Available online: journal.ipb.ac.id/index.php/jphpi DOI: http://dx.doi.org/10.17844/jphpi.v20i2.17907

AKTIVITAS ANTIBAKTERI DARI MELANIN TINTA SOTONG DAN CUMI-CUMI

Yuspihana Fitrial*, Iin Khusnul Khotimah
Program Studi Teknologi Hasil Perikanan, Fakultas Perikanan dan Kelautan, Universitas Lambung Mangkurat. Jl. A.Yani Km 36 Kotak Pos 6 Banjarbaru 70714, Kalimantan Selatan
Telepon/Fax: 0511-4772124
*Korespodensi: *yuspihana@gmail.com*
Diterima: 30 Mei 2017/ Disetujui: 6 Agustus 2017

Cara sitasi: Fitrial Y, Khotimah IK. 2017. Aktivitas antibakteri dari melanin tinta sotong dan cumi-cumi. *Jurnal Pengolahan Hasil Perikanan Indonesia*. 20(2): 266-274.

Abstrak

Kelas Cephalopoda (seperti cumi-cumi dan sotong) memiliki tinta sebagai pertahanan dirinya. Penelitian ini bertujuan untuk membandingkan aktivitas antibakteri melanin dari tinta sotong (*Sepia* sp.) dengan tinta cumi-cumi (*Loligo* sp.) terhadap *Escherichia coli*. Ekstraksi dan pemurnian terhadap tinta sotong dan cumi-cumi dilakukan untuk mendapatkan melanin dengan menggunakan HCl 0,5 M secara mekanik. Melanin yang diperoleh diuji aktivitasnya terhadap *E. coli* dengan metode kontak langsung antara melanin dan *E. coli* di dalam *nutrient broth*. Total mikroba dihitung dengan metode hitungan cawan. Tinta yang berasal dari *Sepia* sp. ataupun *Loligo* sp. juga diuji aktivitasnya. Hasil penelitian menunjukkan bahwa melanin dari tinta sotong dan cumi-cumi memiliki aktivitas penghambatan pada konsentrasi 10 mg/mL dan 20 mg/mL, secara berturut-turut mencapai 99,99% terhadap *E. coli*. Tinta dari kedua jenis Cephalopoda tersebut pada konsentrasi yang sama dengan melanin,tidak menunjukkan adanya aktivitas penghambatan terhadap *E. coli*. Melanin dari *Sepia* sp. memiliki aktivitas antibakteri terhadap *E. coli* lebih tinggi dibandingkan melanin dari *Loligo* sp.

Kata kunci: aktivitas antibakteri, *E. coli*, *Loligo* sp., melanin, tinta, *Sepia* sp.

Antibacterial Activity of Melanin from Cuttlefish and Squid Ink

Abstract

Class Cephalopods (such as squid and cuttlefish) have ink as are notable for their defences. This study aims to compare the antibacterial activity of melanin from cuttlefish ink (*Sepia* sp.) with squid ink (*Loligo* sp.) against *E. coli*. Extraction and purification studies were carried out on *Sepia* and *Loligo* melanin using a hydrochloric acid 0,5M treatment under mechanical.The melanins were obtained and further evaluated their activity by direct contact methods between melanin and *E. coli* in nutrient broth.Total microbes was counted by total plate count.Both inks also was tested their activity against *E. coli*. The results showed that melanin from cuttlefish and squid inks had inhibitory activity at concentrations of 10 mg/ml and 20 mg/ml, respectively reaching 99.99% against *E. coli*.The inks of both Cephalopods at the same concentration as melanin, did not show any inhibitory activity against *E. coli*. The melanin of *Sepia* sp. have a higher antibacterial activity than the melanin of *Loligo* sp.

Keywords: antibacterial activity, cuttlefish, *E. coli*, *Loligo* sp., melanin, *Sepia* sp.

PENDAHULUAN

Kelas Cephalopoda seperti sotong (*Sepia* sp.) dan cumi-cumi (*Loligo* sp.) merupakan komoditi hasil tangkapan perikanan laut yang pemanfaatannya masih sangat terbatas, sementara untuk sotong hanya dikonsumsi dalam bentuk segar. Tinta cumi-cumi ataupun sotong di daerah Kalimantan Selatan yang menjadi daerah pengambilan sampel biasanya dibuang atau tidak dimanfaatkan sebagai bagian dari olahan cumi-cumi.

