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# ISOLATION AND CULTURE OF *ACINETOBACTER BAUMANNII*

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## ABSTRACT

The present work deals with physiological studies on bacterial L-asparaginase. It includes studies on the productivity of L-asparaginase by different *Acinetobacter baumannii* is isolated and cell immobilization. Six isolates under shaken condition were investigated for their abilities to produce L-asparaginase enzyme from them *Acinetobacter baumannii* S4 isolated from sputum, samples gave the highest production level of enzyme. The maximum activity ( 46.70 µ /ml) of L-asparaginase was obtained using M9 medium supplemented with 0.5% soluble starch as carbon source and 0.5% L-asparaginase as nitrogen source , with initial pH 8 , inoculated with 2 ml of 26 h old culture and incubated at 37 ºC for 26 h . Cell immobilization was recorded by physical immobilization and sawdust was the most favorable for the production of the highest L-asparaginase activity 41.2 µ / ml. Entrapment immobilization was the most suitable with sodium alginate by which L-asparaginase activity reached 34.9 µ /ml. Wood shaves as carriers 21 × $10^{6}$ bacterium / ml and efficiency loading of 88.6 µ / ml . One gram of wood shaves produced 88.8 µ / ml while contious L-asparaginase production by recycling immobilized cells could be achieved till 10 cycles with maximum enhancement of 140.2 % after 4 cycles.

**Key words:** *Acinetobacter baumannii*, L-asparaginase, bacterial enzymes.

## 1. INTRODUCTION

*Acinetobacter baumannii* can be an opportunistic pathogen in humans, affecting people with compromised immune systems and is becoming increasingly important as a hospital derived infection (nosocomial) (Ahmed et al., 2012). The predominant site of colonization, in hospitalized patients is the skin, but respiratory tract or digestive systems may also be colonized (Bergogne and Tower, 1996). It has been isolated from soil and water samples, also

it isolated in low numbers from fresh fruit and vegetables (Fornier et al., 2013). *Acinetobacter baumannii* has been noted for its apparent ability to survive on artificial surfaces for an extended period of time therefore allowing it to persist in the hospital environment; this is thought to be due to its ability to form biofilms (Ahwany and Youssef, 2007).

*Acinetobacter baumannii* infections were acquired through hospitalization, while only 4 % of infections were obtained from setting outside the hospital (Fournies and Richet, 2013). Most of the *Acinetobacter baumannii* infections resulted in pneumonia, urinary tract, blood stream and surgical wound infections (Henwood et at., 2002; APIC, 2010).

L-asparaginase catalyzes the hydrolysis of L- asparagine into L- asparagic acid and ammonia as the several types of tumor cells require (Fornier et al., 2013). L- asparagine is an essential amino acid for protein synthesis, they are dervied of an essential growth factor in the presence of L-asparaginase, (Shah et al., 2010) . It has received increased attention in recent years for its anticarcinogenic potential. The clinical action of this enzyme is attributed to the reduction of L-asparagine, since tumor cells unable to synthesize this amino acid are selectively killed by L-asparagine deprivation (Theantana et al., 2007). The L-asparaginase is present in many animal tissues, bacteria, plants and in the serum of certain rodents but not in mankind, whereas microbial L-asparaginase has attracted considerable attention (El-Bessoumy et al., 2004; Siddalingeshwara and Lingappa, 2011). Although this enzyme is produced by a large number of microorganisms such as *Enterobacter* , *Aerobacter* , *Bacillus* , *Erwinia* , *Pseudomonas* , *Serratia* , *Xanthomonas* , *Photobacterium* , *Vibrio* and *Proteus* (El-Bessoumy et al., 2004; Ebrahiminezhad and Amini, 2011). Also wide range of fungi, yeast, Actionomycetes and algae are efficient producer of L- asparaginase, but the enzymes isolated from E. coli and *Erwinia carobovora* are now being used in the treatment of acute lymphoblastic leukemia. However, due to the prolonged and administration of L-asparaginase, the corresponding antibodies are produced in man, which causes an anaphylactic shock or neutralization of the drug effect (Ebrahiminezhad and Amini, 2011; Ahmad et al., 2012). Therefore there is a continuing need to screen newer organisms in order to obtain strains capable of producing new and high yield of L-asparaginase. The recovery and reusability of free cells as catalysts are not convenient, so that immobilization of microbial cells was attempted for solving such problem. Moreover, cells immobilization was considered helpful for stabilizing the whole – cell biocatalyst (Chen et al., 2004).

