Enhanced Production of Bacillus sp. R2 Chitinase through Cell Immobilization

5 authors, including:

Ben Amar Cheba
Université des Sciences et de la Technologie …
71 PUBLICATIONS **28** CITATIONS
SEE PROFILE

Taha Zaghloul
Alexandria University
37 PUBLICATIONS **287** CITATIONS
SEE PROFILE

Original Research Article

Enhanced Production of *Bacillus* sp. R2 Chitinase through Cell Immobilization

Ben Amar Cheba [1,*], Taha Ibrahim Zaghloul[2], Ahmad Rafik EL-Mahdy[3], Mohammad Hisham EL-Massry[2]

[1]Department of Biotechnology -Faculty of Sciences-University of Sciences and Technology-Oran-Algeria
[2] Department of Biotechnology, Institute of Post Graduate Studies and Research, University of Alexandria- Alexandria –Egypt
[3] Department of Food Science and Technology-Faculty of Agriculture-University of Alexandria- Alexandria-Egypt
[*] Author for Correspondence
E-mail address : c31b28@maktoob.com
Phone : +213-699156795

Received: 12/10/2010, Revised in Revised form: 25/12/2010, Accepted: 11/01/2011

Abstract

Chitinases (EC 3.2.1.14), which hydrolyze β-1,4-glucosidic bonds of chitin are widely distributed in the biological word and have received an increased attention during the last two decades due to their wider range of biotechnological applications specially in the agricultural, medical, industrial and environmental fields. To improve the chitinase production of *Bacillus sp.* R_2 ,two cell immobilization strategies were conducted. The physical adsorption on different carriers showed that the highest chitinase activity was obtained by the carrier luffa pulp followed by flaked crab shell chitin with enhancement of 1.42 and 1.27 fold respectively. The entrapment immobilization in different gel matrices indicated that cell entrapment in 2% agar and 2% calcium alginate gave the highest chitinase activity 44 U/ml and 42.51 U/ml, respectively. The successive reuse of immobilized cells was investigated for 7 cycles over a period of one month. The results revealed that agar beads retained 100% or more of its original chitinase activity after 4 cycles and 86.5%, 75.5% and 60.9% of activity after the 5th, 6th and 7th cycle respectively. On the other hand the alginate beads 2% and 4% retained more than 65% and 79.5% of their initial activity after 4 cycles. The effect of storage stability of beads on cell viability and chitinase production also was examined. The result showed that the longer shelf life was attained with agar beads which retain 50% of its initial activity after one month. From these optimizations, we recommend the physical adsorption immobilization on luffa pulp carrier for chitinase continuous production.

***Keywords**: Bacillus* sp.R2; chitinase; cell immobilization; physical adsorption; entrapment

Introduction

Chitin, a β- (1-4) homopolymer of N-acetyl - D- glucosamine (Glc NAc), is the second most abundant polysaccharide existing in nature after cellulose. It is a major structural component of most biological systems such as mollusks, insects, crustaceans, fungi, algae and marine invertebrates [1] . Chitin and its derivatives are of commercial and biotechnological interest because they have various biological activities and wide range of applications in areas ranging from waste water treatment to agrochemical and biomedical uses[2,3,4,5]. Chitinases (E.C.3.2.1.14) are found in variety of organisms such as viruses, bacteria, actinomycetes, yeasts, fungi, plants, animals and also in human beings [6, 7]. During the last decade, chitinases have received an increased attention due to their wider ranges of biotechnological applications especially in the biocontrol of fungal phytopathogens [8] and harmful insects [9]. They have also been used as vaccine [10], antitumor [11], tumor marker [12] and useful biomarker in Gaucher disease [13] and used in the preparation of sphaeroplasts and protoplasts from yeasts and fungal species which can be used further for biotechnological uses [14]. The application of immobilized whole cells for the production of metabolites by microorganisms has been extensively studied and several products of value such as alcohols[15],organic acids, amino acids, antibiotics, steroids, and enzymes[16,17,18] has been successfully obtained. Whole cells immobilization for extracellular enzymes production offers many advantages, such as increase of productivity due to high cell concentration, reduction of washout of microorganisms in continuous operation, easy cell separation from the culture broth for repeated use, and improvement in mass and heat transfer [19]. In the previous studies, *Bacillus* sp.R2 was screened as hyper chitinase producer and identified, furthermore, its chitinase production was optimized. In the present work we reported the improvement of chitinase production through two cell immobilization strategies, the physical adsorption on different carriers and entrapment in various gel matrices.