Tinta cumi-cumi maupun tinta sotong mengandung melanin, protein, lemak dan

glikosaminoglikan. Tinta cumi-cumi dapat berperan sebagai obat pelindung sel pada pengobatan kanker dengan cara kemoterapi, melalui peningkatan jumlah sel leukosit dan sel nucleat sumsum tulang, yang jumlahnya menurun akibat penggunaan obat pembunuh sel tumor tersebut. Melanin dari tinta cumi-cumi mempunyai aktivitas anti-tumor dengan menghambat aktivitas plasmin untuk meningkatkan thromboxan dan meningkatkan sistem imun untuk membunuh sel kanker (Zhong *et al.* 2009). Melanin juga berperan sebagai antioksidan (Lei *et al.* 2007[a]), anti-radiasi (Lei *et al.* 2007[b]), dan anti-rotavirus (Rajaganapathi *et al.* 2007).

Hasil penelitian menyebutkan bahwa tinta sotong dan atau cumi-cumi memiliki aktivitas antibakteri (Nair *et al.* 2011). Aktivitas melanin sendiri sebagai antibakteri belum banyak diungkap. Beberapa peneliti telah melakukan pengujian aktivitas antibakteri hanya terhadap ekstrak dari tinta sotong dan atau cumi-cumi. Nithya *et al.* (2011) meneliti aktivitas antibakteri ekstrak heksan tinta sotong (*Sepia* pharaonis) yang dipurifikasi dengan dietil eter. Hasil penelitian ini menunjukkan ekstrak tersebut memiliki aktivitas penghambatan terhadap *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae* dan *E. coli*. Yuvaraj *et al.* (2015) membuktikan bahwa tinta cumi-cumi (*Loligo* duvauceli) tidak memiliki aktivitas penghambatan terhadap *E. coli*. Hasil penelitian tersebut, dilakukan pengujian aktivitas antibakteri dengan melakukan pendekatan terhadap kemampuan komponen tinta yaitu melanin dalam mengkelat logam. Hasil penelitian Chen *et al.* (2009) menunjukkan bahwa melanin dari tinta cumi-cumi (*Ommastrephes bartrami*) memiliki kemampuan menyerap Cd(II) dan Pb(II) oleh gugus fungsi yang terdapat di molekul melanin. Gugus fungsi tersebut adalah fenolik hidroksil (OH), karboksil (COOH) dan amina (NH). Kemampuan melanin menyerap ion logam inilah yang akan diamati melalui pengujian aktivitasnya terhadap pertumbuhan sel bakteri terutama bakteri Gram negarif seperti *E. coli*. Bakteri Gram negatif pada membran terluar selnya mengandung ion Mg^{2+} dan Ca^{2+} yang berperan penting dalam melindungi kestabilan struktur luar sel.

E. coli merupakan bakteri Gram negatif, bersifat patogen bagi manusia dan umumnya bukan merupakan bakteri indigenous pada ikan (Arias 2009). Adanya *E. coli* pada daging ikan akibat kontaminasi selama pemanenan, pengolahan ataupun penyimpanan. Meskipun demikian beberapa ahli menggolongkannya sebagai salah satu bakteri yang menyebabkan pembusukan pada bahan pangan (Dave dan Ghaly 2011). Penelitian terkait aktivitas antibakteri melanin dari tinta sotong dan cumi-cumi terhadap *E. coli* penting dilakukan untuk mendapatkan informasi tentang potensi melanin jika akan dikembangkan sebagai pengawet alami untuk produk perikanan.

BAHAN DAN METODE

Bahan dan Alat

Bahan yang digunakan adalah (tinta) cumi-cumi dan sotong yang diperoleh dari Pelabuhan Perikanan Muara Kintap Kabupaten Tanah Laut, Kalimantan Selatan. Bahan lain yang digunakan antara lain HCl 0,5M (Merck), aseton (Merck), akuades, Nutrient Broth (NB) (Merck), Nutrient Agar (NA) (Merck), EMBA (Merck), *Syringe filter sterile*-EO (Sartorius Minisart pore size 0,20 µm), Microbact TM GNB12A/B/E, 24E Identification Kits (oxoid) dan alkohol 70%.

Alat yang digunakan pada penelitian ini adalah *refrigerated centrifuge* (Labogene Scanspeed 1580R), *Freeze dryer* (Model Christ alpha 2-4 LD Plus), autoklaf (Pressure Steam Sterilizer Electric Model No.25X-2), *laminar flow* (Biobase), inkubator (Memmert), *coloni counter* (Quebec), *incubator shaker* (Wisd), spektrofotometer (Genesys 10uv) dan peralatan gelas lainnya.

Metode Penelitian

Tahapan penelitian yang dilakukan meliputi isolasi *E. coli* dari daging ikan busuk, ekstraksi dan purifikasi melanin dari tinta sotong dan cumi-cumi. Analisis rendemen dilakukan terhadap ekstrak kasar tinta dan pengujian aktivitas melanin dilakukan terhadap pertumbuhan bakteri uji yaitu *E. coli* serta uji kebocoran sel bakteri uji.

