلبة من بكتريا *Bacillus licheniformis* (Bs 18)

امنة قحطان خلف* , سناء برهان الدين

قسم علوم الحياة, كلية العلوم, جامعة بغداد, بغداد, العراق

الخلاصة

اختيرت 30 عزلة من بكتريا *Bacillus* لكشف قدرتها على انتاج انزيم pullulanase في الاوساط الصلبة والسائلة وانتخبت العزلة (*Bs 18 Bacillus licheniformis*) لكونها الاكثر انتاجا للانزيم وتم تحديد الظروف المثلى لانتاج الانزيم من العزلة المنتخبة باستخدام نظام تخمرات الحالة السائلة ولوحظ ان اعلى انتاجية للانزيم تكون في: وسط حاوي على النشا + pullulan كمصدر كاربوني بالاضافة الى وجود البيبتون كمصدر نتروجيني وبحجم لفاح 2 مل والحضن بدرجة حرارة 40 م° برقم هيدروجيني 7 لمدة 48 ساعة. كما تم تحديد الظروف المثلى لانتاج الانزيم من العزلة المنتخبة باستخدام نظام تخمرات الحالة الصلبة حيث لوحظ ان اعلى انتاجية للانزيم تكون عند اضافة 2 مل من مزرعة البكتريا في وسط نخالة الذرة + نخالة الرز المرطب بدارئ الفوسفات بنسبة ترطيب 1:2 (w/v) وبرقم هيدروجيني 6 وحضنها بدرجة 37 م° لمدة 48 ساعة , كما لوحظ ان ايونات الكالسيوم بتركيز 2mM يؤدي الى زيادة فعالية الانزيم الى 170% , وكان انتاج الانزيم في وسط التخمر الصلب اعلى من انتاجه في الوسط السائل.

Introduction

Pullulan is a linear polymer of maltotriose, linked with α - (1 \rightarrow 6) glycosidic linkages and used in various industries [1]. Pullulanases a debranching enzyme (pullulan-6-glucanohydrolase [EC 3.2.1.41]) are classified as type I or II (amylopullulanase) depending on their ability to degrade α -d-(1 \rightarrow 4) glycosidic linkages in starch, amylopectin, and related oligosaccharides. Both pullulanase types I and II attack α -d-(1 \rightarrow 6) glycosidic linkages in pullulan, producing maltotriose. Unlike type II, pullulanase type I is unable to attack α -d-(1 \rightarrow 4) glycosidic linkages. All pullulanases known to date are unable to degrade cyclodextrins. In contrast, pullulan hydrolase type I (neopullulanase) and pullulan hydrolase type II (isopullulanase) are able to cleave α -d-(1 \rightarrow 4) glycosidic linkages in pullulan, releasing panose and isopanose, respectively, and are highly active on cyclodextrins [2]. Recently, pullulan hydrolase type III was described. This archeal enzyme attacks α -d-(1 \rightarrow 4) as well as α (1 \rightarrow 6) glycosidic linkages in pullulan forming maltotriose, panose and maltose [3]. Bioprocessing of starch into maltose and maltooligosaccharides by enzymatic means is gaining importance, because of their potential use in food, pharmaceutical, beverage and fine chemical industries [4, 5]. A high value is placed on thermostable and thermoactive, amylolytic enzymes in the bioprocessing of starch, since the bioprocessing of starch at elevated temperature improves the solubility of starch, decreases its viscosity, limits microbial contamination, reduces reaction times and becomes more economical. [6]. It is considered advantageous to have microorganisms that produce thermostable enzyme having properties of both amylase and pullulanase, because it cleaves both α -1, 4 and α -1,6 linkages, respectively [7]. Since, the bacterial systems are increasingly investigated for the production of enzymes and metabolites by solid-state fermentation (SSF) [8]. The SSF has numerous advantages over submerged fermentation (SmF), including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build-up [8]. Earlier, the SSF has been employed for the production of thermostable α -amylase by *Bacillus licheniformis* [9,10], no need for complex machinery and sophisticated control

systems, which could reduce the cost of downstream processing and subsequent waste treatment; usage of simple and cheap media for fermentation; and often a high product yield; lower risk of contamination due to the inability of most contaminants to grow in absence of free flowing water [11].

The purpose of the present study was to investigate the production of pullulanase by LSF and SSF condition by *B. licheniformis* (Bs18), strain which was isolated and identified.

Materials And Methods

Collection of Samples:

Twenty samples were collected from different sources of soil, each sample were collected in sterile containers and transported to the laboratory until usage.

Isolation of *Bacillus* spp.

One gm of each soil sample was added to 9 ml of sterile water and mixed vigorously, and then heated to 80 °C for 15 min in water bath. Serial dilutions were made for each sample by using sterile water. 0.1ml of each dilution was spreaded on a nutrient agar plates, and incubated aerobically at 37°C for 24 hrs. Bacterial isolated were obtained and identified as *Bacillus* spp. according to the morphological and microscopic examination.

Microscopic and Morphological Characteristics

The morphology, size, and shape of the bacterial isolate on nutrient agar plate were studied. a loopful of the bacterial suspension was mixed on a slide, and stained by Gram stain to examine Gram reaction, shape and spore forming

Determination of Pullulanase Production Semi-quantitative Method (12)

The activated bacterial isolates were plated on pullulan agar medium consisted of (w/v) 1% pullulan, 0.5% yeast extract, 0.03% K₂HPO₄, 0.02% MgSO₄.7H₂O, 0.1% (NH₄)₂SO₄, 0.02% CaCl₂.2H₂O, 0.001% FeSO₄.7H₂O, 0.0001 MnCl₂.4H₂O and 3.5% agar agar. The pH was adjusted to 6.2 and sterilized at 121°C for 10 min. pullulan as unique carbon source for the screening of hydrolyzing pullulan strains. The area of degradation of pullulan was observed by incubation the growth plates at 37°C for 24 h

followed by the plates were incubated at 37°C for 18 h before scraping the colonies from the agar surface and flooding twice with ethanol, in order to identify pullulan degradation area, which become apparent as a bright halo.

Quantitative Method

Two methods were used for production of pullulanase enzyme:

1. Liquid State Fermentation (LSF) (13)

The activated bacterial isolates (O.D =0.8 at 600 nm) were inoculated in liquid medium composed (g/l) 10 starch, 5 yeast extract, 0.3 K₂HPO₄, 0.2 MgSO₄·7H₂O, 1 (NH₄)₂SO₄, 0.2 CaCl₂·2H₂O, 0.01 FeSO₄·7H₂O and 0.001 MnCl₂·4H₂O. The pH was adjusted to 6.2, and incubated at 37°C for 48hr. the enzyme was extracted by centrifugation for 20 min, the enzyme activity, protein concentration were assayed in the supernatant (crude enzyme), and specific activity was measured.