2-Materials and Methods

Chemicals

Peptone, tryptone, and yeast extract were obtained from (Oxöd Hampshire, England). Crab shell chitin flakes (Win-lab, UK). Colloidal chitin was prepared according to Monreal and Reese, (1969) [20]. Sodium alginate, Acryl amide, N.N-Methylene- bis acryl amide, N-acetyl glucosamine, agarose were from (Sigma -USA).Luffa pulp (*Luffa cylindrica*) was purchased from local market. All other chemicals used were of the highest grade available.

Bacterial strain and culture condition

Bacillus sp.R2 was newly marine bacterial strain isolated from red sea Egypt, identified by biochemical tests followed by 16S rRNA methodology (strain accession number in NCBI GenBank was: DQ923161). For chitinase production several single colonies of the strain *Bacillus* sp. R2 were used to inoculate 5 ml marine Lauria and Bertani medium (MLB) (sea water,10g tryptone,5g yest extract,pH :7)supplemented with 0.5% colloidal chitin. This bacterial culture was allowed to grow at 37ºC for 24 h using orbital shaking incubator, Overnight seed culture 2.5% (v/v) was used to inoculate 50 ml production medium (75% sea water, 0.5% colloidal chitin, 0.5 % yeast extract and 0.5% glucose, pH 7.5). The culture was incubated at 30ºC for 24 h at 180 rpm shaking.

Cell immobilization through physical adsorption[21]

The experiment was performed using different solid supports (granular clay, crab shell chitin flasks and luffa pulp). Ten grams of granular clay, 2g flaked chitin and 2g of cut and washed small cubic pieces of luffa pulp were added separately to 50 ml production medium dispensed in 250 ml Erlenmeyer flasks. The cultures were incubated at 30ºC and 180 rpm shaking for 24 h.

Cell immobilization through entrapment

Agar or agarose (2%)[22] Gels were prepared separately by dissolving 1g agar or agarose in 40 ml of 50% sea water. After sterilization and cooling to 45ºC 10 ml of washed fresh cell suspension was added and mixed well. Twenty ml of this mixture were aseptically poured into sterile Petri dish. After solidification, the gels were cut into small beads with sterile cork

poorer (6 mm diameter). Ten ml of washed beads were used to inoculate 50 ml of production medium.

Polyacrylamide (7.5%) [23] Entrapment was performed by mixing 12.5 ml of polyacrylamide solution (30% [w/v] acrylamide + 0.8% [w/v] bisacrylamide) with 10 ml of washed fresh cell suspension and 24.5 ml of 0.9% (w/v) sterile saline solution. After degassing 2.5 ml of 1.5% (w/v) freshly prepared ammonium persulphate and 25 µl of TEMED were added and mixed well. Twenty ml of this mixture were poured into sterile Petri dish and were allowed to polymerize. The solidified gel was cut into small bead as described above. Ten ml of washed beads were used to inoculate 50 ml production medium.

Alginate (1, 2 and 4%)[24] Sodium alginate solution with different concentrations was prepared separately by dissolving 0.5, 1 and 2g in 40 ml 50% sea water and autoclaved at 121° for 10 min. After cooling, 10 ml of washed fresh cell suspension was added with gentled mixing in aseptic condition. Twenty ml of this mixture were added with sterile syringe and were allowed to drop through the needle into precooled 100 ml of 2 % $CaCl_2$ as cross-linking solution and under mild stirring. The formed bead was left for maturation in $CaCl_2$ for 20 min before collecting. Ten ml of washed beads from each concentration were used to inoculate separately 50 ml production medium.

