Journal of Basic & Applied Mycology (Egypt) 5 (2014): 25-34

Extracellular enzymes production by microbiota isolated from human eye infections in Taiz City, Yemen

M. A. Abdel-Sater[1,2*], A. H. Al- Zubeiry[2] and K. A. Badah[2]

[1]Department of Botany and Microbiology, Faculty of Science, Assiut University, Egypt
[2]Department of Microbiology, Faculty of Science, Taiz University, Taiz, Yemen

[*]Corresponding author: masater59@yahoo.com

Received 2/4/2014, Accepted 2/5/2014

Abstract: A total of 13 isolates representing 7 species of 4 fungal genera isolated from human eye infections in Taiz City, Yemen were screened for their abilities to produce some extracellular enzymes. All tested isolates were able to produce lipase and catalase, while only 10 and 4 isolates exhibited protease and urease respectively. Only 1 isolate could produce coagulase enzyme. Isolates related to *Aspergillus flavus, A. oryzae, A. niger* and *Cochliobolus lunatus* exhibited high enzyme production. On the other hand, most of the 95 tested bacterial isolates could produce catalase (89.5% of the isolates), protease (86.3%) and coagulase (53.7%). Other enzymes such as urease and lipase were detected in 42.1% and 30.5% of the isolates respectively. Isolates belonging to *Staphylococcus aureus, S. epidermidis, S. saprophyticus, Pseudomonas aeruginosa* and *Bacillus cereus* showed high enzyme activities.

Key Words: Microbial enzymes, eye infections, fungi, bacteria.

Introduction

Extracellular enzymes are proteins made by living cells to promote specific metabolic reactions. There are more than 3000 known enzymes in the human body. The body's ability to function and repair itself is directly related to the strength and number of enzymes that are present. Every second, they are changing and renewing, sometimes at unbelievable rates (Gnanadoss *et al.* 2011, Kranthi *et al*. 2012).

Lipases catalyze the hydrolysis of acylglycerides and other fatty acid esters under aqueous conditions and the synthesis of esters in organic solvents (Ueda *et al*. 2002). Many lipolytic enzymes, including lipases, esterases or carboxylesterases and various types of phospholipases, have been found in a wide range of organisms from fungi and bacteria to humans (Moharram 1989, Treichel *et al.* 2010, Mus and Adebyo-Tayo 2012).

Several studies have suggested the possible involvement of proteases in the pathogenesis (Tomee *et al*. 1997). Some fungi were able to produce protease such as *Fusarium culmorum, F. avenaceum* and *F. oxysporum* (Urbanek and Yirdaw 1978). *A. oryzae* was observed to produce acid protease (Aleksieva *et al*. 1981). Also, isolates of *Micrococcus candidus, M. caseolyticus, M. freudenreichii, Pseudoalteromonas aeruginosa, Staphylococcus aureus, Bacillus subtilis* had maximal proteolytic activity at 22ºC (Abou-Elela *et al.* 2011, Smita *et al.* 2012, Krishnaveni *et al*. 2012).

Urease enzyme degrades urea to ammonia and carbon dioxide (Cheesbrough 1992). Among *Trichophyton* spp. examined for urease production, *T. rubrum* was negative, whereas *T. mentagrophytes* appeared to be the most active species (Oki *et al.* 2010).

Catalase seems to be the main regulator of hydrogen peroxide metabolism. Hydrogen peroxide at high concentrations is a toxic agent, while at low concentrations it appears to regulate some physiological processes such as signaling in cell proliferation and carbohydrate metabolism (Sirbu 2011). *A. niger* has four catalases, two of which are produced only under conditions of stress (Witteveen *et al*. 1992). Also, three catalases and two superoxide dismutases have been found in *A. fumigatus* (Crameri *et al*. 1996). In *Aspergillus nidulans* three monofunctional catalases have been described and a fourth catalase activity was observed in native polyacrylamide gels (Scherer *et al*. 2002). Catalase enzyme is also produced by *Pseudomonas fluorescens* and *Bacillus cereus* (Kimoto *et al.* 2012).

