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# *In vitro* cultures of *Silybum marianum* and silymarin accumulation

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**KEYWORDS**

*Silybum marianum*;
*In vitro* cultures;
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Silymarin

**Abstract** In this study, a protocol for initiation of callus and shoot cultures from leaves and shoot tips explants of different silybium genotypes collected from different locations in Egypt was established. Callus cultures were initiated from leaves explants and exposed to different concentrations of the precursor (coniferyl alcohol). Shoot cultures were initiated from shoot tips explants. Moreover, the produced plants of the different Silybium shoots as well as intact plants were subjected to protein screening using SDS–PAGE analysis.

Results obtained revealed that the optimum medium for growth and maintenance of friable callus was MS medium supplemented with 0.25 mg $L^{-1}$ 2,4-Dichlorophenoxy acetic acid (2,4-D) + 0.25 mg $L^{-1}$ Kinetin (Kin). The best medium for proliferation of high number of shoots was MS-medium with 0.25 mg $L^{-1}$ each of Benzyl Adinine (BA) and Naphthalene Acetic Acid (NAA). Coniferyl alcohol in concentration of 30 μM caused an increase in accumulation of silymarin contents in most callus cultures. SDS–PAGE of different Silybium shoots revealed that the protein profiles of 100% of *in vitro* produced plantlets similar to their control.



## 1. Introduction

The milk thistle *Silybum marianum* L. Gaertn belongs to Asteraceae family. It is an annual or biannual herbaceous plant that is widespread in temperate American countries, Australia and areas of Mediterranean climate. In Egypt it grows wild in most districts especially in Nile Delta [1].

The great importance of this plant and consequently its active ingredients can be easily recognized from the list of diseases in which the plant is used such as anorexia disease, cancer disease, demulcent in catarrh and pleurisy, diabetes,

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estrogen-related diseases, hemorrhoids, hydrophaints, malaria, and spleen disease. A number of chemical compounds of herb are now being shown to have this protective effect on liver cells [2]. The most common commercial medicines in use nowadays to treat liver diseases are Legalon-70 and Dura-silymarin. Both of them containing silymarin (extracted from *S. marianum* plant seeds) are expensive and imported medicines. Nowadays silymarin, the purified extract of the fruits and its main constituents, silybin, and the isomers silydinin and silicristin and related compounds are used in the treatment of various liver diseases [1].

Unfortunately, traditional agriculture of silybum plants has many agricultural problems which causes reduction of the total yield and that is due to the leaves of the plant having spiny margins and flowers are spiny also so, it is very difficult to manipulate the manual treatment with the plant during different stages of growth particularly during harvesting. Also, using herbicides creates a problem with the contamination of the fruits (seeds) with toxins. Moreover, the plant is cultivated in rows so, using the combine machine causes damage in the crop yield reach to 40% loss in total yield during the harvesting time [2].

Advanced microbial and chemical methods can synthesize medicinal and aromatic compounds, but the cost in many cases is expensive. However, synthetic medicine can cause side effects. However, traditional methods for propagation of medicinal and aromatic crops have several constraints. In spite of all the above mentioned problems, *In vitro* propagation system can be immensely useful to overcome these constraints for propagation of these crops [3].

Studies on the production of silymarin using *in vitro* cultures reported the production of flavonolignan from callus and cell suspension cultures derived from cotyledons of *S. marianum* L. Higher accumulation of flavonolignan was observed in cell suspension cultures than in callus cultures [4]. Explants from seedlings of *S. marianum* were used to produce either transformed (hairy root culture) or untransformed (root culture). Silybin, isosilybin, silychristin and silydianin were found in untransformed root cultures while only isosilybin and traces of silychristin and silydianin were identified in hairy root cultures [5]. Regeneration from leaf, shoot apex and nodal segment of *in vivo* and *in vitro* seedlings, and enhancement in silybin content have been achieved in *S. marianum* [6].

The aims of this study is to establishment a protocol for both regeneration and calli cultures from *S. marianum* and assess the effect of precursor feeding on silymarin content in calli cultures. Use SDS–PAGE to evaluate variation between tissue culture raised plantlets and *in vivo* plants.