Cell leakage determination: All bacterial cultures, inoculated with the entrapped cells and the control (free cells), were incubated for 24h at 30ºC and 180 rpm. The chitinase activity and CFU/ml were monitored as described earlier. The cell leakage from the various entrapping gels or beads were determined by monitoring the bacterial growth spectrophometrically at 660 nm each hour for 10 hrs.

Effect of immobilized cell repeated use on chitinase production: The experiment was carried out by batch wise reuse method as follows: at the end of each production cycle beads were collected by sintered glass filter funnel and washed with 0.9 % saline under aseptic condition and were added to fresh production medium.

Effect of beads storage time on cell viability and chitinase activity:The beads shelf life was evaluated weekly for one month by inoculating the stored beads in fresh production medium and monitoring the cell viability (CFU/ ml) as well as chitinase activity as mentioned before.

Analytical procedures

Chitinase assay

Chitinase activity was analyzed by estimating the released reducing ends of sugar according to the method of Miller(1959) [25] using N-acetyl - D-glucosamine (NAG) as standard .One unit of chitinase activity was defined as the amount of enzyme required to release 1 µ mol of NAG per minute during these conditions.

Protein assay

Soluble proteins were determined as described by Bradford (1976) [26] using bovine serum albumin as standard.

Growth monitoring

Colony forming units (CFU) was determined [27]. On the other hand, viable bacterial count of immobilized cell by entrapment was determined by dissolving six alginate beads containing cells in 3 ml potassium phosphate buffer (1.5% K_2HPO_4 and 0.23% KH_2PO_4), and for agar, agarose and polyacrylamide, the beads were disrupted in 3 ml physiological saline and the procedure was carried out as above. Moreover, bacterial growth was monitored spectrophotometrically by measuring the absorbance of the cultures at 660 nm.

Scanning electron microscopy (SEM)

Cells grown in marine LB were harvested by mild centrifugation, washed several time with Tris Hcl ,pH 7.5 and fixed with 2 % glutaraldehyde . The preparation was treated and followed by 1% osmium tetroxide . After complete fixation, samples were washed in the above buffer solution and dehydrated in ascending order of ethanol concentrations. The samples were dried completely in critical point dryer, and finally coated with gold in JEOL- Fine coat JEC-1100E ion-sputter-coater (Japan).The specimens were viewed in JEOL-SEM 5300 scanning electron microscope (Japan) operated at 25 KV with a beam angle of 45◦.

3-Results and Discussion

Cell immobilization for chitinase production

Physical adsorption immobilization

The suitability of different carriers for cell immobilization and chitinase production was investigated. The highest chitinase specific activity was obtained by the carrier luffa pulp followed by flaked crab shell chitin with enhancement of 1.42 and 1.27 fold, respectively. On the other hand, the lowest activity (less then free cell) was obtained using granular clay (Table 1). The scanning electron microscopy technique also demonstrated that, the highest cell adsorption was observed on luffa pulp 6.106cell/ mm2 (Figure 1, A) compared to chitin flakes 2.105 cell/ mm2 (Figure 1B). These may be due to the open structure of the luffa pulp which permitted the diffusion of oxygen and nutrients through their fiber net. Table (1) and Figures 1A and B revealed that the good bacterial adsorption was well correlated with the good chitinase yield.