Isolasi *E. coli* dari daging ikan yang busuk

Daging ikan busuk dilarutkan dalam larutan garam fisiologis 0,85% dengan perbandingan 1: 10. Larutan daging sebanyak 1 mL di tumbuhkan dalam media EMB (*Eosin Methylene Blue*) Agar, kemudian diinkubasi selama 24 jam pada suhu 37 °C. Media EMBA merupakan media selektif dan diferensiasi. Eosin akan membedakan antara dua koliform utama, yaitu *E. coli* (koloni kecil dan hijau metalik) dan *Enterbacter aerogenes* (koloni berukuran besar, berwarna merah jambu). *Methylene Blue* secara selektif menghambat Gram positif, sehingga yang dapat tumbuh di media tersebut hanya Gram negatif.

Ekstraksi dan purifikasi melanin dari tinta cumi-cumi

Ekstraksi dan purifikasi melanin pada tinta cumi-cumi dan sotong dilakukan menurut metode Magarelli *et al.* (2010). Tahapan ekstraksi dan purifikasi dilakukan dalam media asam. Preparasi tinta dilakukan dengan mengambil habis tinta dari kantong tinta segar. Tinta sebanyak 50 g ditambahkan 100 mL HCl 0,5M dalam kondisi kedap cahaya. Larutan diaduk dengan menggunakan *magnetic stirrer* selama 30 menit, selanjutnya disimpan selama 24 jam pada suhu 10 °C. Endapan dipisahkan dari supernatan dengan menggunakan sentrifius (10.000 rpm pada suhu 5 °C selama 15 menit). Endapan (padatan) dicuci atau disuspensikan kembali dengan larutan HCl 0,5M sebanyak 3 kali, dilanjutkan dengan akuades, aseton dan terakhir dengan akuades. Tahap selanjutnya dilakukan liofilisasi selama kurang lebih 24 jam untuk memisahkan pelarut hingga diperoleh melanin kering dan di simpan dalam *freezer* sebelum dilakukan pengujian lebih lanjut. Perlakuan tinta (kontrol), tinta diambil dari kantong tinta, lalu dilakukan liofilisasi dengan *freeze dryer* seperti sampel melanin.

Pengujian aktivitas melanin terhadap bakteri uji

Pengujian aktivitas melanin dilakukan dengan metode kontak langsung antara melanin dengan bakteri uji dalam media cair nutrient broth (NB) (modifikasi dari Murhadi (2002)). Pengujian dilakukan dengan membuat seri pengujian di dalam tabung kecil berisi 2,970 mL NB steril ditambah 0,030 mL suspensi bakteri uji sehingga total larutan dalam tabung uji 3,000 mL. Melanin (dalam bentuk serbuk) ditambahkan ke dalam tabung uji sehingga konsentrasi melanin dalam tabung 0,000; 0,002; 0,006; 0,010 g/mL. Pembuatan seri tabung uji ke-1 (konsentrasi melanin 0,000 g/mL), digunakan 2,970 mL NB steril + 0,000 g melanin. Tabung seri ke-2 (konsentrasi melanin 0,002 g/mL), dibuat dengan cara menambahkan 2,964 mL NB steril + 0,006 g melanin, dan seterusnya.

Bakteri uji yang telah disegarkan kemudian disiapkan dan diinkubasi 24 jam (10^8-10^9 CFU/mL) pada 37 °C, lalu diencerkan 10 kali. Tabung uji tersebut diinokulasikan dengan 0,030 mL suspensi bakteri uji, dikocok dengan alat vortex selama 1-2 menit, kemudian diinkubasi pada *incubator shaker* suhu 37 °C selama 24 jam. Perhitungan jumlah bakteri dilakukan dengan metode hitungan cawan (TPC, *Total Plate Count*).

Persentase penghambatan bakteri ditentukan dengan modifikasi metode Cappaso *et al.* (1995) yang dinyatakan: 100 – (Nt x 100/No), Nt adalah jumlah bakteri CFU/mL dalam perlakuan penambahan melanin, sedangkan No adalah jumlah bakteri CFU/mL dalam kontrol (inokulum awal).

Pengujian aktivitas melanin terhadap pertumbuhan *E. coli*

Pengujian penghambatan melanin terhadap pertumbuhan *E. coli* dilakukan dengan cara yang sama dengan pengujian aktivitas melanin di atas, yaitu dengan konsentrasi 0,010 g/mL (yaitu konsentrasi melanin dimana persen penghambatan relatif nya terhadap jumlah mikroba awal mendekati 100%). Pengamatan dilakukan per tiga jam selama 24 jam.

Pengujian kebocoran sel bakteri uji

Pengujian ini untuk melihat akibat dari aktivitas melanin tinta sotong terhadap mikroba uji mengacu pada Bunduki *et al.* (1995). Analisis kebocoran sel dilakukan dengan menggunakan Spektrofotometer *Double Beam* pada panjang gelombang 280

dan 260 nm. Panjang gelombang 280 nm digunakan untuk mengukur kadar nitrogen dari protein sel, sedangkan panjang gelombang 260 nm untuk mengukur kadar nitrogen asam nukleat sel.