2. Solid State Fermentation (SSF) (14)

Five grams of rice bran, corn bran, respectively, was hydrated with 1:1.5 (w/v) pepton. Yeast extract (PYE) medium containing (g/L): NH₄Cl, 1.0; MgCl₂·6H₂O, 0.2; KH₂PO₄, 0.3; NaHPO₄·7H₂O, 2.0; peptone, 10.0; yeast extract, 3.0; starch, 2; in Roasting bags, autoclaved at 121°C for 20 min, and inoculated with (2ml) of 24hr activated bacterial suspension (OD=0.8 at 600nm) and incubated at

37°C for 48hr. the enzyme was extracted by added (50ml) of tap water to the solid substrate culture and well mixed by agitation for (5 min), then filtered through a cloth, the filtered was centrifuged at (8000rpm) for (20min), the supernatant was used as crude enzyme, the activity, protein concentration, and specific activity were assayed.

Determination of Pullulanase Activity (15)

pullulanase activity was assayed by measuring the amount of reducing sugar released from pullulan. A reaction mixture (3 mL) composed of 0.5 mL pullulan (1% w/v) and 0.5 mL of crude enzyme source in 2 mL of sodium phosphate buffer (0.1 M, pH 6.5), and 0.5 ml CaCl₂ (0.02% w/v) was added to the reaction mixture. After incubation at 40 °C for 20 min, the reaction was stopped by cooling the tubes in an ice bath and reducing sugar released by enzymatic hydrolysis of pullulan was determined by addition of 1 mL of 3, 5-dinitrosalicylic acid reagent, then incubated in a boiling water bath for 5 minutes, and measured the enzyme activity at (540nm). One unit of pullulanase is defined as the amount of enzyme which released one micro mole of reducing sugars as glucose per min under standard assay conditions.

Protein concentration in the supernatant was determined by method described by Lowry *et al* [16].

Enzyme activity (U/ml) = O.D (540 nm) / (slope x volume of enzyme x incubation period)

Protein concentration (mg/ml) = O.D (600 nm) / (slope x 1000)

Calculation of specific activity

The specific activity of the enzyme was calculated as following

$$\text{Specific activity (U/mg protein)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

Determination of Optimum Conditions for Pullulanase Production by Liquid State Fermentation (LSF):

Effect of Incubation Temperature on Enzyme Production

LSF medium was inoculated with 2ml of activated bacterial suspension (OD=0.8 at 600 nm) and incubated at different temperature (37, 40, 45, 50, and 60°C) for 48 hr. the cells were precipitated by centrifugation at 8000rpm.

Supernatant were assayed for enzyme activity, protein concentration, and specific activity was measured.

Effect of Incubation Period on Pullulanase Production

LSF medium (10ml) was inoculated with 2ml of activated bacterial suspension (OD=0.8 at 600 nm) and incubated at 37°C for different times (24, 48, and 72hr). The cells were

precipitated by centrifugation at 8000rpm. Supernatant were assayed for enzyme activity, protein concentration, and specific activity was measured.

Effect of Initial pH on Enzyme Production

LSF medium (10ml) was prepared at different PH values (4-9) adjusted with 1N HCL or 1N NaoH. The medium was inoculated with 2ml of activated bacterial suspension (OD=0.8 at 600 nm) and incubated at 37C° for 48 hr. the cells were precipitated by centrifugation at 8000rpm. Supernatant were assayed for enzyme activity, protein concentration, and specific activity was measured

Effect of Inoculum Size on Pullulanase Production

LSF medium (10ml) was inoculated with (0.5, 1, 1.5, 2, and 2.5 ml) of 24hr activated bacterial suspension (OD=0.8 at 600 nm) and incubated at 37C° for 48 hr at PH 7. the cells were precipitated by centrifugation at 8000rpm. Supernatant were assayed for enzyme activity, protein concentration, and specific activity was measured.

Effect of carbon sources on pullulanase production

LSF medium (10ml) was inoculated with (2ml) of activated bacterial suspension (OD=0.8 at 600 nm), at different carbon sources (pullulan, starch, pullulan+starch, glucose, sucrose) and incubated at 37C° for 48 hr at PH 7. the cells were precipitated by centrifugation at 8000rpm. Supernatant were assayed for enzyme activity, protein concentration, and specific activity was measured.

Effect of Nitrogen Sources on Pullulanase Production

LSF medium (10ml) was inoculated with (2ml) of activated bacterial suspension (OD=0.8 at 600 nm), at different nitrogen sources (peptone, yeast extract, tryptone, gelatin) and incubated at 37C° for 48 hr at PH 7. the cells were precipitated by centrifugation at 8000rpm. Supernatant were assayed for enzyme activity, protein concentration, and specific activity was measured.

Determination of Optimum Conditions for Pullulanase Production by Solid State Fermentation

Effect of Various Complex Organic Solid Substrates on Enzyme Production

The isolate was grown in Roasting bags containing 10 g of each various solid substrates such as wheat bran , corn bran , rice bran , soya bean, and mixed two substrate to gather in the same time, coarse as well as fine types and rice bran, then hydrated with 1:1.5 phosphate buffer, sterilized, inoculated with 2ml of activated bacterial suspension (OD=0.8 at 600 nm) and incubated at 37 °C for 48 hr. the cells were precipitated by filtration . Supernatant was centrifuged at 8000rpm and enzyme activity, protein concentration, and specific activity was measured.

Effect of Incubation Temperature on Enzyme Production

The rice bran + corn bran as solid substrate was inoculated with 2ml of activated bacterial suspension (OD=0.8 at 600 nm) and incubated at different temperature 37, 45, 50, and 60°C for 48 hr. the cells were precipitated by filtration. Supernatant was centrifuged at 8000rpm and enzyme activity, protein concentration, and specific activity was measured.

Effect of Incubation Period on Pullulanase Production

The rice bran +corn bran as solid substrate was inoculated with 2ml of activated bacterial suspension (OD=0.8 at 600 nm) and incubated at 37C° for different times (24 , 48 , and 72hr) . The cells were precipitated by filtration. Supernatant was centrifuged at 8000rpm and enzyme activity, protein concentration, and specific activity was measured.

Effect of Initial pH on Enzyme Production

The rice bran + corn bran as solid substrate was prepared at different pH values (4-9) adjusted with 1N HCL or 1N NAOH. The medium was inoculated with 2ml of activated bacterial suspension (OD=0.8 at 600 nm) and incubated at 37C° for 48 hr. the cells were precipitated by filtration. Supernatant was centrifuged at 8000rpm and enzyme activity, protein concentration, and specific activity was measured.