Cell immobilization by entrapment

1. Effect of different immobilization gel matrices on chitinase production

Several gel matrices were used to immobilize strain R2 cells by entrapment matrices were listed in Table 2. Data indicated that entrapment in 2% agar or 2% calcium alginate was optimal for chitinase production. However, these results were still lower than those obtained from the physical adsorption immobilization. This could be due to the fact that R2 cells are aerobes. It was noticed also from Table 2 that, 2% alginate was the most appropriate concentration for good gel porosity which was responsible for diffusion and transfer of oxygen, nutrients and metabolite products to and from the gel beads . It seemed that low gel concentration probably allowed better release of the enzyme to surrounding medium. However, 1% Calcium alginate beads showed fragile nature and high cell leakage Table 3. In contrast 4% Ca-alginate and 7.5% polyacrylamide gave the lowest activity and the lowest cell leakage. This may be due to the low porosity and cell growth inside the beads which made an additional barrier affecting the diffusion. Other factors such as pressure stress and morphological changes may also modify cell metabolism and growth rate [28- 30].

2. Effect of immobilized cells repeated use on chitinase production

The successive reuse of immobilized cells was investigated for 7 cycles (two days each). The results presented in Figure 2 illustrated that, the agar beads retained 100% its original chitinase activity after 4 cycles and 86.5%, 75.5% and 60.9% of activity after the 5^{th}, 6^{th} and 7^{th} cycles, respectively. On the other hand, the alginate beads (2% and 4%) retained more than 65% and 79.5% of their initial activity after 4 cycles, respectively. Moreover, the alginate beads (2%) were destructed by shaking while the 4% beads retained 25.5% its original activity after 7 cycles. Generally, it could be concluded that, the agar beads were the most appropriate beads for the continuous production of chitinase enzyme.

3. Effect of beads storage time on cell viability and chitinase production

Storage stability of immobilized cells in form of beads was tested as described in the materials and methods. The highest chitinase activity and cell viability were obtained with agar bead which exhibited the longer shelf life and retained about 50% of its initial activity after one month Table 4. On the other hand, the shortest storage stability was observed in 4% alginate beads which failed to retain its cells and enzymes due to beads destruction by shaking and also due to autolysis of cells during the washing process.

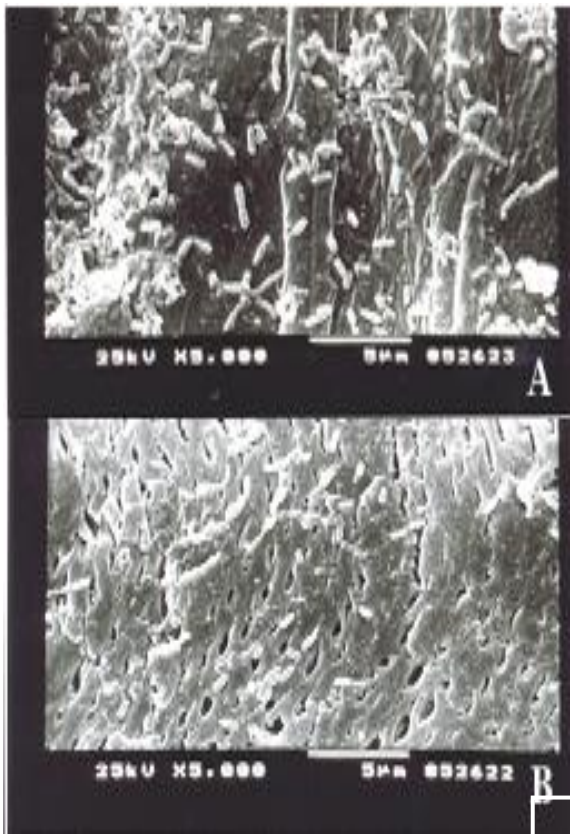

Figure 1: Scanning electron micrographs of immobilized Bacillus sp. R_2 on luffa pulp (A) and chitin flakes (B).