Kultur murni sebanyak 10 mL disentrifus pada 10.000 rpm selama 10 menit. Filtrat dibuang lalu ditambahkan 5 mL larutan garam fisiologis (0,85% NaCl) dalam endapan sel pada tabung reaksi, kemudian divorteks agar sel homogen dalam larutan fisiologis. Selanjutnya ditambahkan melanin dengan konsentrasi 0; 0,005; 0,010; 0,015; 0,020 g/mL dan dibiarkan pada suhu kamar selama 24 jam. Suspensi kemudian disentrifus pada 10.000 rpm selama 10 menit dan supernatan disaring dengan kertas saring (*Syringe filter sterile* 0,20 μm) untuk memisahkan selnya. Analisis dilakukan dengan mengamati OD (*Optical Density*) dari supernatan bebas sel.

HASIL DAN PEMBAHASAN

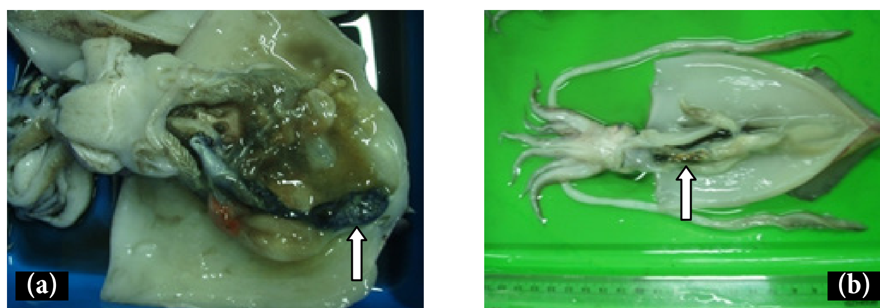
Isolasi *E. coli*

Koloni yang diduga *E. coli* yaitu yang berwarna hijau metalik diuji sifat biokimianya dengan menggunakan *microbact identification kits* (oxoid). Hasil pengujian dengan *microbact kit* menunjukkan lisin (+), ornitin (+), H_2S (-), glukosa (+), manitol (+), xilosa (+), ONPG (+), Indol (+), urease (-), V-P (-), citrate (-), dan TDA (-). Hasil identifikasi menunjukkan *Escherichia coli* (96,39%).

Rendemen Tinta dari *Sepia* sp. dan *Loligo* sp.

Sepia sp. memiliki kantong tinta yang panjang dan besar, sementara kantong tinta *Loligo* sp. berukuran kecil sehingga tinta yang dihasilkan juga lebih sedikit (Gambar 1). Tinta yang terdapat dalam kantong sangat ditentukan oleh kondisi terakhir sebelum ditangkap, jika sebelum ditangkap sudah banyak tinta yang dikeluarkan maka hanya sedikit yang tersisa di kantong. Analisis rendemen dari tinta berdasarkan perbandingan berat kantong (berisi tinta) terhadap berat badan per ekor yang dihasilkan oleh kedua sampel. Berat utuh *Loligo* sp. yaitu 116,6±40,36 g dan berat kantong tinta 0,6±0,1 g, sedangkan *Sepia* sp. memiliki berat utuh 173,0±19,6 g dan berat kantong tinta 4,0±1,4 g. Hasil analisis rendemen terlihat bahwa jenis *Sepia* memiliki rendemen tinta yang lebih besar (2,3%) dibandingkan tinta dari jenis *Loligo* (0,5%).

Nair *et al.* (2011) menyatakan bahwa tinta *Sepia* terdiri atas granula melanin dalam media yang kental tidak berwarna. Pigmen melanin diolah dalam sel mature kelenjar tinta, terutama pada bagian dasar kantong tinta yang terus-menerus memproduksi tinta. Akhir proses pematangan, sel-sel kelenjar tinta menyimpannya dalam kantong tinta yang berperan sebagai penampung. Setiap kantung tinta *Sepia* mengandung ~ 1 g melanin (Derby 2014), dan banyaknya melanin ~ 15% dari berat basah total tinta (Wang *et al.* 2014). Melanin *Sepia* terbentuk oleh banyak kelompok agregat. Agregat-agregat ini terbentuk juga oleh butiran bola kecil dengan distribusi ukuran yang berbeda. Diameter butiran kecil berkisar 100-200 nm (Mbonyiryivuze *et al.*2015). Ukuran butiran bola melanin pada cumi-cumi, berkisar antara



Gambar 1 Jenis chepalopoda dan kantong tinta yang digunakan pada penelitian ini. (a) jenis sotong (*Sepia* sp.), kantong tinta dari jenis *Sepia* sp., (b) jenis cumi-cumi (*Loligo* sp.), kantong tinta dari jenis *Loligo* sp.