The aim of this study is to optimize medium conditions for increasing the activity of L-asparaginase in free cell cultures of *Acinetobacter baumannii*, then to compare free and immobilized cell cultures for enzyme production.

## 2. MATERIAL AND METHODS

### 2.1. Sample collection

The study included a total of 80 samples of which 40 were from sputum and 40 were from blood collected from patients at different hospitals in Baghdad city.

### 2.2. Bacteriological analysis

The collected microbial sources were transported to the laboratory. Samples were plated primarily on to blood agar and MacConkey agar which was incubated at 37 ºC for 48 h. the bacterial isolates were observed for morphological characters and identified by using the tests guided by Berge's Manual of systemic bacteriology. Suspicious isolates were presumptively identified by using colony morphology, negative oxidase test, positive catalase test inability to motile coccobacilli (Constantinu et al., 2004). Further, the *Acinetobacter baumannii* .Isolate was confirmed by using API20NE biochemical kit according to the manufacturer's instruction.

### 2.3. Screening for L-asparaginase production

#### 2.3.1. Quantitative analysis

All bacterial isolates were evaluated for their ability to produce L-asparaginase by growing on agar based M9 medium composition for 1 L. ( 6.0g $Na_2HPO_4.2H_2O$ ; 3.0g $KH_2PO_4$ ; 0.5g NaCL ; 2.0g L-asparagine , 0.5g $MgSO_4.7H_2O$; 0.014g $CaCl_2.2H_2O$;2.0 lactose (w/v) and 20g agar ) pH= 7.0 supplemented with phenol red (few drops )as an indicator . The inoculated agar plates were incubated at 37 c in an incubator .Pink zone radius and colony diameter was measured from positive isolates after incubation for 24 h. (Prakasham et al., 2010).

#### 2.3.2. Quantitative analysis

The selected bacterial isolates were grown in M9 medium without addition of agar and incubated on the rotary shaker at 150 rpm at 37 c for 24 h. After removal of cells by centrifugation at 8000 xg for 25 min., the clear supernatant was used as the crude extracellular enzyme source and the amount of L-asparaginase produced was assayed.

### 2.4. Enzyme assay

The enzyme was assayed by direct nesslerization method according to the method of () as follows :0.1 ml enzyme preparation was added to 1.7 ml of 0.5 M Tris – HCl buffer (pH 7.5) and the reaction was initialed by adding 0.2 ml of 0.1 M L-asparagine for 30 min. at 37 ºC . Reaction was stopped by the addition of 0.5 ml of 1.5 M trichloro-acetic acid and precipitated protein was removed by centrifugation (8000 rpm) at 4 ºC. The reaction mixture was diluted to 6.5 ml with deionized water and 0.5 ml of nessler reagent was added , and kept for 16-18 min. at 20 ºC before estimation of ammonia by determinating the OD. At 500 nm. Standard curve was prepared with ammonium sulfate. On L-asparaginase unit (IU) is defined as that amount of enzyme, which liberates 1 µM of ammonia / min. under the optimal assay conditions.

### 2.5. Estimation of protein content

The protein content of the enzyme was determined by using Bradford dye method with BSA as a standard (Bradford, 1976).

### 2.6. Optimization of the culture condition for L-asparaginase production

Various process parameters that enhance the yield of L-asparaginase by *Acinetobacter baumannii under* submerged state fermentation were investigated the impact of incubation time (18-130) h. , incubation temperature (25-55) ºC , initial pH (4-11 adjusted with 1N HCl or 1 N NaOH ) , inoculum age (18-30) h. inoculums concentration ( 0.5-3.5) ml were evaluated . In addition , the effect of additional carbon sources (glucose , maltose , sucrose , galactose , mannose , lactose and soluble starch at 0.5 % (w/v) , nitrogen sources (L-asparagine , ammonium nitrate , ammonium sulfate , yeast extract , malt extract , urea , peptone , tryptone and sodium nitrate at 0.5 % (w/v) were studied (Suresh and Raju, 2012) .