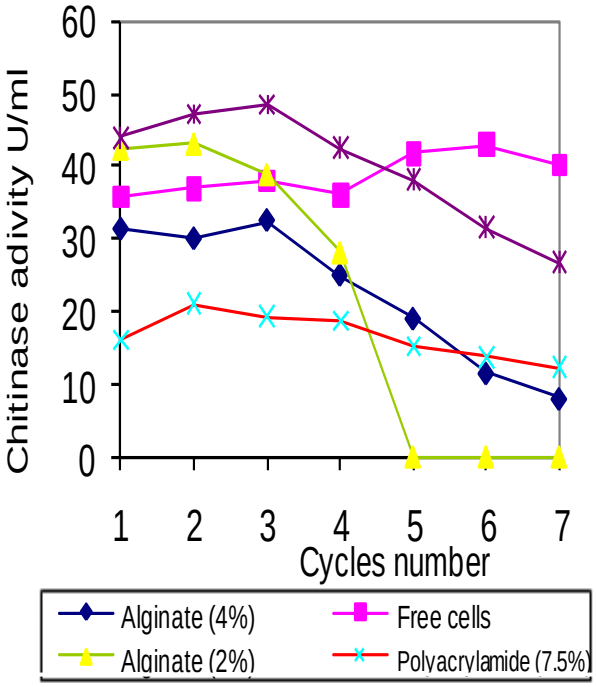

Figure 2: Effect of different immobilization supports on chitinase production

Table 1: Effect of different immobilization supports on chitinase production

Supports	Protein content (mg/ml)	Chitinase activity	
		Activity (U/ml)	Specific activity (U/mg)
1- Granular clay	0.072	28.6	397.22
2- Luffa pulp	0.089	58.22	654.15
3- Flaked crab shell chitin (win lab)	0.091 0.098	53.22 45	586.92 459.18
4- (Free cells) control			

Table 2: Effect of different immobilization gel matrices on chitinase production

Matrices (concentration)	**CFU/ml after 24 h**		**Chitinase activity (U/ml)**
	In beads	**Out of beads**	
Agar (2%)	2.5×10^9	5.5×10^7	44
Agarose (2%)	0.8×10^9	4.7×10^7	39.75
Polyacrylamide (7.5%)	1.5×10^9	0.21×10^7	16
Alginate (1%)	2.9×10^9	9.8×10^7	36.80
Alginate (2%)	3.5×10^9	6.2×10^7	42.51
Alginate (4%)	5.1×10^9	2.3×10^7	21.33
Control (free cells)	–	–	35.67

Table 3: Effect of incubation time on cell leakage from various immobilization matrices

Hours after inoculation	Free cells (OD_{660nm})	Liberated cells from various matrices (Abs. 660 nm)				
		Poly-acrylamide (7.5%)	Agar 2%	Alginate		
				1%	2%	4%
0	0	0	0	0	0	0
2	0.317	0	0.09	0.108	0.07	0
3	0.625	0.08	0.103	0.271	0.151	0.05
4	0.803	0.102	0.222	0.394	0.294	0.104
5	1.067	0.186	0.315	0.542	0.372	0.172
6	1.140	0.232	0.407	0.680	0.461	0.289
7	1.221	0.253	0.583	0.759	0.680	0.335
8	1.623	0.280	0.760	0.891	0.795	0.481
9	1.9	0.285	0.830	1.253	0.901	0.610
10	1.8	0.271	1.102	1.402	1.124	0.731

Table 4: Effect of beads storage time on cell viability and Chitinase activity

Storage time (weeks)	Matrices					
	Agar 2%		Polyacrylamide 7.5%		Alginate 4%	
	CFU/ml	Activity	CFU/ml	Activity	CFU/ml	Activity
1	2.5×10^{9}	44	1.5×10^{9}	16	5.1×10^{9}	31.33
2	0.2×10^{9}	42.5	2×10^{9}	18.9	0.4×10^{8}	25
3	0.8×10^{8}	27.1	0.3×10^{8}	16.3	0.0	0.0
4	3×10^{7}	22.3	9×10^{7}	13	0.0	0.0