## 2. Materials and methods

### 2.1. Plant materials and culture conditions

Seeds of different silybium genotypes collected from different environmental locations (Kafr-El-Shiekh, Asuiet, El-Menia, Beni-Sewaf, El-Fayuim and Agricultural road of Alexandria) were surface sterilized with 70% ethanol for 1 min, followed by 30% commercial Clorox (contained 5.25% sodium hypochlorite) for 20 min. After three successive rinses in sterile distilled water, sterilized seeds were cultured on MS [7] basal medium supplemented with 30 g $L^{-1}$ sucrose, 100 mg$L^{-1}$ myo-inositol and solidified with 7 g$L^{-1}$ agar.

All media were autoclaved (121 °C and a pressure of 1.2 kg $cm^{-2}$ for 20 min) and the pH was adjusted to 5.8 (using 1 M NaOH or HCl). All cultures were incubated in the growth chamber for 1 month at 25 ± 2 °C under a 16-h photoperiod (irradiance of about 40 mmol $m^{-2}$ $s^{-1}$ provided by cool white fluorescent lamps).

### 2.2. Establishment of callus cultures

When the seedling reached 30 days old, leaves (about 0.25 cm in length) of all genotypes were excised from different *S. marianum* seedlings and then cultured on MS-basal-medium supplemented with 0.25 mg $L^{-1}$ 2,4-D + 0.25 mg$L^{-1}$ Kin. Cultures were incubated in a growth chamber for one month for callus initiation. Calli cultures were subcultured monthly for 6 months on that medium for increasing of calli tissues for further experiments.

### 2.3. Establishment of shoot cultures

Shoot tips of genotype A, (Collected from Agricultural road of Alexandria) and genotype B, (Collected from El-Fayuim) were isolated from aseptically germinated seeds and transferred to MS-medium supplemented with 0.0, 0.25, 0.5 or 1.0 mg$L^{-1}$ NAA in combination with 0.0, 0.25, 0.5 or 1.0 mg$L^{-1}$ BA. These experiments were set up in a completely randomized design and repeated two times. Each treatment has three replications. Shoot number (longer than 1 cm), and shoot length (cm), were recorded after one month of cultivation. All cultures were maintained in the same environmental conditions mentioned before. The number of shoots per explant and shoot lengths were recorded after one month. Data obtained were subjected to statistical analysis [8].

### 2.4. Precursor feeding experiment

Calli tissues 0.25 mg of the genotypes collected from different environmental regions were grown in media containing 0.25 mg $L^{-1}$ 2,4-D + 0.25 mg$L^{-1}$ Kin (control) and the same medium containing different concentrations (10, 30 and 60 μM coniferyl alcohol) as a precursor for flavonoliganan biosynthesis. Calli cultures were subcultured on the same medium containing precursor for 3 months. Calli tissues were collected and dried in oven under 60 °C overnight followed by analyzing using HPLC [9].

### 2.5. Electrophoresis (SDS–PAGE) Sodium dedocyl sulfate–Polyacrylamide gel electrophoresis

Soluble proteins of five randomly selected tissue-culture derived Silybum shoots and their intact plants (collected from Mansoria, El-Fayum, Bani-Sewaf, Asuiet and El-Menia) were extracted from leaf tissues of (0.5 g) by homogenizing in sodium phosphate buffer, pH 6.8. A 30 mg portion of protein was separated in 10% SDS–PAGE [10]. The separation was carried out using EC mini gel unit at 60 V for 4 h. Gels were stained with Coomassie brilliant blue (R-250), distained with high methanol solution (40% methanol in 10% acetic acid), photographed and the molecular weights of polypeptide bands were calculated from a calibration curve of low molecular weight marker standards of Pharmacia.

## 3. Results and discussion

### 3.1. ***In vitro*** *callus cultures*

The optimum medium for growth and maintenance of healthy and friable calli was MS medium supplemented with 0.25 mgL$^{-1}$ 2,4-D + 0.25 mgL$^{-1}$ Kin (incubated in darkness) as shown in Fig. 1 (1) [9]. This medium was therefore used for maintenance of calli tissues and subculture was done for 4 months to increase the amount of callus tissues.