### 2.7. Production of L-asparaginase by immobilized cells

#### 2.7.1. Cell immobilized by adsorption method

Batch adsorption studies were carried out by adding 1 ml of prepared inoculums of bacterial cells to 1g of different supports, namely chitin, pectin, synthetic sponge, sawdust, and then transferred to fresh culture media; the resulted matrixes were inoculated to 50 ml of culture medium portions. After incubation at 37ºC for L-asparaginase activity was assayed.

### 2.7.2. Cell immobilization by entrapment

A sterile 20 ml syringe with a wide bore needle (2 mm diameter) was used to add the alginate – bacterial cell mixture drop wise into 3% $CaCl_2$ solution for cross – linkage beads of approximately 2mm in diameter were immediately formed in the solution. Alginate beads entrapping bacterial cells were washed with sterile distilled water and suspended in fresh culture medium on a rotary shakers 160 rpm / min. at 37 ºC and L-asparaginase activity was assayed by agarose or alginate .Entrapment was performed using 3% concentration of agar. The liquid culture was centrifuge for 10 min. at 6000 xg and the supernatant was discarded. The pellet was resuspended with previously autoclaved solution of 3% sodium alginate.

Entrapment agar or agarose was performed by dissolving 3 g agar or agarose in 80 ml water , sterilization of the solution , and adding about 20 ml cell suspension with mixing 10ml of this mixture were aseptically poured into petri-dish. After solidification, the gel was cut with a sterile cutter into small cubes to be transferred into culture medium (El-Ahwany and Youssef, 2007) .After incubation.

### 2.7.3. Optimization of some conditions for immobilization on wood waste

To investigatethe influence of support size on the L-asparaginase activity , 1ml of a clerical isolate was mixed with different forms of wood waste such as wood chippes , shavings and sawdust .The resulted matrixes were inoculated to 50ml of culture medium portions after incubation.

### 2.7.4. Effect of Biomass load (Efficiency load)

1.0g of wood shaves was mixed with different bacterial concentrations (3,12.5,21, 33, 46.5)×**10** bacterium ml/g wood shaves .The resulted matrix with different bacterium concentrations were used for the inoculation of 50ml culture medium portions .After incubation.

### 2.7.5. Effect of matrix concentration

In this experiment different concentrations of wood (0.5, 1, 1.5, 2, 2.5, 3) g/50ml culture medium were used for the immobilization process. In all cases, equal amounts of inoculum size were used .After incubation, L-asparaginase activity was measured.

### 2.7.6. Repeated batch process for production of L-asparaginase by immobilized cells

The present experiment was carried out to investigate the production of L-asparaginase by the immobilized cells in repeated batch process for 10ml.

## RESULTS AND DISCUSSION

Out of 80 samples collected, 6 (7.5%) *Acinetobacter baumannii was isolated.* Among which 4/6 (67%) were from blood , 2/6(33%) were from blood .The high isolation rate of *Acinetobacter baumannii* isolates from sputum is consisted with their association with lower respiratory tract infections (Humpherys & Towner,1997). A small majority of the isolates belonging to other groups were recovered from blood, indicating that they also cause serious infections, albeit less frequently than isolates of the *Acinetobacter baumannii*, however, this association with blood could also reflect the fact than non- *Acinetobacter baumannii* isolates are ubiquitous members of the normal human skin flora and are therefore prone to cause contamination of blood cultures (Bergogne & Tower, 1996).

*Acinetobacter spp*. isolates are mostly implicated in various nosocomial infections like respiratory tract infections, blood stream infections, wound infections and urinary tract infections (Wayne, 2006). In study done (Bhattncharyya *et al.,* 2013) , *Acinetobacter spp*. constituted 16.9% of all nosocomial bacterial isolates .Infection is facilitated by the ability of the bacterium to colonise hospital equipment and to persist on animate surfaces for prolonged periods of time ranging from 3 days to 5 months , and *Acinetobacter* spp. can be detected on various equipments including bedrails , curtains , ventilation equipments (Wayne,2006) . *Acinetobacter baumannii* was the most commonly found species in isolates from clinical sources, followed by *Acinetobacter* species (Bouret *et al.,* 1990). In addition (Brock *et al.,* 2009) found that *Acinetobacter baumannii* was most frequently isolated from sputum samples (48%) and wound samples (19%) , while *Acinetobacter lwoffui* was mainly isolatated from blood samples or intravascular lines (42%).