Coagulase is an extracellular protein, which binds to prothrombin in the host to form a complex called staphylothrombin. The protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin. Coagulase is a traditional marker for identifying *Staphylococcus aureus* in the clinical microbiology laboratory (Kenneth 2005). The presence of coagulase maintained the viability of capsule-lacking *Staphylococcus aureus* in the peritoneal cavity. Coagulase leads not only to

maintained viability, but also to its proliferation in the peritoneal macrophages (Salamah 1992). Ten different serotypes of staphylocoagulases have been reported to date (Yigit *et al.* 2011).

The aim of the present study was to test the ability of fungal and bacterial isolates, identified from human eye infection in Taiz City, Yemen, for extracellular enzymes production.

Materials and Methods

Screening for Extracellular Enzymes

Thirteen fungal isolates and 95 bacterial isolates, previously identified by Abdel-Sater *et al.* (2012) from diseased human eyes in hospitals of Taiz City, Yemen (Al-Gumhoury Hospital, Al-Thawra Hospital, Al-Askary Hospital, Al-Gabaly Hospital, Al-Taawoon Hospital and Al-Noor City Hospital) were screened for their abilities to produce extracellular enzymes. The following fungi were tested: *Aspergillus oryzae, A. awamori, A. flavus, A. niger, Candida albicans, Cochliobolus lunatus* and *Penicillium griseofulvum.*

Bacteria tested were: *Staphylococcus aureus, S. epidermidis, S. saprophyticus, Streptococcus viridans, St. pyogenes,* Enterococci, *Nocardia* sp., *Bacillus cereus, B. coagulans, Pseudomonas aerginosa, Escherichia coli, Alcaligenes* sp., *Acinetobacter* sp. and *Proteus mirabilis***.**

1-Lipase

The lipolytic activity was measured using the method of Ulman and Blasins (1974). The formation of lipolytic enzymes was seen as a visible precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by the enzyme.

2- Protease

(a) Fungi: The fungal proteolytic activity was tested using casein hydrolysis medium (Paterson and Bridge 1994). This medium contains skim-milk that gives an opaque final medium. Hydrolysis of the casein, which may also be due to acid production, results in a clear zone around the fungal colony.

(b) Bacteria: Modified O'reilly and Day (1983) solid medium was employed for testing proteolytic activity of bacteria. After autoclaving the medium, sterile skim-milk (10% solution of powdered defatted milk in water was sterilized separately) was added to the medium at the rate of 5 ml per 100 ml of medium before pouring the medium into Petri-plates. The tested bacteria were inoculated in the center of Petri-plates and incubated at 35ºC for 24-48 hours. After incubation complete degradation of milk protein was seen as a clear zone in the somewhat opaque agar around colonies. The diameter of the clear zone (mm) was measured.

3- Urease

(a) Fungi: The ability of fungal strains to produce urease enzyme was detected using urease medium described by Paterson and Bridge (1994). It consists of an agar medium containing urea and a pH indicator. Hydrolysis of urea results in free ammonia turning the medium from yellow to red or purple.

(b) Bacteria: The urease activity of bacterial isolates was tested in nutrient broth medium. Isolates capable of producing urease turned the colour of the medium to red-pink colour (Cheesbrough 1992).

4- Catalase

It was detected by pouring 2-3 ml of hydrogen peroxide solution into test tube. Then a good growth of the test bacteria or fungi was removed using a sterile needle and immersed in the hydrogen peroxide solution. Evolution of O2 bubbles indicates a positive result (Cheesbrough 1992).

5- Coagulase

Coagulase causes plasma to clot by converting fibrinogen to fibrin. The ability of fungal and bacterial isolates to produce coagulase enzyme was detected by using diluted human plasma according to the method described by Cheesbrough (1992). Free coagulase produced by bacteria or fungi converts fibrinogen to fibrin by activating a coagulase-reacting factor found in plasma. Free coagulase is detected by the appearance of a fibrin clot in the test tube. If no clotting occurred the tubes were examined every 30 min. for up to 6 hours. Most strains of *S. aureus* produce a fibrin clot within 1 hour of incubation. There should be no fibrin clot in the negative control tube.