### 3.2. *Sylibium marianum shoot cultures*

Data in Table 1 and Fig. 1(2 and 3) showed mean number of shoot per explant, and shoot length of *S. marianum* genotype A, (Agricultural road of Alexandria) and genotype B, (El-Fayuim), respectively as affected by NAA and BA concentrations. It could be observed that increasing levels of BA and NAA caused decrease in the number of shoots per explant for two genotypes. However, the best medium for initiation of the highest number of shoots was MS-medium with 0.25 mgL$^{-1}$ each of BA and NAA. Concerning shoot length it could be noticed that the best shoot length was recorded with shoots grown on growth regulators free medium, while shoots grown on media with high levels of BA and NAA gave the lowest shoot length. Also, the shoots grown on medium with 0.25 mg L$^{-1}$ each of BA and NAA gave a suitable plant height for the produced shoots. A significance increase in the number of shoots was observed between shoots grown on media with low level and shoots grown on media with high levels of BA and NAA. Also, in shoot length of shoots grown on media with low levels of BA and NAA than shoots grown on media with high levels of BA and NAA.

In general the best medium for proliferation of high number of shoots was MS-medium with 0.25 mg L$^{-1}$ each of BA and NAA, while medium without hormones gave the tallest shoots.

In this connection, a recent report indicates that regeneration from leaf, shoot apex and nodal segment of *in vivo* and *in vitro* seedlings, and enhancement in silybin content have been achieved in *S. marianum* [6]. Callus was induced within 4 weeks on MS-medium. The callus differentiated multiple shoots followed by rooting in 100% cultures. Plantlets could be successfully transplanted to soil. Yield of silybin content in the cultured tissues varied with age and composition of the medium and maximum yield resulted after 8-weeks on media supplemented with zeatin.

Also, concerning *in vitro* regeneration, the highest percentage of direct organogenesis (from leaf explants) was observed with medium contained 1 mg/l BA + 2 mg/l NAA. However, transferring callogenic explants to 2 mg/l kin + 2 mg/l NAA containing medium resulted in the highest percentage of indirect organogenesis presented as shoot formation in *S. marianum* L. Gaertn [11].

### 3.3. *Electrophoresis (SDS–PAGE) Sodium dedocyl sulfate–Polyacrylamide gel electrophoresis*

Total soluble proteins of five randomly selected tissue-culture derived silybium shoots were extracted and subjected to protein electrophoresis, in order to compare the protein banding patterns of plantlets or shoots produced from *in vitro* culture

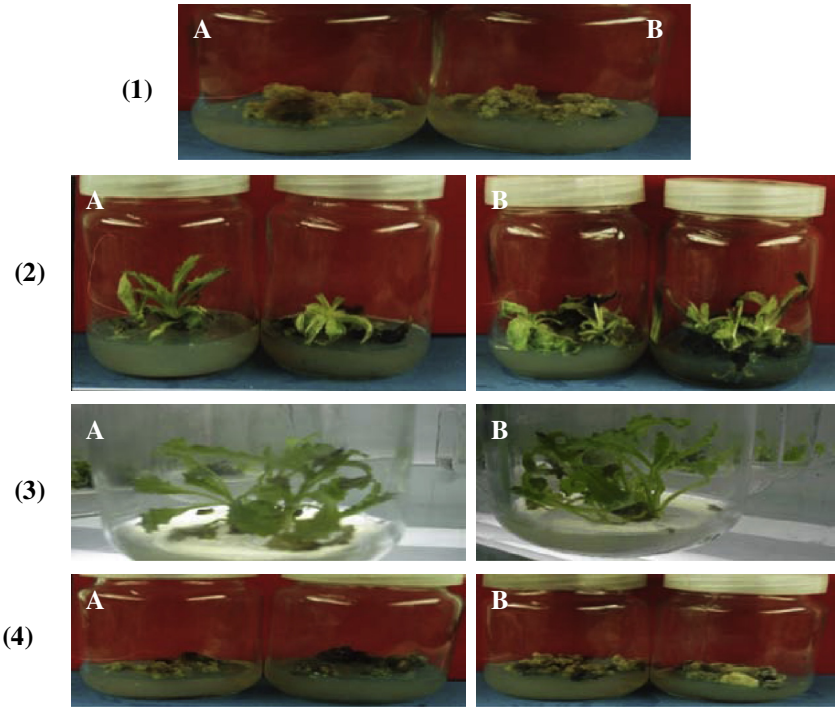


**Figure 1** (1) Callus tissues of Silybum marianum of genotypes, Agricultural road of Alexandria (A), (left) and El-Fayuim (B) (right) and grown in MS-medium supplemented with 0.25 mg/l 2,4-D + 0.25 mg/l Kin. (2) Shoot cultures of genotype A and genotype B, respectively after one month grown in MS-medium containing 0.25 mg/l NAA and 0.25 mg/l BA. (3) Multiplication of shoots from genotype A (left) and genotype B (right). (4) Callus tissues of genotypes (A) and (B) grown in culture medium containing coniferyl alcohol.