### Assay for asparaginase

### 1-Qualitative analysis

All *Acinetobacter baumannii* isolates could grow on L-asparaginase agar with phenol red, a dye indicator that change from yellow (acidic condition) to pink (alkaline condition). The pink zones around Acinetobacter baumannii colony indicate the pH alteration which originated from ammonia accumulation in the medium. For agar plate assay, all 6

*Acinetobacter baumannii* isolates gave positive test, 4 isolates demonstrated pink zone around colonies and 2 isolates demonstrated pink zone within colonies .*Acinetobacter baumannii* S4 exhibited the large zone red when compared with their colony diameter (Table-1). This method is not complicated and L-asparaginase production can be examined by plate inspection .It is easy to screen for L-asparaginase Acinetobacter *baumannii*. For further evaluation by spectrophotometric method.

**Table 1: Colony diameter zone redii and L-asparaginase activities of *Acinetobacter baumannii* isolates.**

| Isolate No. | Colony diameter (Cm) | Zone radius (Cm) | Enzyme unit (Unit/ml) |
|---|---|---|---|
| S1 | 1.3 | 0.2 | 2.47 |
| S2 | 1.4 | 0.3 | 6.61 |
| S3 | 1.7 | 0.5 | 13.11 |
| S4 | 1.3 | 0.8 | 13.53 |
| B5 | 1.0 | 0.4 | 4.98 |
| B6 | 1.5 | 0.7 | 9.13 |

**2-Quantitative analysis**

All *Acinetobacter baumannii* isolates that exhibited pink zone around and within colonies were tested for L-asparaginase production in liquid condition by Nesslerization these isolates revealed L-asparaginase activity between 2.47-13.53 Unit/ml (Table-1). *Acinetobacter baumannii* S4 demonstrated high L-asparaginase activity 13.53 Unit/ml.There are many reports regarding the presence of L-asparaginase in various distinct bacterial sources such as *E.coli* , *Erwinia aroideae* , Pseudomonas flourescens (Ahmad *et al.,* 2012) . Maximum production of the enzyme has been found in stationary phase of growth and mostly activity has been associated with the E.R. (Triantafillou *et al.,* 1988).

**Optimization of the culture condition for L-asparaginase production**

**1. Effect of incubation period**

Optimum incubation period for L-asparaginase production was determined by conducting experiments with different time interval from 18h. to 36h. with a variation of 2h. (Figure-1). It can be concluded that there were variations in enzyme level produced rise from an initial of

13.53 U/ml at 18h. Giving its peak activity of 17.88 U/ml at 26.h. of fermentation. Fermentation beyond 28h. Showed a decrease in enzyme production, which could be either due to the inactivation of the enzyme because of the presence of some kind of proteolytic activity or the growth of the organism, might have reached a stage from which it could no longer balance its steady growth with the availability of nutrient source. (Shah *et al.*, 2010) revealed that by using the optimized fermentation parameters, the enzymatic activity was at lowest values in the log phase and increasing in the exponential phase, at 26 hours it reached to the maximum values and in the early stationary phase (up to 30h.) of the growth cycle the activity was stable and continued in decreasing at late stationary phase.