50–150 nm (Chen *et al.* 2009). Berdasarkan hasil pengamatan, tinta *Loligo* sp. memiliki tekstur yang halus, sedangkan tinta *Sepia* sp. memiliki tekstur yang kasar.

Aktivitas Melanin Tinta *Sepia* sp. dan *Loligo* sp.

Pengaruh tinta dan melanin dari *Sepia* sp. dan *Loligo* sp. pada beberapa konsentrasi terhadap pertumbuhan *E. coli* dapat dilihat pada Tabel 1. Konsentrasi melanin semakin tinggi menghasillkan aktivitas penghambatan terhadap *E. coli* juga semakin besar. Melanin *Sepia* sp. pada konsentrasi 0,002 g/mL terlihat tidak ada aktivitas penghambatan, sementara pada konsentrasi yang lebih tinggi yaitu 0,006 g/mL terlihat jumlah koloni setelah inkubasi 24 jam tidak berbeda dengan jumlah awal (sebelum inkubasi). Hal ini menunjukkan pada konsentrasi 0,006 g/mL sudah terjadi penghambatan terhadap pertumbuhan sel bakteri, hingga 24 jam inkubasi tidak terjadi peningkatan yang berarti pada jumlah sel bakteri.

Konsentrasi yang lebih tinggi yaitu 0,010 g/mL, melanin mampu membunuh bakteri sehingga setelah inkubasi 24 jam hanya ada 1 sel yang hidup (penghambatan mencapai 99,99%). Melanin dari *Sepia* sp. memiliki aktivitas penghambatan yang lebih besar dibandingkan dengan melanin dari *Loligo* sp. Pada konsentrasi yang lebih rendah (1/2 dari konsentrasi melanin *Loligo* sp), melanin dari *Sepia* sp. mampu menghambat hampir 100%. Tabel 1 menunjukkan *E. coli* lebih sensitif terhadap melanin dari *Sepia* sp. dibandingkan dengan dari *Loligo* sp.

Tinta *Sepia* sp. dengan konsentrasi yang lebih tinggi dibandingkan dengan melaninnya tidak memiliki aktivitas penghambatan, sementra tinta *Loligo* sp. pada konsentrasi yang sama dengan melanin tidak memiliki aktivitas penghambatan terhadap *E. coli*. Hal ini disebabkan karena yang terkandung di dalam tinta tidak hanya melanin, melainkan ada komponen lain misalnya protein, lemak dan glikosaminoglikan yang diduga tidak memiliki aktivitas penghambatan terhadap *E. coli*, akan tetapi sebaliknya dapat meningkatkan pertumbuhan *E. coli*.

Tabel 1 Pertumbuhan *E. coli* pada media NB yang mengandung tinta dan melanin

Sumber		Konsentrasi (g/mL)	Jumlah *E. coli* (CFU/mL)		% Penghambatan relatif terhadap jumlah mikroba awal [100-(Ntx100/No)]	Log Peng-hambatan
			Inkubasi 0 jam (Nol)	Inkubasi 24 jam (Nt)		
Sepia sp.	Tinta	0	$8,6 \times 10^5$	$1,6 \times 10^{10}$	-	-4,27
		0,013	$8,6 \times 10^5$	$6,2 \times 10^9$	-	-3,86
		0,017	$8,6 \times 10^5$	$5,6 \times 10^9$	-	-3,81
		0,020	$8,6 \times 10^5$	$4,4 \times 10^9$	-	-3,71
	Melanin	0,002	$8,6 \times 10^5$	$1,2 \times 10^{10}$	-	-5,14
		0,006	$8,6 \times 10^5$	$6,0 \times 10^5$	30,23	0,16
		0,010	$8,6 \times 10^5$	$1,0 \times 10^0$	99,99	5,93
Loligo sp.	Tinta	0	$8,6 \times 10^5$	$3,1 \times 10^{10}$	-	-4,56
		0,013	$8,6 \times 10^5$	$8,6 \times 10^9$	-	-4,00
		0,017	$8,6 \times 10^5$	$2,3 \times 10^9$	-	-3,43
		0,020	$8,6 \times 10^5$	$1,0 \times 10^9$	-	-3,06
	Melanin	0,013	$8,6 \times 10^5$	$2,1 \times 10^4$	97,56	1,61
		0,017	$8,6 \times 10^5$	$2,1 \times 10^3$	99,76	2,61
		0,020	$8,6 \times 10^5$	$1,4 \times 10^2$	99.87	3,79

Keterangan: (-) nilai negatif artinya tidak ada penghambatan dan terjadi peningkatan