**Table 1** Mean number of shoots per shoot tip, and shoot length of silybum marianum genotypes A, (Agricultural road of Alexandria) and B, (El-Fayuim) as affected by NAA and BA concentrations, incubated in the growth chamber for 1 month at 25 ± 2 °C under a 16-h photoperiod (irradiance of about 40 mmol $m^{-2}$ $s^{-1}$ provided by cool white fluorescent lamps). Mean ± SE, $n = 5$.

| Growth regulator (mg/l) | | Number of shoots per shoot tip | | Shoot length (cm) | |
|---|---|---|---|---|---|
| NAA | BA | A | B | A | B |
| 0.0 | 0.0 | 1.6 ± 0.33[a] | 1.33 ± 0.33[a] | 11.33 ± 0.60[a] | 11.16 ± 0.61[a] |
| 0.25 | 0.25 | 5.0 ± 0.57[b] | 4.00 ± 0.57[b] | 10.46 ± 0.46[a] | 9.70 ± 0.55[a] |
| 0.50 | 0.50 | 4.0 ± 0.57[bcd] | 3.00 ± 0.57[b] | 5.60 ± 0.55[b] | 6.70 ± 0.49[b] |
| 1.0 | 1.00 | 3.0 ± 0.57[d] | 2.33 ± 0.33[b] | 6.50 ± 0.56[b] | 5.50 ± 0.43[b] |

Data (mean ± SE) sharing the same letter in the same column is not significantly different ($P < 0.05$).

with control plants. Screening of different Silybium shoots using SDS–PAGE revealed that the protein profiles of 100% of *in vitro* produced plantlets are similar to their control and no detectable differences were observed. The group of protein which characteristics of the produced plantlets are in approximately 94 and 14 kDa as shown in Fig. 2.

Molecular characterization is a prerequisite for the identification of the *in vitro* produced plantlets. SDS–PAGE protein analysis in many published reports was used to prove the identity of regenerated plants *in vitro* to their intact plants also to assess the variations which could occur. [12,13]. On the other hand, in the study on genetic stability of transgenic potato [14] expressing *cry1Aa7* gene reported that, protein banding profiles were not sufficient to detect variations among transgenic and non-transgenic lines.

### 3.4. Precursor feeding experiment

In this experiment callus tissues 0.25 mg of six genotypes Kafr-El-Shiekh, Asuiet, El-Menia, Beni-Sewaf and El-Fayuim and Agricultural road of Alexandria were grown in MS-medium containing 0.25 $mgL^{-1}$ 2,4-D + 0.25 $mgL^{-1}$ Kin (control) and also were grown on media containing different concentrations of the precursor coniferyl alcohol for 3 months.

Data in Table 2 show calluses of the genotypes, Kafr-El-Shiekh, Asuiet, El-Menia, Beni-Suif, El-Fayium and Agricultural road of Alexandria which were grown in media containing various concentrations of the precursor (coniferyl alcohol) for 3 months. It could be noticed that all samples under investigation contain Silychristin (Sc.) in concentrations fluctuating between 0.09 and 2.01 mg $g^{-1}$, except that of the calluses obtained from Asuiet. While Silydianin (Sd.) was only found in the range between 0.02 and 0.25 mg $g^{-1}$. Calluses of *S. marianum* of Beni-suif grown in media containing 10 μM of the precursor (coniferyl alcohol) for 3 months were found to contain the highest content of Sd. (0.25 mg $g^{-1}$). Complete absence of Sd. was observed in all samples of calluses of El-Menia genotypes. Silybinin was found in samples of calluses grown in media containing various concentrations of the precursor in the range 0.01–0.61 mg $g^{-1}$, the highest content, 0.61 $mgg^{-1}$was found in El-Faium calluses grown in media containing 60 μM of the precursor. The highest content of total silymarin was observed in El-Fayium calluses (2.1 mg $g^{-1}$ dry weight) followed by El-Menia calluses 1.81 (mg $g^{-1}$dry weight) while the lowest content of total silymarin was found