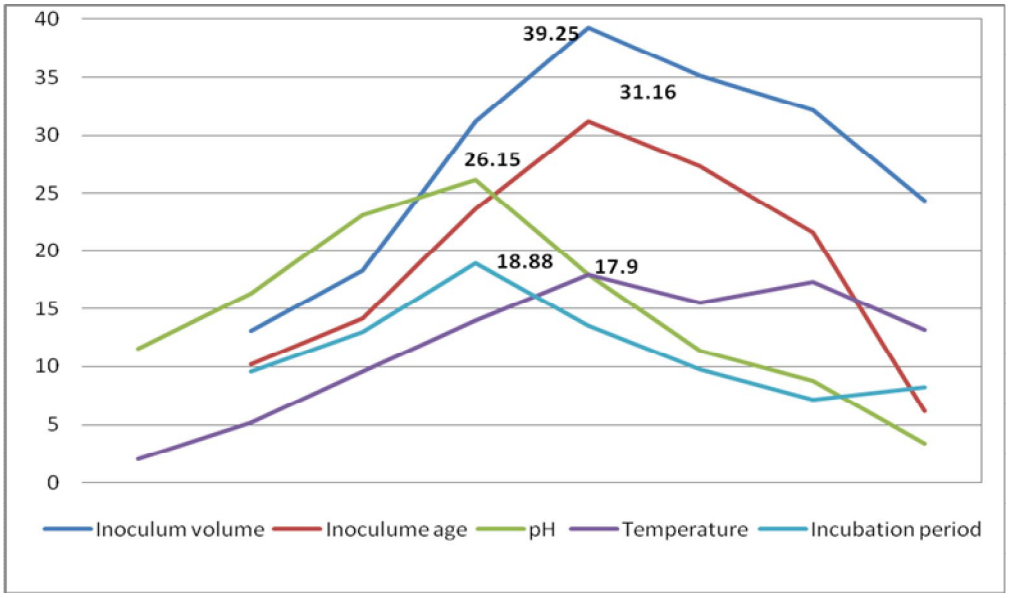


**Figure 1: Effects of some factors on L-asparaginase production**.

## 2. Effect of temperature

Incubation temperature has a profound effect on enzyme production. So the fermentation was carried out at different temperatures ranging from 25 to 55 ºC by *Acinetobacter baumannii* S4 under submerged culture conditions. The maximum enzyme activity of 17.90 U/ml was obtained at 37 c (Figure-1) .The enzyme production reduced gradually with further increase in incubation temperature. This may be due to the denaturation of microbial strain at higher temperatures. These results were in agreement with those reported by (shah *et al.*, 2010).

## 3. Effect of initial pH

Experiments were executed to find out the optimum pH in order to maintain the favorable conditions for increased L-asparaginase production .The fermentation medium pH was adjusted accordingly with 1N HCl /NaOH from 4-11. The significance of pH on the

production of L-asparaginase was observed. The maximum L-asparaginase production of 26.15U/ml was obtained at pH 8.0 (Figure-1). This may be attributed to the balance of ionic strength of plasma membrane. The similar results were reported by (shah *et al.*, 2010).

**4. Effect of inoculum age**

M9 medium was inoculated with inoculum ages ranging from 18-28h. old cultures to optimize the effect of incubation age (Figure-4) . It was observed that maximum L-asparaginase production of 31.16 U/ml was obtained with 26h. Old inoculum of *Acinetobacter baumannii* S4. Further increase in the age , resulted in decrease of enzyme production , which may be due to the occurrence of microbial death phase .Inoculum age of 21 hours was found to be the most suitable for maximum L-asparaginase production by *E. coli* (Kumar *et al.*, 2012).

**5. Effect of inoculum volume.**

This experiment was carried out with different inoculum volumes varying from 0.5 to 3.5ml for a period of 26h. To study its effect on the production of L-asparaginase (Figure-1) .Maximum value of 39.25U/ml was obtained with 2ml of inoculum. With further increase in inoculum volume, there was a gradual decrease in the enzyme production and microbial activity, which might be attributed to the nutrient limitations. The inoculum size of 10% was the best for L-asparaginase production in *E. coli* (Kumar *et al.*, 2012).

**6. Effect of carbon source**

To determine the effect of carbon sources on L-asparaginase production, different carbon sources were tested which include lactose, glucose, maltose, sucrose, galactose, mannose and soluble starch. Each of them at a concentration of 0.5 % (w/v) with other optimized conditions was supplemented to the production medium of *Acinetobacter baumannii* S4 and they have exerted a considerable effect on the biosynthesis of L-asparaginase (Figure-2). Maximum enzyme production was promoted by soluble starch with activity of 44.86 U/ml. This may be attributed to the positive influence of starch as a co-metabolic agent for enhanced enzyme biosynthesis. The synthesis of L-asparaginase in *E. coli* was almost completely suppressed if glucose was added to the growth medium. This was because glucose caused catabolite repression and catabolite inhibition of the components involved in lactate transport and lactate stimulated L-asparaginase synthesis.