Results and Discussion

Thirteen fungal isolates representing 7 species of 4 genera, isolated from human eye infections at Taiz City, Yemen, were tested for their abilities to produce some extracellular enzymes. All isolates were able to produce lipase and catalase, while only 10 and 4 isolates exhibited protease and

urease abilities respectively. Only 1 isolate could produce coagulase enzyme (Table 1). In this respect, Muhsin *et al*. (1997) tested 123 isolates of 14 species of dermatophytes and yeasts for their ability to produce several enzymes and found that lipase and phospholipase were detected in all the species except *Trichophyton violaceum*. In another study of Muhsin and Salih (2001), five species of dermatophytes and eleven species of other fungi were screened for keratinase, proteinase, lipase and amylase. Three dermatophytes were capable of producing lipase, protease and amylase, but the non-dermatophytic species produced more activities than the dermatophytes particularly *Curvularia* isolates which showed the highest protease and amylase activities and *A. parasiticus* showing the highest activity of lipase and amylase.

The ability of 95 bacterial isolates to produce extracellular enzymes was also tested. Most of these isolates could produce catalase (89.5% of total isolates), protease (86.3%) and coagulase (53.7%). Urease and lipase were detected only in 38.9% and 30.5% of the isolates respectively (Table 2). In this respect, Feng *et al.* (2011) screened 56 bacterial strains isolated from the intestine of silkworm larvae for lipase production and noticed that only nine were lipase producers. Mus and Adebyo-Tayo (2012) screened 37 bacteria and 17 fungi isolated from different environmental samples for lipase production and observed that *Acinetobacter* sp. and *Trichoderma* sp. had the highest activity.

1- Lipase production:

From the positive fungal isolates, 10 (76.9%) exhibited high lipase production and these were related to *A. flavus, A. oryzae, A. niger* and *C. lunatus*. Two isolates belonging to *A. awamori* and *P. griseofluvum* were moderate producers whereas *Candida albicans* isolate was weak (Table 1). In this respect, Barakat and Abdel-Sater (1999) testing 105 fungal isolates isolated from butter, found that 69 isolates showed lipolytic activity among which 14 exhibited the highest production. Twenty-four could produce the enzyme with moderate degree of activity and 31 were weak. Many of fungi were also able to produce lipolytic enzymes such as *A. fumigatus* (Hankin and Anagnostakeis 1975), *Penicillium* species (El-Sheikh 1976), *Aspergillus* spp. (Mohawed *et al*. 1985), *A. niger* (Hofelman *et al*. 1985), *Mucor* sp. and *Candida* sp. (Yasuhiro 1985), *Acremonium implicatum, Cephaliophora tropica, Cladosporium cladosporioides, Fusarium equiseti, F. oxysporum, Graphium putridinis, Mucor circinelloides, M. hiemalis, Penicillium martensii* and *Syncephalastrum racemosum* (Moharram 1989), *Rhizopus oryzae* (Salah *et al*. 2006), *A. niger, A. fumigatus* and *A. flavus* (Brooks and Asamudo 2011).

The current results showed also that only 29 bacterial isolates out of 95 were lipase producers among which 22 isolates exhibited low activity, 6 showed high and 1 moderate activity (Table 2).

Many of bacterial species showed lipolytic activities such as *Pseudomonas aeruginosa* (Morkbak *et al*. 1999), *P. fluorescens* (Al-Saleh and Zahran 1999), *Bacillus megaterium* (Ruiz *et al*. 2002), *Photobacterium lipolyticum* (Ryu *et al*. 2006), *Bacillus, Brevibacterium, Corynbacterium, Staphylococcus, Klebsiella* and *Stenotrophomonas* Feng *et al.* (2011). Evidence exists that staphylococcal lipases like other bacterial lipases, possess a lid-like domain that might be involved in the interfacial activation of these enzymes (Rosenstein and Gotz 2000).). *Helicobacter pylori* is a significant and wide-extended pathogen which produces lipase(s) and phospholipases that seem to play a role in mucous degradation and the release of pro-inflammatory and cytotoxic compounds (Cristian *et al*. 2007).