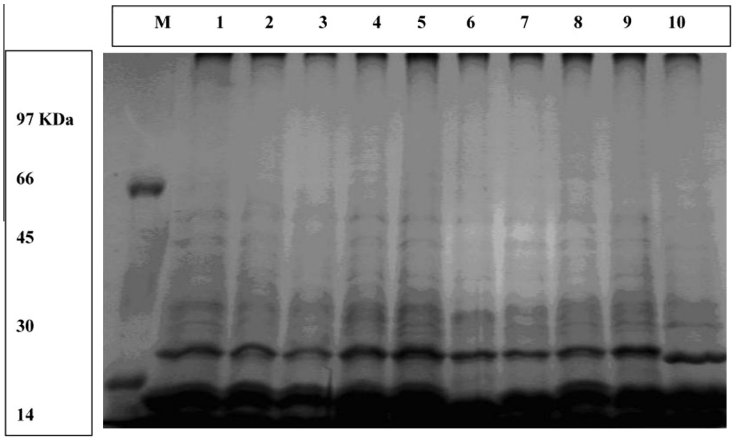


**Figure 2** SDS–PAGE of different *Silybum murianum* L. shoots produced from tissue culture and their control samples. Lane M refers to low molecular weight standard protein marker. Lane 1 refers to the genotype El-Mansoria – Giza (violet). Lane 2 refers to the genotype El-Mansoria (control). Lane 3 refers to the genotype El-Fayum (violet). Lane 4 refers to the genotype El-Fayum (control). Lane 5 refers to the genotype Bani-Sewaf (violet). Lane 6 refers to the genotype Bani-Suif (control). Lane 7 refers to the genotype Asuiet (violet). Lane 8 refers to the genotype Asuiet (control). Lane 9 refers to the genotype El-Menia (samalot – violet). Lane 10 refers to the genotype F.l-Menia (control).

**Table 2** Silymarin contents of Kafr-El-Shiekh, Asuiet, El-Menia, Beni-Suif, El-Fayium and Agriculture road of Alexandria calluses grown in media containing various concentrations of the precursor (coniferyl alcohol) for 3 months (samples by HPLC analyses mg/g dry weight).

| Coniferyl alcohol (μM) | Silymarin content (mg/g dry weight) | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Kafr-El-Shiekh | | | | Asuiet | | | | El-Menia | | | | Beni-Suif | | | | El-Fayium | | | | Agri. road of Alex. | | | |
| | 1 | 2 | 3 | T | 1 | 2 | 3 | T | 1 | 2 | 3 | T | 1 | 2 | 3 | T | 1 | 2 | 3 | T | 1 | 2 | 3 | T |
| 0.0 | 0.09 | 0.06 | 0.21 | 0.45 | – | 0.11 | – | 0.211 | 0.27 | – | 0.11 | 0.51 | 0.25 | 0.02 | – | 0.35 | 0.71 | 0.1 | – | 1.1 | 0.09 | 0.1 | – | 0.96 |
| 10 | 2.01 | – | – | 0.26 | – | – | 0.156 | 0.49 | 0.16 | – | – | 0.49 | 0.91 | 0.25 | – | 1.4 | 0.11 | – | 0.17 | 0.86 | 1.4 | – | – | 1.1 |
| 30 | 0.65 | 0.16 | 0.25 | 1.2 | – | 0.07 | 0.36 | 1.1 | 0.93 | – | 0.38 | 1.81 | 1.20 | – | 0.01 | 1.6 | 0.63 | – | 0.09 | 2.1 | 0.72 | 0.04 | 0.2 | 1.7 |
| 60 | 0.21 | 0.09 | 0.01 | 0.64 | – | – | 0.12 | 0.67 | 0.61 | – | – | 0.91 | 0.88 | – | 0.06 | 1.3 | 0.36 | – | 0.61 | 1.6 | 0.32 | – | 0.35 | 1.45 |

1, 2, 3 and T refer to Silychristin, Silydianin, Silybinin (A,B) and Total silymarine, respectively.

in samples of Asuiet and Kafr-El-Shiekh calluses, 0.21 and 0.26 (mg $g^{-1}$dry weight), respectively.