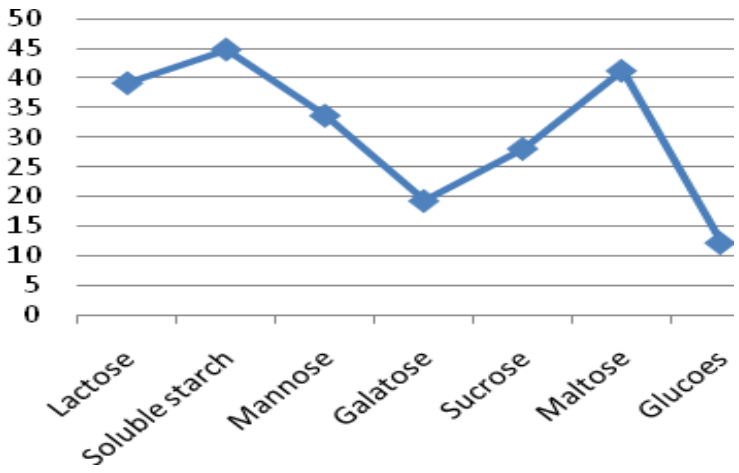


**Figure 2: Effect of carbon sources on L-asparaginase production.**

### 7. Effect of nitrogen source

The supplementation of additional nitrogen sources (either organic or inorganic ) such as L-asparagine , ammonium nitrate , ammonium sulfate , yeast extract , malt extract , urea , peptone , tryptone and sodium nitrate to the production medium had show a profound impact on the production of L-asparaginase by *Acinetobacter baumannii* S4 (Figure-3) . Among the various nitrogen sources tested , L-asparagine in the medium promoted enhanced growth of microorganism and consequently the L-asparaginase production with activity of 46.70 , followed by yeast extract (45.09) (Geckil *et al.*,2006). Yeast extract was important for the cell mass formation and L-asparaginase synthesis in high concentration .L-asparaginase production was inhibited (Verma *et al.*, 2007).Organic acids and amino acids such as L-methionine and L-asparagine were found to enhance production of L-asparaginase in *E. coli* (Netral *et al.*, 1977).

### Production of L-asparaginase by immobilized cells

To enhance L-asparaginase production, immobilization technique used to compare enzyme production by immobilized cells in comparison to free cells under optimum conditions. Adsorption of *Acinetobacter baumannii* S4 cells on different supports such as chitin, pectin, sawdust any synthetic sponge were examined. The results showed that sawdust and chitin were the most suitable for cell immobilization and afforded highest enzyme productivity than pectin and synthetic sponge as shown in (Table-2).

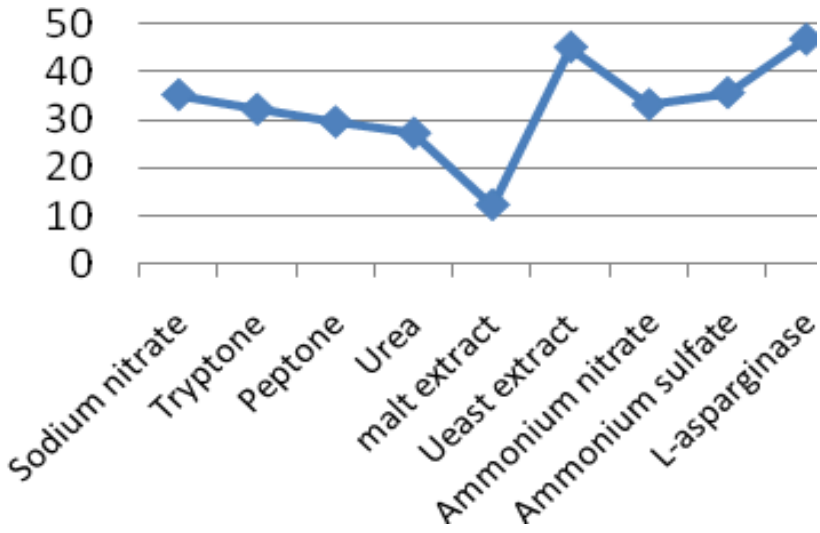


**Figure 3: Effect of nitrogen sources on L-asparaginase production.**

**Table 2: Production of L- asparaginase by immobilized cell *Acinetobacter baumannii* S4 by adsorption.**

| Carrier | Protein content (mg/ml) | L- asparaginase activity (Unit/ml) |
|---|---|---|
| Free cell | 4.17 | 46.7 |
| Synthetic sponge | 2.99 | 17.7 |
| Chitin | 1.95 | 33.9 |
| Sawdust | 3.79 | 41.2 |
| Pectin | 1.12 | 12.6 |

The bacterial cell immobilization on sawdust was the most efficient and yielded active L-asparaginase (41.2 U/ml) which was less active than that produced by free cells (46.7 U/ml).Entrapments was performed using 3% concentration of agar, agarose or alginate. The results in (Table-3) showed that immobilization on sodium alginate was the most favorable (34.9) U/ml. This was less active than immobilized cells on sawdust.