Aktivitas Melanin *Sepia* sp. terhadap Pertumbuhan *E. coli*

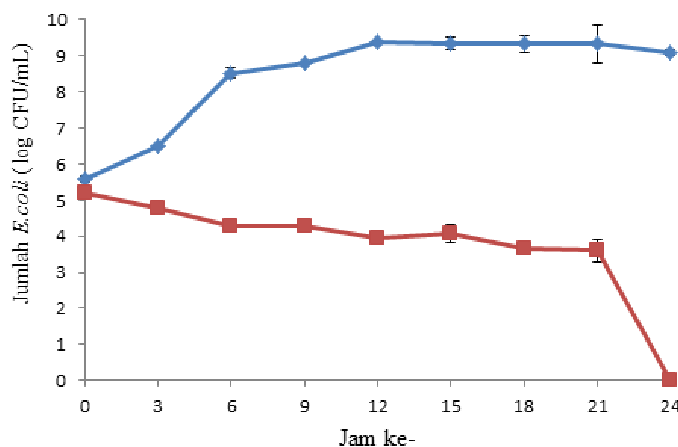
Aktivitas melanin dari *Sepia* terhadap pertumbuhan *E. coli* dapat dilihat pada Gambar 2. Pertumbuhan *E. coli* yang diberi perlakuan melanin dari *Sepia* sp. sebanyak 10 mg/mL terlihat jumlah koloninya yang hidup mengalami penurunan lebih dari 1 $\log_{10}$ setelah 6 jam inkubasi dan penurunan tersebut terus berlanjut hingga 2 $\log_{10}$ setelah 21 jam inkubasi. Akibat aktivitas melanin, terjadi perpanjangan fase adaptasi dan menyebabkan terjadinya penurunan jumlah koloni yang hidup, setelah 24 jam inkubasi, tidak ada lagi koloni yang hidup (Gambar 2). Hal ini menunjukkan bahwa selain memperpanjang fase adaptasi, melanin juga mempercepat fase kematian pada sel bakteri.

Pertumbuhan *E. coli* tanpa perlakuan melanin terjadi peningkatan jumlah koloni yang hidup lebih dari 4 $\log_{10}$ (dari 10^5 menjadi 10^9). Pertumbuhan pada 3 jam pertama memasuki fase adaptasi dan pertumbuhan awal yang dilanjutkan dengan fase logaritmik setelah 6 jam inkubasi. Setelah 6 jam terjadi peningkatan jumlah koloni yang cepat hingga 2 $\log_{10}$, dan terus meningkat hingga jam ke-12 walaupun hanya sedikit terjadi pertambahan populasi (kurang dari 1 $\log_{10}$). Pertumbuhan memasuki fase stasioner setelah jam ke-12 hingga jam ke-24.

Vasantharaja *et al.* (2014) melaporkan bahwa ekstrak metanol tinta *Sepiella inermis* dengan menggunakan GC-MS menunjukkan adanya campuran dari struktur oligomer yang merupakan gabungan antara dihidroksi indol-2-asam karboksilat dan dihidroksiindol. Ekstrak metanol ini memiliki aktivitas penghambatan terutama terhadap bakteri Gram negatif misalnya *Proteus vulgaris*, *Pseudomonas aeruginosa* dan *E. coli*. Neifar *et al.* (2009) melaporkan bahwa dihidroksiindol dan asam dikarboksilat dari *Sepia officinalis* memiliki aktivitas penghambatan terhadap mikroba.

Melanin merupakan tirosinase yang telah diidentifikasi terdapat di dalam tinta cumi-cumi (Derby 2014). Tinta cumi-cumi terdiri atas suspensi granula eumelanin di dalam media yang viscous dan tidak berwarna. Eumelanin bersifat heterogen, umumnya polimer yang tidak larut yang berkembang melalui oksidasi enzimatis dari asam amino tirosin. Produksi eumelanin di dalam sel pigmen terjadi di dalam organel khusus yang disebut melanosome. Eumelanin tersusun dari unit 5,6-dihidroksiindol (DHI) sekitar 20% dan unit 5,6-dihidroksiindol-2-asam karboksilat (DHICA) (Magarelli *et al.* 2010). Eumelanin alami dilaporkan merupakan molekul pigmen yang dapat mengadsorpsi logam pada konsentrasi tinggi. Kemampuan berikatan eumelanin dengan sisi dari logam merupakan parameter penting untuk memahami komplek logam-melanin (Lei *et al.* 2008; Chen *et al.* 2009).

Dinding sel bakteri mengandung banyak jenis kation termasuk Mg^{2+}, Ca^{2+}, Na+, dan K^+. Ion-ion ini bertanggung jawab atas berbagai aktivitas bakteri, termasuk kerja enzim,



Gambar 2 Kurva pertumbuhan *E. coli* yang diinkubasi dengan melanin *Sepia* sp. ◆ = kontrol (tanpa melanin), ■ = ditambah melanin.