Effect of Hydration Ratio on Enzyme Production

The rice bran + corn bran as solid substrate was moistened by varying the substrate to moisture Ratio (w/v) with 1:1.5, 1:2, and 1:2.5 of (0.02M) phosphate buffer (pH=6), the medium was inoculated with 2ml of activated bacterial suspension (OD=0.8 at 600 nm) and incubated at 37°C for 48 hr. the cells were precipitated by filtration. Supernatant was centrifuged at 8000rpm and enzyme activity, protein concentration, and specific activity was measured. protein concentration, and specific activity was measured.

Effect of Hydration Solution on Pullulanase Production

Different solution were used for production of pullulanase from the corn bran + rice bran as solid substrate including Tap water, phosphate buffer, and peptone yeast extract medium PYE, The medium was inoculated with 2ml of activated bacterial suspension (OD=0.8 at 600 nm) and incubated at 37°C for 48 hr. the cells were precipitated filtration. Supernatant was centrifuged at 8000rpm and enzyme activity, protein concentration, and specific activity was measured.

Effect of Inoculum Size on Pullulanase Production

The corn bran + rice bran medium was inoculated with 0.5, 1, 1.5, 2, and 2.5 ml of 24hr activated bacterial suspension (OD=0.8 at 600 nm) and incubated at 37°C for 48 hr. the cells

were precipitated filtration. Supernatant was centrifuged at 8000rpm and enzyme activity, protein concentration, and specific activity was measured.

Results and Discussion

Isolation and Identification of *Bacillus* spp. (17)

Thirty isolates of *Bacillus* were obtained from different sources of soil in Iraq. bacterial isolates were identified as *Bacillus* spp. according to the morphological and microscopic examination in which the growing colonies on the nutrient agar medium showed opaque with dull to rough surface, mounds and lobes consisting largely of slime often accumulate on colony. The growing colonies were purified by sub culturing on nutrient agar for many time until pure culture was obtained then stored at 4°C as stock culture and recultured every two weeks .

Screening for Pullulanase Producing *Bacillus*

Semi-quantitative Screening

Pullulan agar medium containing 1% pullulan as a carbon source was used for screening the pullulanase production. The results showed that 20 isolates from 30 isolates were able to produce pullulanase with different diameter of pullulan hydrolysis zones at 37°C incubation. The ratio of pullulan hydrolysis zones * (diameter of the clear zones/diameter of the colony) ranged between 0.1-5.5 , as shown in Table 1

Table 1- The ability of *Bacillus* isolates to produce pullulanase and their pullulan degradation on the pullulan agar medium after incubated at 37°C for 24hrs.

Code number of isolates	* Ratio of pullulan hydrolysis zone	Code number of isolates	Ratio of pullulan hydrolysis zone	Code number of isolates	Ratio of pullulan hydrolysis zone	Code number of isolates	Ratio of pullulan hydrolysis zone
BS1	0.1	BS6	1.8	BS11	3.4	BS16	0.3
BS2	0.3	BS7	0.3	BS12	0.8	BS17	2.5
BS3	0.1	BS8	3.2	BS13	3.8	BS18	5.5
BS4	2.1	BS9	2	BS14	2.5	BS19	3.7
BS5	0.6	BS10	1.6	BS15	0.9	BS20	0.1

The diameter and clearance of hydrolytic zone varied among *Bacillus* isolates according to genetic expression of pullulanase and species of *Bacillus*.

Bacillus is known as a producers of a wide range of useful extracellular enzymes such as protease , glucanase , amylase ,and pullulanase. [18].

Quantitative Screening

Five isolates (BS18, BS13, BS19, BS11, and BS8) which have largest pullulan hydrolysis zones were selected for quantitative screening of pullulanase production. The results showed that the activity of pullulanase produced by these isolates ranged between 10-18 U/mg proteins Table 2, these differences in the production of enzyme among isolates may be due to different sources of isolates or the variation in genes coded pullulanase synthesis

Table 2- The production of pullulanase quantitatively by *Bacillus* isolates after incubation at 37°C for 4hrs

Code of isolates	Specific activity(U/mg protein)
BS 18	18
BS 13	14
BS 19	16
BS 11	13
BS 8	10

According to the results indicated that *Bacillus* BS18 showed the highest specific activity for the production of pullulanase (18U/mg protein) with largest pullulan hydrolysis ability (5.5), thus it was selected for further study.

Identification of *Bacillus* BS18 Isolate

The strain BS18 which showed the highest pullulanase activity in the plate test was chosen for further analysis. The isolated BS18 strain was a spore forming, motile; rod shaped, aerobic, anaerobic growth, gram-positive bacterium. According to Bergey's Manual of determinative bacteriology [19], the isolates* BS18 belong to the genus *Bacillus*. The spores of this strain were ellipsoidal in shape and terminal in position, with a swollen sporangium. The strain grew at the pH of 4-9 and the temperature of 40–70 °C, was positive in the Voges–Proskaver test and produced catalase and H₂S. It produced acid from mannitol and glucose, reduced nitrate and utilized citrate but not propionate. BS18 hydrolyzed gelatin, which is the main diagnostic character of this strain with *Bacillus licheniformis*, Table 3.

Table 3- Morphological and Biochemical characteristics of the *Bacillus* BS18 isolate.

Characteristics	<i>Bacillus licheniformis</i>
Cell shape	Rod
Spore shape	Ellipsoidal
Spore site	Terminal
Motility	+
Gram stain	+
Catalase	+
Oxidase	+
Methyl Red	-
Voges – Proskauer	+
Starch hydrolysis	+
Gelatin liquefaction	+
Indol test	-
Citrate utilization	+
Anaerobic growth	+
Growth at 50°C	+
Carbohydrate fermentation	
glucose	+
Sucrose	+
Maltose	+
Fructose	+

+ = positive result. - = negative result.

Determination of Optimal Conditions for Pullulanase Production in Liquid State Fermentation (LSF)

The result Table 4 showed that the media containing starch and pullulan as carbon source

with peptone as nitrogen source then inoculated with 2ml of bacterial suspension and incubation at 40°C for 48hrs at pH 7 was the best media for pullulanase production with specific activity 20.6U/mg protein.