2- Protease production:

Among 13 fungal isolates tested, 9 isolates could produce protease in low quantity and these belong to *Aspergillus flavus* and *A. oryzae* and one exhibited moderate activity belonging to *A. niger* (Table 1). Some fungi were reported as able to produce protease such as *Fusarium culmorum, F. avenaceum, F. oxysporum* (Urbanek and Yirdaw 1978) and *A. oryzae* (Aleksieva *et al*. 1981). *A. niger* and *Mucor mucedo* (Gnanadoss *et al.* 2011), *Aspergillus* spp. (Radha *et al*. 2012). On the other hand, *C. albicans* most likely does not produce extracellular protease in the human oral cavity (Germaine and Tellefson 1981). Gopinathan *et al*. (2001) observed extracellular proteases produced in-vitro by corneal fungal pathogens, namely *A. flavus* and *F. solani* when collagen was provided as the sole nitrogen source. The extracellular proteases of *A. flavus*, from a severe case of kerititis, were identified (Kranthi *et al*. 2012, Rani and Prasad 2012). Eman and Abdel-Sater (2003) tested 73 isolates for protease production and observed that 30 exhibited strong protease production of which those related to *A. niger, A. flavus, A. terreus* and *A. sydowii* were the most potent, while 19 isolates were moderate producers including *Fusarium oxysporum*, *Cladosporium* and *Penicillium* species, whereas 13 isolates were weak producers. Like the alkaline proteinase of *A. fumigatus*, this enzyme was able to degrade human fibrinogen, and thus may act as a mediator of the severe chronic bronchopulmonary inflammation from

which cystic fibrosis patients suffer (Reichard *et al*. 2006).

Most bacterial isolates tested (82 isolates out of 95) in the current study showed protease activity. Forty isolates were high producers and these were related to *S. aureus* (33 isolates) and *P. aeruginosa* (3) and 1 isolate of each of *Staphylococcus epidermidis*, *Streptococcus viridans*, enterococci and *Bacillus cereus*. Twenty-two isolates exhibited moderate productivity and most of these belonged to *S. aureus*, *S. saprophyticus* and *P. aeruginos*a. The remaining 20 positive isolates showed low-producing ability and most of these were related to *S. epidermidis* and *E. coli*. Two species of bacteria did not show any proteolytic activity on solid medium namely *Alcaligenes* species (3 isolates) and *Acinetobacter* species (1 isolate) (Table 2).

Some protease enzymes were reported earlier by *Bacillus subtilis* (Kirshnaveni *et al*. 2012), *B. cereus* (Abou-Elela *et al*. 2011, Smita *et al*. 2012). The finding that clinical isolates of group B streptococci which elaborated high levels of extracellular protease may indicate that the production of several different factors may determine the virulence of these organisms (Straus *et al*. 1980). Some authors regarded protease as the main virulence factor among all of the extracellular factors. It has been suggested that proteolytic enzymes of fish pathogens such as *Flavobacterium columnare* (Griffin 1987) participate in causing massive tissue damage in the host and contribute to the invasion characteristic of the pathology.

Staphylococcus aureus produces four major extracellular proteases: staphylococcal serine protease, cysteine protease, metalloprotease and staphopain (Anna and Staffan 2002). *S. aureus* strains isolated from the colonized skin lesions of patients with acute-phase atopic dermatitis were reported to produce various extracellular proteolytic enzymes and these results imply that staphylococcal proteinases may contribute to the pathogenicity of atopic dermatitis (Miedzobrodzki *et al*. 2002).

An extracellular protease was purified from *Pseudomonas* strains (Zeng *et al*. 2003). Also, alkaline protease has been derived from *Bacillus* strains (Adinarayana and Ellaiah 2004). *P. aeruginosa* protease causes corneal epithelial erosions, indicating its likely activity as a virulence-promoting factor in *Pseudomonas* keratitis (Mary *et al*. 2005).

3- Urease production:

The 4 positive fungal isolates had low or moderate enzyme production and these belong to *A. flavus, A. oryzae* and *P. griseofulvum* (Table 1). Urease was detected earlier in *A. niger* (Smith *et al*. 1993), *Sporobolomyces roseus* (Jahns 1995), *T. mentagrophytes* (Mahmoud *et al*. 1996), *Aspergillus* species, *Alternaria alternata* and *Candida* (Fariba *et al*. 2006). Ammonia and enzymatically active urease released from spherules during the parasitic cycle of *Coccidioides posadasii* contribute to host tissue damage, which exacerbates the severity of coccidioidal infection and enhances the virulence of this human respiratory pathogen (Fariba *et al*. 2006).