Tabel 2 Nilai OD dari supernatan bebas sel *E. coli* yang diinkubasi dengan melanin dari *Sepia* sp.

Konsentrasi melanin (g/ml)	OD pada 260 nm	OD pada 280 nm
0	0,015	0,018
0,005	0,212	0,234
0,010	0,277	0,307
0,015	0,314	0,384
0,020	0,398	0,464

pengaturan metabolik dan menjaga integritas lapisan luar. Mg^{2+} dan ion Ca^{2+} khususnya, berperan penting dalam melindungi kestabilan struktur luar (Ferrero *et al.* 2007; Peshenko *et al.* 2007). Lapisan paling luar dari membran luar pada bakteri Gram negatif adalah lipopolisakarida (LPS), secara individu, molekul ini bermuatan negatif. Kation divalen membantu menstabilkan dan menjaga integritas membran luar dengan mengikat molekul LPS yang berdekatan. Kation *divalent* ini berfungsi sebagai jembatan garam berikatan dengan molekul lipid yang bermuatan negatif (Raetz *et al.* 2007). Membran luar pada sel bakteri berfungsi sebagai penghalang masuknya senyawa-senyawa yang tidak diperlukan sel (seperti bakteriosin, enzim dan senyawa hidrofobik). Jika kation tersebut dapat diadsorpsi oleh gugus fungsi melanin, maka sistem metabolisme sel bakteri akan terganggu, akibatnya pertumbuhan sel bakteri juga terganggu.

Sahalan *et al.* (2013) menjelaskan ion Ca^{2+} dan Mg^{2+} berperan melindungi membran terluar pada sel bakteri terhadap Polymyxin B yang berinteraksi dengan kation divalent, dengan mengganti kation dari tempat pengikatannya di molekul lipopolisakarida (LPS). Hal ini menyebabkan disorganisasi komponen membran luar bakteri Gram negatif, akibat lepasnya komponen LPS dari permukaan bakteri yang menyebabkan kebocoran membran dan akhirnya menyebabkan kematian sel. Ca^{2+} telah terbukti lebih efektif dalam melindungi sel bakteri dibandingkan Mg^{2+}.

Pengikatan gugus fungsi dari melanin yaitu gugus hidroksil fenolik (OH), karboksil (COOH) dan grup amina (NH) (Chen *et al.* 2009) terhadap ion Ca^{2+} dan Mg^{2+} pada membran luar mengakibatkan kebocoran pada membran bakteri Gram negatif dalam hal ini diwakili oleh *E. coli*. Kebocoran yang disebabkan oleh melanin terhadap sel *E. coli* dapat dilihat dari dihasilkannya supernatan bebas sel isi sel bakteri setelah diinkubasi selama 24 jam dengan melanin tinta Sepia sp. (Tabel 2). Tabel 2 menunjukkan hubungan antara konsentrasi melanin dengan supernatan bebas sel dari sel *E. coli*. Analisis kebocoran sel dilakukan dengan menggunakan Spektrofotometer *Double Beam* pada panjang gelombang 280 dan 260 nm. Panjang gelombang 280 nm digunakan untuk mengukur kadar nitrogen dari protein sel, sedangkan panjang gelombang 260 nm untuk mengukur kadar nitrogen asam nukleat sel. Semakin tinggi nilai OD baik pada panjang gelombang 260 nm maupun 280 nm menunjukkan semakin besarnya kebocoran sel akibat melanin. Semakin tinggi konsentrasi melanin, semakin besar tingkat kebocoran sel.

Hasil pengamatan terhadap spektrum Infra Red (IR) menggunakan FTIR spektrofotometer dari melanin tinta *Sepia* sp. dan *Loligo* sp. (data tidak dipublikasikan) menunjukkan kedua melanin memiliki pola spektrum yang sama, mengandung gugus fenolik, amina dan karboksil. Intensitas dari masing-masing gugus aktif tersebut yang berbeda diantara keduanya. Gugus fenolik, amina dan karboksil dari melanin *Sepia* sp. memiliki intensitas yang lebih tinggi dibandingkan dengan melanin dari *Loligo* sp. Intensitas ini menunjukkan konsentrasi dari gugus aktif tersebut di dalam melanin. Hal inilah yang diduga mengakibatkan perbedaan aktivitas kedua melanin tersebut terhadap *E. coli*.

KESIMPULAN

Melanin dari tinta sotong (*Sepia* sp.) dan cumi-cumi (*Loligo* sp) memiliki aktivitas penghambatan terhadap *E. coli*. Aktivitas penghambatan terhadap *E. coli* dari melanin tinta sotong lebih tinggi dibandingkan melanin dari cumi-cumi. Tinta sotong dan cumi-cumi pada konsentrasi 0,013-0,020 g/mL tidak memiliki aktivitas penghambatan terhadap *E. coli*.