Table 4- Optimal conditions for pullulanase production by using liquid state fermentation medium

parameter	Optimum for production	enzyme activity (U/mg protein)
Carbon sources	Starch+ pullulan	18
Nitrogen sources	Peptone	18.4
Temperature	40C°	18.7
Inoculum size	2ml	18.9
Incubation time	48hr	19.5
pH	7	20.6

Studies provided that production of pullulanase can be enhanced by pullulan, maltose, and maltotriose [20].

Many bacteria and archaea, *B. sterothermophilus*, *Clostridium* sp., *Thermotoga* produce pullulanase utilizing starch and related carbohydrate [21]. It has high ability to produce pullulanase enzyme during growth the *Raoultella* on starch as compared to maltose [22].

The highest value of pullulanase activity was achieved in medium containing peptone thus indicated that peptone could be one of favorable organic nitrogen source that enhance the cell growth of the culture as well as the pullulanase production [22].

The optimum pH at enzyme pullulanase was 7 is comparable to those of pullulanase from other

bacteria, *B. sterothermophilus* [23], while the optimum condition with the production of glucoamylopullulanase from *Bacillus subtilis* showed at pH 9.5 [24], other worker were reported that a high level of pullulanase production observed at pH 5 from *Bacillus* sp. [1].

The highest value for glucoamylopullulanase production from *Bacillus subtilis* was 70°C [24], other results were reported for *Aspergillus niger* at 35°C [25].

Similar results were reported from *Bacillus* sp. in the production of amylase enzyme was 48h [26], other worker showed a high level of enzyme production from *Anoxybacillus flavithermus* was 72h [27].

Comparison between solid state fermentation SSF medium and liquid state fermentation LSF medium for the production of pullulanase enzyme



Figure 1- Pullulanase activity by solid state fermentation medium and liquid state fermentation medium

The use of LSF medium in the optimum condition and SSF medium composed of wheat bran as a substrate, hydration ratio 1:2(w/v), inoculated with 2ml, phosphate buffer as a hydration agent, and were incubated at 37°C for 48hrs at PH7. The results Figure 1 observed that SSF was more efficient in pullulanase production than LSF, the highest specific activity for enzyme (24 U /mg protein) by SSF, while LSF had the highest specific activity for enzyme (10.5 U /mg protein). Solid substrate fermentation (SSF) have been defined as the microbial transformation of biological materials

in their natural state, in contrast with liquid or submerged fermentation that is carried out in dilute solution or slurries [28]. The solid substrate in SSF provides a rich and complex source of nutrient that may be sufficient or sometimes in sufficient and in compete with respect to the overall nutritional requirement of that particular microorganism that is cultivated on that substrate [28]. SSF is preferred to LSF because of simple technique, low capital investment, lower levels of catabolic repression and end product inhibition, low waste output, better product recovery, and high quality production [29].

Optimal Conditions for Pullulanase Production by Using Solid State Fermentation medium

Effect of Various Complex Organic Solid Substrates

Different solid substrates were used as solid substrate media for pullulanase production from *B. licheniformis*. (BS18), the result showed in Figure 2 that the enzyme gave highest activity in media containing mixed of rice bran and corn bran as a substrate in SSF in mixed rice bran and corn bran, the specific activity was reached to (29 U/mg), while the media containing rice bran and soya bean gave the lowest specific activity (7U/mg), similar results were reported for *Anoxybacillus flavithermus* in the production of amylase was used of rice husk as the best substrate [27], Other worker showed a high level of enzyme production from *B.pumilus* by using wheat bran as a substrate [30,31]., The optimum condition for amylase production by *B. cereus* was observed at wheat bran + gingely oil cake gave the highest enzyme activity [29]. The nature of solid substrate is the most important factor in SSF. this not only supplies the nutrient to the culture but also serves as an anchorage for microbial cells. Therefore, the particle size and the chemical compositions of substrate are of critical importance [32].

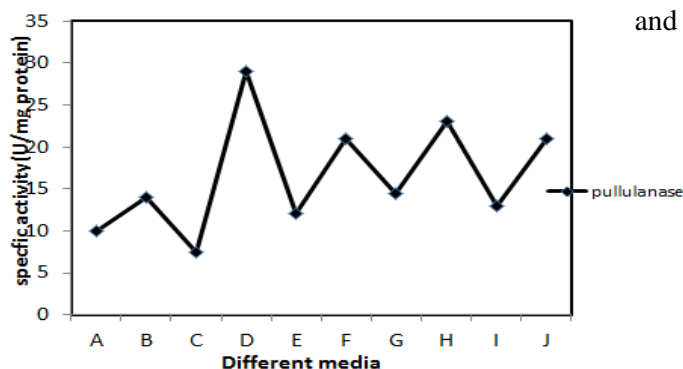


Figure 2-Effect of solid substrate on pullulanase production by *B.licheniformis*.BS18.in SSF after incubation at 37°C for 48 hr. A: rice bran ,B: rice bran + wheat bran , C: rice bran + soya bean, D: rice bran + corn bran, E: corn bran + soya bean , F corn bran + wheat bran , G:wheat bran + soya bean , H: wheat bran , I: soya bean, J: corn bran

Effect of Incubation Temperature

The result showed that the optimum temperature for enzyme production from *B. licheniformis*.(BS18) was 37°C, the specific activity was (33U/mg), then the production of enzyme was decreased with increasing incubation temperature to reach (20U/mg) at 60°C Figure 3 .

Similar results were reported for *Aspergillus niger* at 35°C [25], and for amylase production by *Bacillus* sp. at 37°C [26], other worker showed a high level of enzyme production from *Raoultella* was 30°C [22], the optimum condition for amylopullulanase production by *Clostridium thermosulfogenes* was observed at 60°C [31].

Temperature is one of the important factors affecting the growth and metabolism of Microorganism it influences the activity of the enzyme and solubility of the substrates, and hence the growth and enzyme production can be improved when the incubation temperature is suitable for the cell metabolism [33], Temperature control in the substrate bed is very important for SSF since growth and production of enzymes or metabolites are usually sensitive to temperature [34, 35]. Large number of weak non covalent bonds maintains the tertiary structure of an enzyme, when it absorbs too much energy the tertiary structure will disrupt and the enzyme will be denaturated [36].