S. epidermidis, S. saprophyticus, B. cereus and *P. aeruginosa* were the most active for producing urease. All isolates of *S. viridans, S. pyogens, B. coagulans* and *Alcaligenes* sp. could not produce urease (Table 2). *Streptococcus thermophilus* possesses a urease which converts urea into ammonia and carbon dioxide (Juillard *et al*. 1988). These results were greatly similar to those obtained by Matteuzzi and Crociani (1973), who reported that most strains (about 74%) of *Bifidobacterium suis* produced urease enzyme. Twenty-four lactose-fermenting, urease-producing strains of beta-hemolytic *Escherichia coli* were isolated from a variety of clinical materials (Lesher and Jones 1978). *Helicobacter pylori* produces strong urease, which is considered to play a role in the pathogenesis of gastritis and peptic ulcers (Suthienkul *et al*. 1995).

4- Catalase production

The 13 fungal isolates tested were positive for catalase. One isolate could produce the enzyme in high activity, 7 in moderate activity (all belong to *Aspergillus flavus*) and 5 isolates exhibited weak activity representing one isolate of *A. niger* and two of each of *A. oryzae* and *C. albicans* (Table 1). *A. niger* has four catalases, two of which are produced only under conditions of stress (Witteveen *et al*. 1992). Catalases have also been described in *A. nidulans* (Scherer *et al*. 2002) and *Beauveria bassiana* (Ali *et al*. 2012). Among 158 fungal strains screened, 132 strains possess intracellular catalase most of them (108) related to *Penicillium* and *Aspergillus* (Sirbu 2011).

Most bacterial isolates tested (85 out of 95 isolates) had the ability to produce catalase whereas isolates of *S. viridans, S. pyogens,* enterococci, *E. coli* and *Alcaligenes* sp. had no ability. From these, 67 isolates were strong producers and these were related to *Staphylococcus aureus, S. epidermidis, S. saprophyticus, Bacillus coagulans* and *P. aeruginosa*. The rest 18 isolates showed moderate (15) or weak (3) activity (Table 2).

Catalase activity was reported earlier in *Pseudomonas fluorescens* (Himelbloom and Hassan 986), *Bacillus cereus* (Horsburgh *et al*. 2001) and *Psychrobacter piscatarii* (Kimoto *et al*. 2012).

Table 1: Number of fungal isolates tested for their enzymatic activity classified according to their degree of activity.

Species	NIT	Lipase*				Protease**				Urease***				Catalase****				Coagulase*****			
		NIP	H	M	L	NIP	H	M	L	NIP	H	M	L	NIP	St	M	W	NIP	F	M	S
Aspergillus awamori Nakazawa	1	1	0	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0
A. flavus Link	4	4	4	0	0	3	0	0	3	1	0	0	1	4	1	3	0	0	0	0	0
A. niger van Tieghem	1	1	1	0	0	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0
A. oryzae (Ahlb.) Cohn	3	3	3	0	0	3	0	0	3	2	0	1	1	3	0	1	2	0	0	0	0
Candida albicans (Robin) Berkhout	2	2	1	0	1	0	0	0	0	0	0	0	0	2	0	0	2	1	0	1	0
Cochliobolus lunatus Nelson & Haasis	1	1	1	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0
Penicillium griseofulvum Thom	1	1	0	1	0	1	0	0	1	1	0	0	1	1	0	1	0	0	0	0	0
Total	13	13	10	2	1	10	0	1	9	4	0	1	3	13	1	7	5	1	0	1	0
Percentage of positive cases		100				76.9				30.8				100				7.7			

NIT= Number of isolates tested; NIP= Number of positive isolates; High activity (H) = more than 11 mm; Moderate (M) = 6-10 mm; Low (L) = less than 5 mm.

*Expressed as the depth of visible precipitate in test tube.

** Expressed as the diameter of the clear zone surrounding a colony growth.

***Expressed as the intensity of red-pink color in the tube.

****Expressed as the intensity of effervescence: (St= strong; M= moderate; W= weak).

*****Expressed as the time required to produce clotting of the plasma: (F= fast; M= moderate; S= slow).

Table 2: Number of bacterial isolates tested for their enzymatic activity classified according to their degree of activity.