In general, it could be concluded that addition of the precursor (coniferyl alcohol) in concentration of 30 (μM) caused an increase in accumulation of total silymarin contents in most calli used of different samples. On the other hand, increasing the concentration of precursors to duplicate (60 μM) caused decrease in total silymarin content. These results may be due to the effect of feedback mechanisms. Feedback inhibition of metabolic enzymes as well as inhibition of membrane transport can be eliminated by the accumulation of synthesized products in a second phase introduced into the aqueous medium [15].

A recent study evaluated the accumulation of kaempferol in undifferentiated callus of *Tylophora indica* through TLC, HPTLC analysis with standard reference compound. Kaempferol is a strong antioxidant and helps to prevent oxidative damage to cells, lipids and DNA. The kaempferol content was enhanced in *T. indica* tissue culture by using precursors like sallicyclic acid, ornithine, cinnamic acid, tyrosin and phenylalanine in different concentrations (10 and 20 $mg^{-1}$ 100 ml). Static as well as suspension culture were used to enhance the kaempferol concentration. A remarkable enhancement in kaempferol content was obtained by using 20 $mg^{-1}$ 100 ml of tyrosin (1.49% dw; control −0.096%dw) in suspension culture, which is more than tenfold increase [16].

## 4. Conclusions

Callus and shoot cultures were established from leaves and shoot tips explants depending on the type and concentration of growth regulators added to MS media. Accumulation of silymarin in callus cultures exposed to different levels of coniferyl alcohol as a precursor was affected. SDS–PAGE analyses cannot detect differences between control and *in vitro produced shootlets*.

## References

[1] V.H. Heywood, J.B. Harborne, The biology and Chemistry of Compositae, Academic Press, London, 1977, p. 414.
[2] F.M. Hammouda, S.I. Ismail, N.M. Hassan, A.K. Zaki, A. Kamel, H. Rimpler, Phytother. Res. 1 (1993) 90–91.
[3] S. Luper, Altern. Med. Rev. 6 (1998) 410–421.
[4] M. Cacho, M. Moran, P. Corchete, J. Fernandez-Tarrago, Plant Sci. 144 (1999) 63–68.
[5] F. Alikaridis, D. Papadakis, K. Pantelia, T. Kephalas, Fitoterapia 4 (2000) 379–384.
[6] S.M.P. Iqbal, S. Srivastava, J. Plant Biotechnol. 2 (2000) 81–87.
[7] T. Murashige, F. Skoog, Physiol. Plant. 15 (1962) 473–497.
[8] G.W. Snedecor, W.G. Cochran, Statistical methods, Univ. Press, Iowa State, 1967, pp. 189–199.
[9] F.M. Hammoda, E.H. Aboutabl, S.I. Imbaby, H.N. Nazief, W.A. Abdallah, M.A. Matter, H.A. Ghareeb, M.M. Saker, M.R. Rady, J. Genetic Eng. Biotechnol. 7 (2009) 5–9.
[10] U.K. Laemmli, Nature 224 (1970) 680–685.
[11] S.A. Bekheet, H.S. Taha, A.M.M. Gabr, J. Appl. Sci. Res. 9 (1) (2013) 860–866.
[12] E.H. Ultrika, M. Lars, V.A. Sara, Physiol. Plant. 88 (1993) 315–321.
[13] J. Hendriks, S. Veries, Current Issues in Plant Molecular and Cellular Biology, in: M. Terzi, R.R. Cella, A. Falavigna (Eds.), Kluwer Academic Publishers, Netherlands, 1995, pp. 359–368.
[14] E.A. Metry, M.A. Ibrahim, S.A. Moustafa, T.M. Nasr El-Din, Arab J. Biotechnol. 1 (2003) 113–124.
[15] H. Dörnenburg, D. Knorr, Enzyme Microb. Technol. 17 (1995) 674–684.
[16] P. Chaturvedi, A. Chowdhary, Adv. Appl. Sci. Res. 4 (2) (2013) 325–330.