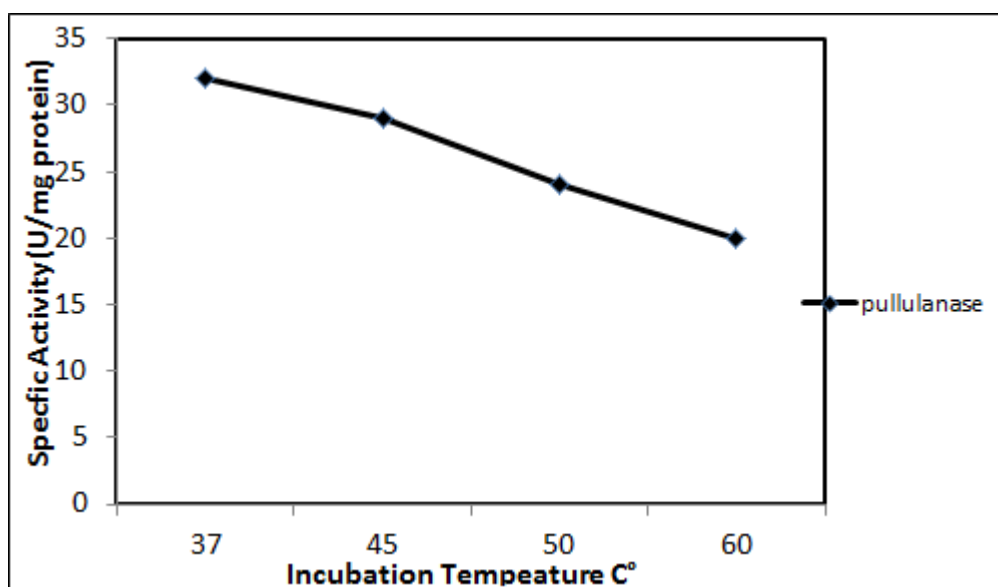


Figure 3- Effect of incubation temperature on pullulanase production by *B. licheniformis*.BS18. Under solid state fermentation, incubated at different temperatures for 48 hr.

Effect on Incubation Time

The results observed that the higher specific activity for pullulanase production was (33U/mg) from *B. licheniformis*.(BS18) appeared after 48h, and decreased gradually with increasing of incubation time to reach (26U/mg) at 72h, Figure 4. Similar results were reported from *Bacillus* sp. in the production of amylase enzyme was 48h [26], other worker showed a high level of enzyme production from *Anoxybacillus flavithermus* was 72h [27], while the optimum condition for the tannase production was observed at 96h [25]. The production of enzyme by using the SSF technique requires less fermentation time, 24–48 h [37]. During the time course study enzyme activity was detected in the culture supernatant from 24 h to 72 h,

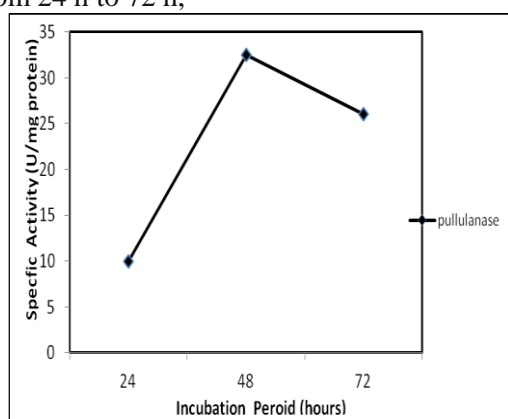


Figure 4- Effect of incubation time on pullulanase production by *B. licheniformis*.BS18. Under solidstate fermentation incubated at 37C° for different times (24, 48, and 72hr).

The reason for this might have been due to the depletion of the nutrients or denaturation of the enzyme caused by the interaction with other components in the medium or a change in the pH of the medium [38].

Effect of pH on Pullulanase Production

The optimum pH for production enzyme from *B. licheniformis* .(BS18) showed at 6, the specific activity was 32U/mg , then the activity was decreased with increased pH value

Figure 5 ,Similar results were worked for *Bacillus* sp. [26], and for *Anoxybacillus flavithermus* [27], while other worker were reported the optimum condition in the production of amylase for *Bacillus cereus* at pH 7 [29].

pH can influence the growth and product formation due to its effect on the solubility of nutrient, ionization of the substrate and its availability to the microorganism [28] , also the acidic or basic media may lead to denaturation of the enzyme [39], An attempt to overcome the problem of pH variability during the SSF process is obtained by the substrate formulation considering the buffering capacity of the different components employed or by the use of buffer formulation with components that have no deleterious Influence on the biological activity [28].

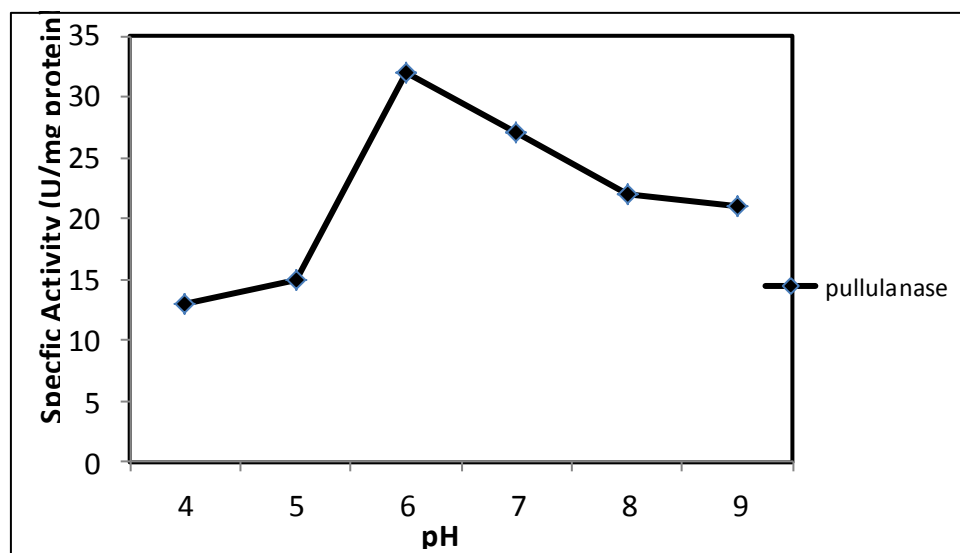


Figure 5- Effect of initial pH on pullulanase production by *B. licheniformis*.BS18. Under solid state fermentation incubated at 37°C for 48 h at different PHS (4-9).