Conclusions

To improve the chitinase production of Bacillus sp. R2 two cell immobilization strategies were conducted. The physical adsorption on different carriers showed that the highest chitinase activity was obtained by the carrier luffa pulp. These may be due to the open structure of the luffa pulp which permitted the diffusion of oxygen and nutrients through their fiber net .Whereas, the entrapment immobilization in different gel matrices indicated that cell entrapment in 2% agar gave the highest chitinase activity. The successive reuse of immobilized cells revealed that agar beads retained 100% or more of its original chitinase activity after 4 cycles. The effect of beads storage stability on cell viability and chitinase production also showed that the longer shelf life was attained with agar beads which retain 50% of its initial activity after one month. Generally, it could be concluded that, the agar beads were the most appropriate beads for the continuous production of chitinase enzyme. So, whole cells immobilization in agar beads for extracellular chitinase production offers many advantages, such as increase of productivity due to high cell concentration, reduction of washout of microorganisms in continuous operation, easy cell separation from the culture broth for repeated use, and improvement in mass and heat transfer. Our finding can be applied for chitinase large scale continuous production as well as further improvement in the enzyme properties will be suitable for various applications in the future.

Acknowledgement

This work was funded by the Algerian ministry of higher education and scientific research. The author also thanks professor Almahdy R.A for kindly providing some chemicals and his useful scientific discussion. We are grateful to Dr. Zaghloul T.I. for his helpful suggestions, critical reading of the manuscript and his invaluable comments. Authors would also like to thank Dr. EL-Massry M.H. for the assistance in electron microscopy and in data analysis and interpretation.