Immobilization of *Acinetobacter baumannii* S4 on different forms of wood wastes showed that wood shaves adsorbed highest amount of bacterial cells and produced maximum L-asparaginase activity (66.4)U/ml compared to other forms of wood waste as shown in (Table-4).

**Table 3: Production of L- asparaginase by immobilized cell *Acinetobacter baumannii* S4 by entrapment.**

| Carrier | Protein content (mg/ml) | L- asparaginase activity (Unit/ml) |
|---|---|---|
| Free cell | 4.17 | 46.7 |
| Agar | 2.0 | 14.5 |
| Agarose | 1.88 | 19.7 |
| Sodium alginate | 3.13 | 34.9 |

**Table 4: Immobilization of *Acinetobacter baumannii* S4 on different forms of wood wastes.**

| Carrier | Protein content (mg/ml) | L- asparaginase activity (Unit/ml) |
|---|---|---|
| Sawdust | 3.79 | 41.2 |
| Wood chippes | 2.57 | 45.8 |
| Wood shaves | 3.49 | 66.4 |

The effect of biomass load (efficiency load) was determined by using different concentration of bacterial inoculum and the results in (Table-5) revealed that was an increase in the enzyme activity with increasing cells load up to 21 bacterium /ml × $10^{7}$ /g carrier ), whereby the maximal enzyme yield was attained (88.6)U/ml . Further increase in spores concentration to decrease in enzyme production .Different concentration from wood shaves were used to determine the optimal concentration of this material. The results in (Table-6) showed that the highest L-asparaginase activity was obtained with matrix concentration of 1.0g wood shaves /50ml.

**Table 5: Effect of biomass loading on wood shaves on immobilized cell *Acinetobacter baumannii* S4 by cell immobilization**

| Efficiency loading (bacterium/ml) | Protein content (mg/ml) | L- asparaginase activity (Unit/ml) |
|---|---|---|
| 3 | 3.17 | 66.2 |
| 12.5 | 3.76 | 72.4 |

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| | | |
|---|---|---|
| 21 | 4.33 | 88.6 |
| 33 | 2.54 | 57.2 |
| 46 | 2.28 | 49.1 |

**Table 6: Effect of different concentration of wood shaves (matrix) on cell immobilization.**

| Wood shaves (g) | Protein content (mg/ml) | L- asparaginase activity (Unit/ml) |
|---|---|---|
| 0.5 | 2.14 | 66.9 |
| 1 | 4.21 | 88.6 |
| 1.5 | 3.79 | 76.6 |
| 2 | 3.02 | 61 |
| 2.5 | 2.44 | 58.8 |
| 3 | 2.11 | 53.2 |

Production of L-asparaginase by immobilized cells of *Acinetobacter baumannii* S4 was investigating in repeated batch process. As indicated by (Table-7) it was observed that L-asparaginase production by immobilized cells attained maximum level at 4$^{th}$ cycle, since enzyme production was increased 140.2% compared to control value. After this L-asparaginase production declined slowly up to 10 cycles. These results indicated that woody materials has a significant role in providing a favorable environment for enzyme production and this may be due to adherence of bacterial cell to the surface as well as into the pores , thus increasing the residence time of cells in the medium .Immobilization protects the organism from inhibitory compounds or metabolites and facilitates the use of dense cell populations without alteration of the rheological properties of the suspending medium , thus high rates of products biosynthesis can be obtained (Keweloh *et al.*, 1989) . Cell immobilization is a common technique for increasing the over –all cell concentration and productivity .The separation of products from immobilized cells is cells is easier compared with suspended cell systems (Beshay,2003).

**Table 7: production of L- asparaginase by immobilized cell of *Acinetobacter baumannii* S4 on wood shaves in repeated batch process.**

| Cycle No. (days) | L- asparaginase activity (Unit/ml) | Relative activity % |
|---|---|---|
| 1 (control) | 88.6 | 100 |
| 2 | 96.01 | 108 |
| 3 | 122.21 | 137.9 |
| 4 | 124.26 | 140.2 |
| 5 | 113.06 | 127.6 |
| 6 | 77.17 | 87 |
| 7 | 44.12 | 49.7 |
| 8 | 28.34 | 31.9 |
| 9 | 22.71 | 25.6 |
| 10 | 11.16 | 12.5 |

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