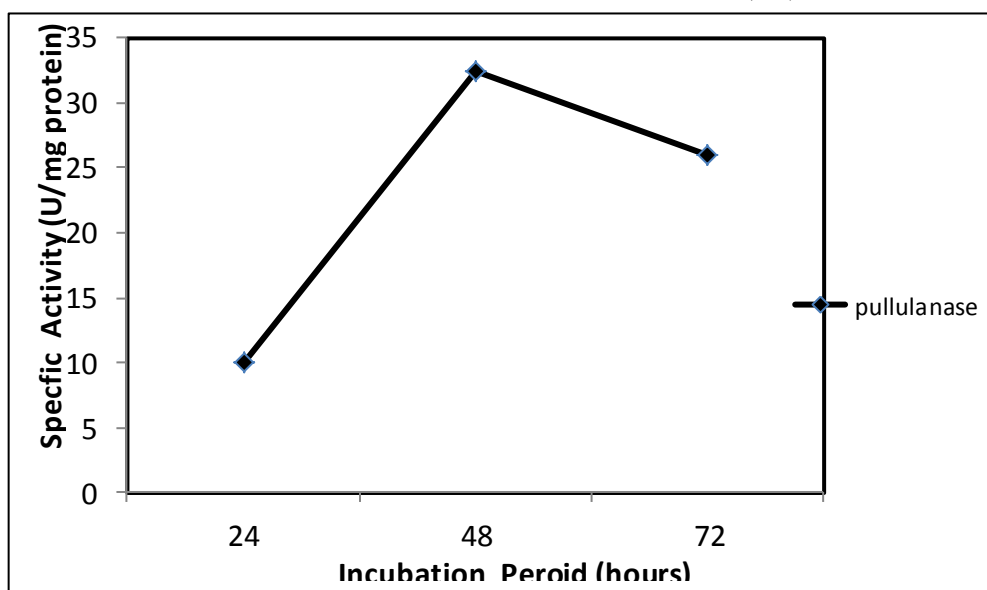


Figure 6- Effect of hydration ratio on pullulanase production by *B. licheniformis*.BS18. Under solid state fermentation incubated at 37°C for 48 h at different ratios(1:1.5,1:2,1:2.5)(w/v)

Effect of Hydration Ratio

The result showed that the optimum hydration ratio for pullulanase production from *B. licheniformis*. (BS18) was 1:2 (w/v), the specific activity reached 32U/mg, while it decreased to 25U/mg, when the hydration ratio was increased to 1:2.5 (w/v) Figure 6. Worker provided the same results in the production of amylopullulanase by *Clostridium thermosulforegenes* was 1:2.25 (w/v) [31], other worker showed that 1:3 ratio (w/v) of substrate to moistening agent is the best for the production of xylanase by *Bacillus pumilus* [30], while the optimum condition for the production

of amylase by *Bacillus* sp. was at ratio of 1:1.5 (w/v) [26].

the critical importance of moisture level in SSF media and its influence on the biosynthesis of enzyme has been attributed to the interference of moisture in the physical properties of solid particles, higher moisture level decreases porosity, limitation of oxygen transfer and addition to that the substrate become more compact at high water content [32]. The moisture content is an important factor that influences the growth and product yield in SSF [9]. Moisture is reported to cause swelling of the

substrates, thereby facilitating better utilization of the substrate by microorganisms [40].

Effect of Hydrating Solution

Different hydration solution was used; the result showed that phosphate buffer (0.2M at pH 6.0) gave highest pullulanase activity than PYE and tap water Figure 7. The specific activity was 32U/mg, 25U/mg, 20U/mg respectively, Tap water or phosphate buffer are the common moistening agents used by various workers. Worker provided that results were showed a high level of enzyme production from *Bacillus cereus* was distill water as a hydration solution [29], while the optimum condition for xylanase

production was high in the using of tap water [30]. The nature of the moistening agent and the level of moisture content in the fermentation medium are the other main factors in SSF which often determine the success of a process [32], however, the enzyme yield from medium hydrated by phosphate buffer was 20 ml of the maximum yield. Phosphate buffer was most suitable for large scale production of enzyme. Therefore, phosphate buffer was used as hydration agents for further research in the present study [26].

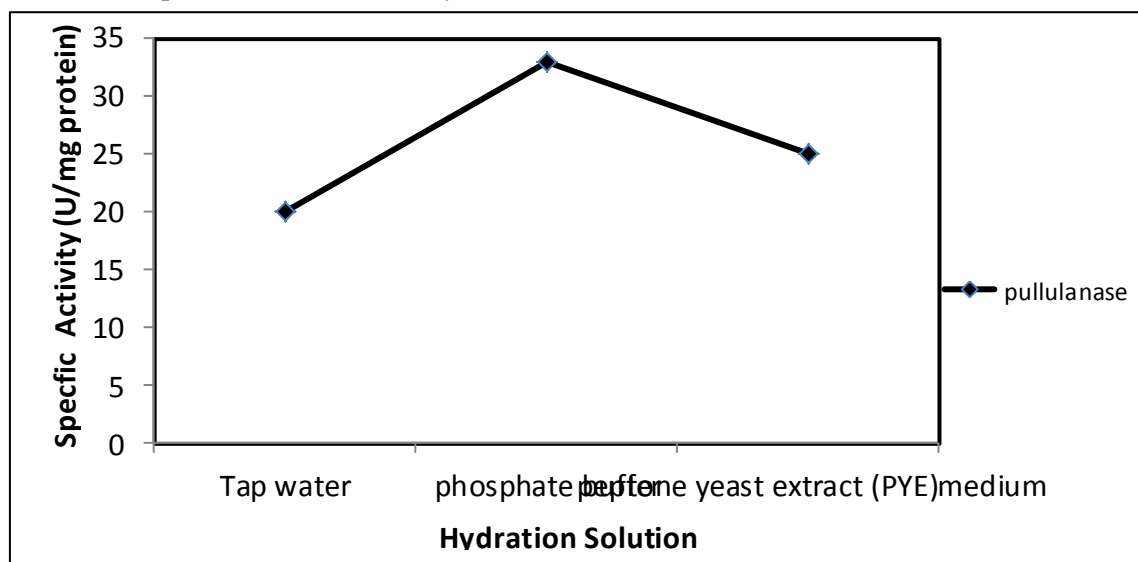


Figure 7- Effect of hydration solution on pullulanase production by *B. licheniformis*. BS18. by solid state fermentation incubated at 37°C for 48 h at different hydration solutions.

Effect of Inoculum size on enzyme production

The result showed that the optimum inoculum size for pullulanase production from *B. licheniformis*. (BS18) was (2 ml), the specific activity reached 31 U/mg, while it decreased to 20U/mg, when the inoculum size was increased to (2.5 ml) Figure 7. Similar results were worked that the optimum condition in the production of amylopullulanase at inoculum size 2ml [8], other worker showed that the maximum enzyme yield at 1.5ml for the production of enzyme [35], while other results observed that the maximum enzyme production was obtained with 4ml of inoculation size by

Anoxybacillus flavithermus sp. [27]. The less enzyme production at lower inoculum level (<2 ml) might be because less number of viable cells in the production medium require more time to grow to an optimum number to utilize the nutrients in substrate and for enzyme production [41]. However, less enzyme production at higher inoculum level (>2 ml) may be due to decreased nutrient availability for the large number of viable cells, or rapid accumulation of toxic metabolites [42].