Species	NIT	Lipase*				Protease**				Urease***				Catalase****				Coagulase*****			
		NIP	H	M	L	NIP	H	M	L	NIP	H	M	L	NIP	St.	M	W	NIP	F	M	S
Staphylococcus aureus Rosenbach	44	13	0	0	13	44	33	11	0	3	0	0	3	44	35	9	0	44	44	0	0
*Staphylococcus epidermidis (*Winslow & Winslow) Evans	20	6	3	0	3	16	1	1	14	17	0	11	6	20	15	4	1	0	0	0	0
Staphylococcus saprophyticus (Fairbr.) Shaw, Stitt & Cowan	5	1	0	0	1	2	0	2	0	4	0	1	3	5	4	0	1	0	0	0	0
Streptococcus viridans (group)	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Streptococcus pyogenes Rosenbach	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Enterococci	1	1	1	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Nocardia sp.	1	0	0	0	0	1	0	0	1	1	1	0	0	1	0	0	1	0	0	0	0
Bacillus cereus Frank. & Frank.	3	1	0	0	1	2	1	0	1	2	1	0	1	3	2	1	0	2	0	0	2
Bacillus coagulans Hammer	1	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	1	0	1	0
Pseudomonas aeruginosa (Schr.) Migula	1	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	1	0	1	0
Escherichia coli (Migula) Castell. & Chalm.	4	0	0	0	0	4	0	1	3	1	1	0	0	0	0	0	0	0	0	0	0
Alcaligenes sp.	3	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	3	1	2	0
Acinetobacter sp.	1	1	1	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0
Proteus mirabilis Hauser	1	1	0	1	0	1	0	1	0	1	1	0	0	1	1	0	0	0	0	0	0
Total	95	29	6	1	22	82	40	22	20	40	10	13	17	85	67	15	3	51	45	3	3
Percentage of positive isolates		30.5				86.3				42.1				89.5				53.7			

Legends are the same as in Table 1.

5- Coagulase production

From 13 fungal isolates tested for coagulase, only *C. albicans* (1 isolate) exhibited moderate activity (Table 1). In this respect, Alghalibi (2000) reported few number of fungal isolates (11 out of 79 isolates) able to produce coagulase and these are related to *A. terreus, A. ochraceus, C. albicans, Candida* species, *Fusarium solani* and *Trichothecium roseum* and most of them had very weak coagulase activity. Also, in the study of Rodrigues *et al*. (2003) all *C. krusei* isolates were coagulase negative, but most *C. albicans* and *C. tropicalis* isolates produced coagulase. Yigit *et al.* (2011) examined the coagulase activity of 48 *Candida* isolates isolated from patients and noticed that all isolates had coagulase activity.

From the 95 bacterial isolates tested for coagulase, 51 isolates were able to produce the enzyme. Forty- five of the positive isolates showed high production for the enzyme. These were *S. aureus* (44 isolates), *B. cereus* (2), *B. coagulans* (1), *P. aeruginosa* (1) and *Alcaligenes* sp. (3). *S. aureus* was the most active for coagulase production (Table 2). Thirty-five coagulase negative *Staphylococcus* isolates cultured from corneal ulcer were speciated and *S. epidermidis* was the commonest (Manikandan *et al*. 2005).

Twenty-four strains of the 27 outbreak-associated Methicillin-resistant *S. aureus* (MRSA) showed low expression of protein A and high expression of coagulase. Conversely, sporadic strains generally gave higher levels of protein A and a wide variety of coagulase reactions (Roberts and Gaston 1987). Both coagulase and pigmentation, however, lead not only to maintained viability of a capsule-lacking *S. aureus*, but also to its proliferation in the peritoneal macrophages. Iron was found to enhance the virulence of this bacterium, but iron enhancement was contingent on the presence of either pigmentation or coagulase or preferably both (Salamah 1992). On contrast, 18 of *S. aureus* strains isolated from clinical specimens showing negative reaction for coagulase (Mlynarczyk *et al*. 1997).

An identifying characteristic of *S. aureus* is the production of coagulase (Carter *et al*. 2003) however, occurrence of coagulase-negative *S. aureus* strains may lead to problems in their identification and the necessity of an application of other methods like clumping factor, protein A or biochemical reactions (Mlynarczyk *et al*. 1998).

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