4-References

[1] B.O.Jung, S.Roseman, J.Park, The central concept for chitin catabolic cascade in marine bacterium Vibrios ,Macromol.Res. 16,1 (2008) 1-5
[2] E.Pascual, M.R.Julia, The role of chitosan in wool finishing, J. Biotechnol. 89 (2001) 289-296
[3] S.Hirano, Chitin biotechnology applications, Biotechnol. Annu. Rev. 2(1996) 237-258
[4] H.Nagahama, R. New, S. Jayakumar,T. Koiwa, H. Furuike, J.Tamur, Novel biodegradable chitin membranes for tissue engineering, Carbohyd.Polym.73 (2008) 295-302
[5] I.Y.Kim,S.Y. Sio, H.S.Moon, M.K.Yoo, I.Y.Park, B.C. Kim, C.S. Cho, Chitosan and its derivatives for tissue engineering applications, Biotechnol.Adv.26:1 (2008)1-21
[6] G.Escott, D.J. Adams,Chitinase activity in human serum and leukocytes, Infec. Imnun. 63:12(1995) 4770-4773
[7] A.J.Hall, S. Morroll,P.Tighe, F. Gotz, F.H. Falcone, Human chitotriosidase is expressed in the lacrimal glands and has an anti bacterial spectrum different from lysozyme, Microb. Infec. 10:1(2008)69-78
[8] M.Rajkuman, K.J. Lee, H. Freitas, Effect of chitin and salicylic acid on biological control activity of *Pseudomonas* sp. against damping off of pepper,South African .J.Botany. 74:2(2008)268-273
[9] C.Wiwat, S. Thaithanun, S. Pantuwatana, A. Bhumiratana, Toxicity of chitinase producing *Bacillus thuringiensis* sub sp. *kurstaki* HD-1 (G) toward *Plutella xylotella* , J. Invert. Pathol. 76(2000) 270-277
[10] R.Adan, B. Kaltmann,W. Rudin, T. Friedrich, T. Marti, R. Lucius, Identification of chitinase as the immuno-dominant filarial antigen recognized by sera of vaccinated rodents , J. Biol. Chem. 271 (1996) 1441-1447
[11] X.Q.Pan, C.C. Shih, J. Harday, Chitinase induce lysis of MCF-7 cells in culture and human breast cancer xenograft B11-2 in SCID mice , Anti cancer Res. 25:5 (2005) 3167-3172
[12] C.S.Diefenbach, et al., Preoperative serum YKL-40 is a marker for detection and prognosis of endometrial cancer, Gynecol. Oncol. 104:2(2007) 435-442
[13] Y.Guo, W. He, A.M. Boer, R.A. Wevers, A.M. De-Bruijn, J.E.M.Groener, C.E.M. Hollak**,** J.M.F.G.Aerts, H.Galjaard, H., O.P.Van- Diggelen, Elevated plasma chitotriosidase activity in various lysosomal strorage disorders , J. Inherit. Metab. Dis. 18(1995) 717-722
[14] H.S.Kelkar, V.Shankar, M.V. Deshpande, Rapid isolation of *Sclerotium rolfsii* protoplasts and their potential application for starch hydrolysis , Enz. Microb. Technol. 12(1990) 510-514
[15] M.J.Nunez,J.M.Lema,Cell immobilization: application to alcohol production , Enz.Microb.Technol. 9(1987) 642-651
[16] R.Jamuna,S.V. Ramkrishna, Continuous synthesis of thermostable α-amylase by *Bacillus* cells immobilized in calicium alginate, Enz.Microb.Technol.14 (1992)36-41
[17] I.I.Sutar,H.G.Vartak,M.C.Srinivasn,H. Sivaraman, Production of alkaline protease by immobilized mycelium of *Conidiobolus* .Enz.Microb.Technol. 8(1986) 632-634
[18] T.I.Zaghloul,H.M.Hendawy,S. El Assar,M.H. Mostafa, Enhanced stability of the cloned *Bacillus subtilis* alkaline protease gene in alginate-immobilized *B. subtilis* cells.Enz. Microb.Technol.30 (2002) 862-866
[19] M.A.Longo,I.S.Novella,L.A.Garcia,M. Diaz, Diffusion of protease in calicium alginate beads .Enz.Microb.Technol. 14(1992)586-590
[20]J. Monreal, and , E.T Reese, The chitinase of Serratia marcescens. Can. J. Microbiol. 15(1969) 689 – 696.
[21] E.J.T.M.Leenen,Description of the support materials.In:Immobilized cells.ed. by: R.H.Wijffels,Springer Verlag, Berlin,(2000)6-14
[22] Houria Ouled Haddar: MSc thesis. Baghdad University, Biotechnology Department; 2001.
[23] Y.Morikawa,K.Ochiai,I.Karube,S.Suzuki,Bacitracin production by whole cells immobilized in polyacrylamide gel,Antimicrobial Agents and Chemotherapy,15,1(1979)126-130
[24] H.Eikmeir, F.Westmeir, G.H.Rehm, Morphological development of *Aspergillus niger* immobilized in Ca-alginate and K- carrageenan ,Appl.Microbial.Biotechnol. 19(1984) 53-57
[25] G.R.Miller, Use of Dinitrosalicylic Acid reagent for determination of reducing sugar, Anal. Chem. 31:3(1959) 426 – 428
[26] M.M.Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem.72 (1976) 248 – 254
[27] M.J.Pelczar, E.C.Chan, Laboratory exercises in microbiology, 4th edition, Mc Graw Hill, Inc. (1977)
[28] A Martinsen, I. Storre, G.Skiak-Break, Alginate as immobilization material. III: Diffusional properties, Biotechnol. Bioeng. 39(1992) 186-194
[29] H.J. Rehm, S.H.Omar, Morphological and metabolic behaviour of immobilized microorganisms. In: Biotechnology (H.J.Rehm, G.Reed, Eds), Weinhein, New-York, (1993)223-248
[30**]** **B.** Gosman, H.J.Rehm, Influence of growth behaviour and physiology of alginate entrapped microorganisms on the oxygen consumption, Appl. Microbial. Biotechnol. 10 :29(1988) 554-559