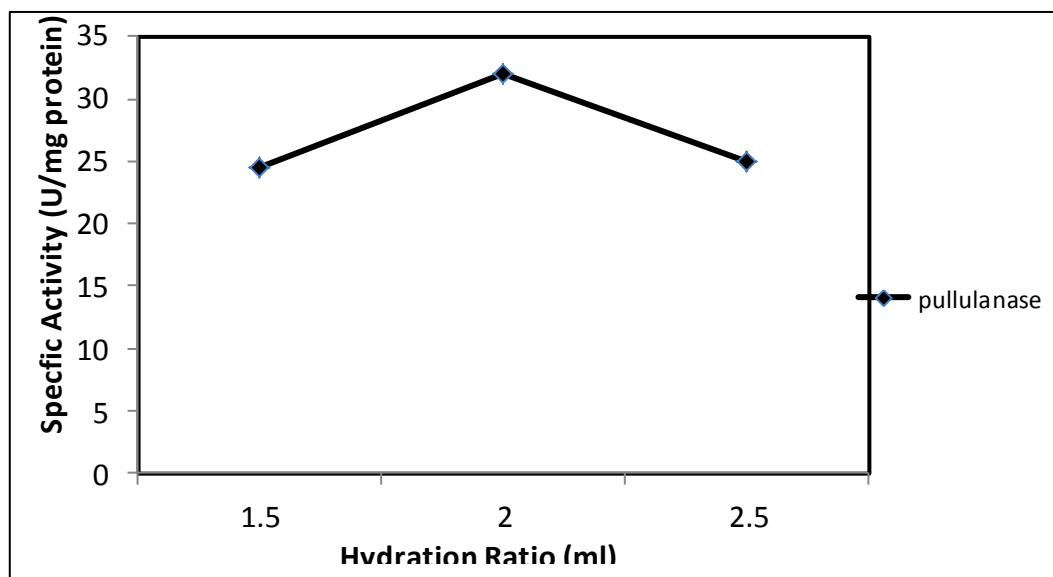


Figure 8- Effect of inoculum size on enzyme production by *B. licheniformis*. BS18. Under solid state fermentation incubated at 37°C for 48 h at different inoculum sizes .

Conclusion

Pullulanase, produced by *B. licheniformis*. BS18, was capable to attack specifically the α -1,6 linkages in pullulan to generate maltotriose as the major end product, as well as the α -1,4 linkages in amylopectin and soluble starch leading to the formation of a mixture of maltose and glucose. *B. licheniformis*. BS18 is an efficient local isolate for pullulanase production, the study of the optimum conditions of solid state fermentation SSF medium, liquid state fermentation medium and comparison between them, indicated that the use of SSF gave higher production of enzyme than LSF, SSF technique is a suitable and economic method for pullulanase production, the enzyme is active at a wide range of pHs (4-9) and temperature (37-60°C), showed its potential for usage in industrial application.

References

- Roy A, Ben Messaoud E, Bejar S. **2003**. Isolation and purification of an acidic pullulanase type II from newly isolated *Bacillus* sp. US149. *Enzyme Microb Technol* ;**33**:720-4.
- Doma nacute-Pytka M, Bardowski J. **2004**. Pullulan degrading enzymes of bacterial origin. *Crit Rev Microbiol* ;**30**:107-21.
- Duffner F, Bertoldo C, Andersen JT, Wagner K, Antranikian G. **2000**. A new thermoactive pullulanase from *Desulfurococcus mucosus*: cloning, sequencing, purification, and characterization of the recombinant enzyme after expression in *Bacillus subtilis*. *J Bacteriol* ;**182**:6331-8.
- Fogarty, W. M. and Kelly, C. T. **1990**. Recent advances in microbial amylases. *In: Microbial enzymes and Biotechnology*. Fogarty, W. M. and Kelly, C. T. (eds.). Elsevier Applied Science Publishers, London. 71- 132.
- Saha, B. C., Jordan, D. B. and Bothast, R. H. **2009**. Enzymes, Industrial (overview). *Encyclopedia of Microbiology*. *In: Schaechter, M. (ed.)*. Elsevier, Oxford. pp. 281-294.,
- Brown, S. H. and Kelly, R. M. **1993**. Characterization of amylolytic enzymes, having both α -1,4 and α -1,6 hydrolytic activity, from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis*. *Applied and Environmental Microbiology* **59**, 2614-2621.
- Nagar, S., Anuradha, M, Davender, K, Lalit K, Ramesh C, and Vijay, K.G. **2011**. Hyper production of alkali stable xylanase in lesser duration by *Bacillus pumilus* SV-85S using wheat bran under solid state fermentation. *Elsevier, New Biotechnology*, volume 28, Number 6:581-587.
- Mrudula, S.1, Gopal Reddy 2 and Seenayya, G. **2011**. Effect of substrate and culture conditions on the production of amylase and pullulanase by thermophilic

- Clostridium thermosulfuregenes* SVM17 in solid state fermentation. Malaysian Journal of Microbiology, Vol 7[1] 2011, pp. 19-25
9. Paranthaman, R., R. Vidyalakshmi and K. Alagusundaram **2009**. Production on Tannin Acyl Hydrolase from Pulse Milling By-Products Using Solid State Fermentation. Academic Journal of Plant Sciences **2** [3]: 124-127.
 10. Özdemir, S., Fatma, M., Veysi, O., Abdurrahman, D., Mehmet, S.U., Mert, Kumru. **2011**. Isolation of a thermophilic *Anoxybacillus flavithermus* sp. nov. and production of thermostable α -amylase under solid-state fermentation (SSF). Ann Microbiol DOI 10.1007/s13213-011-0385-4.
 11. Segel, I. **1976**. Biochemical Calculations. John Wiley and Sons. USA.
 12. Sodhi, H. K., Sharma, K., Gupta, J. K. and Soni, S. K. **2005**. Production of a thermostable α -amylase from *Bacillus* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. Process Biochemistry **40**, 525- 534.
 13. Ling, H. S., Tau, C. L., Rosfarizan, M., Arbakariya B. A., **2009**. Characterization of Pullulanase Type II from *Bacillus cereus* H1.5. American Journal of Biochemistry and Biotechnology **5** [4]: 170-179, 2009 ISSN 1553-3468.
 14. Swamy, M. V. and Seenayya, G. **1996**. Thermostable pullulanase and α -amylase from *Clostridium thermosulfurogenes* SV9: Optimization of culture conditions for enzymes production. Process Biochemistry **31**, 157-162.
 - JZarein, S., Khajeh, K., Ranjbar, B., Dabimanesh, B., Ghollasi, M. and Mollania, N. **2010**. Purification and characterization of a novel amylopullulanase that converts pullulan to glucose, maltose, and maltotriose and starch to glucose and maltose. Enzyme and Microbial Technology **46**, 57-63.
 16. Archana, A.; Satyanarayana, T. **1997**. Xylanase production by thermophilic *Bacillus licheniformis* A99 in solid-state fermentation. Enzyme. Microb. Technol. **21**:12±17
 17. Nicholson, W.L. **2002**. Roles of *Bacillus* Endospores in the Environment. Cell. Mol. Life Sci. **59**: 410-416.
 18. Ramesh, M.V.; Lonsane, B.K., **1990**. Critical importance of moisture content of the medium in alpha-amylase production by *Bacillus licheniformis* M27 in a solid-state fermentation system. Appl. Microbiol. Biotechnol. **33** : 501±505
 19. Ramesh, M.V.; Lonsane, B.K.. **1991**. Ability of a solid-state fermentation technique to significantly minimize catabolite repression of α -amylase production by *Bacillus licheniformis* M27. Appl. Microbiol. Biotechnol. **35**: 591±593
 20. Gessesse, A. and Mamo, G. **1999**. A novel xylanase production by an alkaliphilic *Bacillus* sp. by using solid-state fermentation. Enzyme and Microbial Technology. **25**, 68-72.
 21. Miller, G.L.: **1959**. Use of dinitrosalicylic acid reagent for determinations of reducing sugars. Anal. Chem. **31** 426±428.
 22. Lowry, O. H.; Rosenbough, N.J.; Farr, A. L. and Randall, R.J. (**1951**). Protein Measurement with Folin-Phenol Reagent. J. Biol. Chem. Vol. **193**: 265-275.
 23. Sivaramakrishnan, S.; Gangadharan, D.; Nampoothiri, K. M.; Soccol, C. R. and Pandey, A. **2006**. α -Amylases from Microbial Sources – An Overview on Recent Developments. Food Technol. Biotechnol. **44** [2]:173–184.
 24. Bergey DH, Holt JG. **1994**. Bergey's manual of determinative bacteriology. Baltimore: Williams&Wilkins;p.xviii,787 p.
 25. Lonsane BK, Ghildyal NP, Budiattman S, Ramakrishna SV **1985**. Engineering aspects of solid state fermentation. Enzyme Microb Technol ;**7**:258–65.
 26. Bull, A. and Bushnell, M. **1976**. Environmental Control of Fungal Growth. In: The Filamentous Fungi. Edited by (Smith, J. and D. Berry) Vol. [2]. 1-26. Edward Arnold, London, U.K.
 27. Babu KR, Satyanarayana T. **1995**. Amylase production by thermophilic *Bacillus coagulans* in solid state fermentation. Process Biochem;**30**:305–309.
 28. Kotwal SM, Gote MM, Sainkar SR, Khan MI, Khire JM. **1998**. Production of β -galactosidase by thermophilic fungus *Humicola* sp. in solid state fermentation and its application in soyamilk hydrolysis. Process Biochem ;**33**:337–43.
 29. Mahanta N, Gupta A, Khare SK **2008** Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa* PseA in solid-state fermentation using Jatropa

- curcas seed cake as substrate. *Bioresour Technol* **99**:1729–1735.
30. Pandey A, Soccol CR, Rodriguez-Leon JA, Nigam P. **2001**. Factors that influence on solid state fermentation. In: Pandey A, editor. *Solid state fermentation in biotechnology: fundamentals and applications*. New Delhi: Asiatech Publishers Inc.; p. 21–9.
 31. Mckee, T. and Mckee, J. **1996**. Enzymes. In: *Biochemistry*: 118-148. Wm.c. Brown publisher. United state .
 32. Kim, J. H., Hosobuchi, M., Kishimoto, M., Seki, T. and Ryu, D. D. Y. **1985**. Cellulase production by a solid state culture system. *Biotechnology and Bioengineering* **27**, 1445-1450.
 33. Kashyap, P., A. Sabu, A. Pandey, G. Szakas and C. Soccol. **2002**. Extracellular L glutaminase production by *Zygosaccharomyce rouxii* under solid state fermentation. *Process Biochem.*, **38**: 307-312.
 34. Zhang, H., Y. Wu and Y. He. **2002** . Effects of inoculum size and age on biomass growth and paclitaxel production of elicitor treated *Taxus yunnanensis* cell cultures. *Appl. Microbiol. Biotechnol.*, **60**: 396-402.
 35. Mrudula, S., R. Kokila. **2010** . Production of thermos table a-amylase by *Bacillus cereus* MK in solid state fermentation :partial purification and characterization of the enzyme. *The Internet journal of Microbiology* .volume 8 Number 1.
 36. Bibel, M., Brettl., C., Gossler, U., Kriegshäuser, G. and Liebl, W. **1998**. Isolation and Analysis of Genes for Amylolytic Enzymes of the Hyperthermophilic bacterium *Thermotoga maritima*. *FEMS Microbiology Letters* **158**: 9-15.
 37. Amitava R, Ben Messaoud, Bejar S. **1987**. Isolation and purification of an acidic pullulanase type II from newly isolated *Bacillus* sp. US149. *J Jpn Soc Starch Sci* ;**34**:38–44.
 38. Brandt CJ, Catley BJ, Awad WM Jr. **1976**. Extracellular and protease released pullulanase . *J Bacteriol* , **125**:501-8.
 39. Bertoldo, C., Antranikian, G., **2002**. Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. *Curr. Opinion Chem. Biol.* **6**, 151–160.
 40. Hii, S., Ling., Tau, C., Ling., Rosfarizan, M., and Arbakariya, B.A. **2009**. Enhancement of extracellular pullulanase production by *Raoultella planticola* DSMZ 4617 using optimized medium based on sago starch. *The open biotechnology Journal*, **3**, 1-8.
 41. Wind, R.D., Buitelaar, R.M., Eggink, G., Huizing, H.J., Dijkhuizen, L., **1994**. Characterization of a new *Bacillus stearothermophilus* isolate: a highly thermostable a-amylase producing strain. *Appl. Microbiol. Biotechnol.* **41**, 155–162.
 42. Asoodeh, A., Lagzian, M., **2012**. Purification and characterization of a new glucoamylopullulanase from thermotolerant alkaliphilic *Bacillus subtilis* DR8806 of a hot mineral spring. *Process Biochemistry* **47**, 806–815.