UCAPAN TERIMA KASIH

Ucapan terima kasih disampaikan kepada Direktorat Riset dan Pengabdian Masyarakat Direktorat Jenderal Penguatan Riset dan Pengembangan, Kementerian Riset, Teknologi dan Pendidikan Tinggi atas bantuan dana penelitian melalui hibah Fundamental (sesuai dengan Surat Perjanjian Penugasan Pelaksanaan Program Penelitian Nomor: 024/SP2H/LT/DRPM/II/2016 tanggal 17 Februari 2016).

DAFTAR PUSTAKA

Arias C. 2009. Chilled and Frozen Raw Fish. Di dalam: Fernandes R (editor) Microbiology Handbook Fish and Seafood. United Kingdom (UK): Leatherhead Food International Ltd.

Bunduki MMC, KJ Flanders and CW Donnelly. 1995. Metabolic and structural sites of damage in heat and sanitizer-injuried population of *Listeria monocytogenes. Journal Food Protect*. 58: 410-415.

Cappaso R, Evidente A, Schivo L, Orru G, Marcialis MA, Cristinzio G. 1995. Antibacterial polyphenols from olive oil mill waste waters. *Journal of Applied. Bacteriology*. 79: 393-398.

Chen S, Xue C, Wang J, Feng H, Wang Y, Ma Q, Wang D. 2009. Adsorption of Pb(II) and Cd(II) by squid *Ommastrephes bartrami* melanin. *Bioinorganic Chemistry and Applications*. 1-7

Dave D, Ghaly AE.2011. Meat spoilage mechanisms and preservation techniques: a critical review. *American Journal of Agricultural and Biological Sciences.* 6(4): 486-510.

Derby CD.2014.Cephalopod ink: production, chemistry, functions and applications. *Marine drugs*. 12: 2700-2730.

Ferrero MA, Martínez-Blanco H, Lopez-VelascoFF, Ezquerro-SáenzC, NavasaN, Lozano S & Rodríguez-Aparicio LB. 2007. Purification and characterization of GlcNAc-6-P 2-epimerase from *Escherichia coli* K92. *Acta Biochimica Polonica*. 54(2): 387-399.

Lei M, Wang JF, Pang L.Wang YM, Chen SG, Xue CH. 2007a. Effects of *Sepia* on the metabolization of blood lipid and antioxidant ability in hyperlipidemia rats. *The Chinese Journalof Marine Drugs.* 3: 30-33.

Lei M, Wang JF,Wang YM, Pang L, Wang Y, Xu W,Xue CH. 2007b. Study of theradio-protective effect of cuttlefish ink on hemopoietic injury. *Asia Pacific Journal of Clinical Nutrition*.16: 239-243.

Lei M, Xue CH, Wang YM, Li ZJ, Xue Y, Wang JF. 2008. Effect of squid ink melanin-Fe on iron deficiency anemia remission. *Journal of Food Science*. 73(8): 207-11.

Mbonyiryivuze A, Nuru ZY, Diop Ngom B, Mwakikunga B, Simon Mokhotjwa Dhlamini SM, Park E, Maaza M. 2015. Morphological and chemical composition characterization of commercial *Sepia* melanin. *American Journal of Nanomaterials*. 3(1): 22-27.

Murhadi. 2002. Isolasi dan Karakterisasi Komponen Antibakteri dari Biji Atung (Parinarium glaberrimum Hassk). [disertasi]. Bogor (ID): Institut Pertanian Bogor.

Nair JR, Pillai D, Joseph SM, Gomathi P, Senan PV, and Sherief PM.2011. Cephalopod research and bioactive substances. *Indian Journal of Geo-Marine Sciences*. 40(1): 13-27.

Neifar A, Rebah FB, Gargouri AF, Abdelmouleh A. 2009. Physicochemical characterization of *Sepia officinalis*ink and the effects of storage conditions on the coagulation process. *Journal of the Marine Biological Association of the UK*. 89(04): 803-807.

Nithya M, Ambikapathy V, Panneerselvam A. 2011. Effect of pharaoh's cuttlefish ink

against bacterial pathogens. *Asian Journal of Plant Science and Research*. 1 (4):49-55

Peshenko IV & Dizhoor AM. 2007. Activation and inhibition of photoreceptor guanylyl cyclase by guanylyl cyclase activating protein 1 (GCAP-1): The functional role of Mg^{2+}/Ca^{2+} exchange in EF-hand domains. *The Journal of Biological Chemistry*. 82(30): 21645-21652.

Raetz C R, Reynolds CM, TrentMS & BishopRE. 2007. Lipid: A modification systems in gram-negative bacteria. *Annual Reviews Biochemistry*. 76: 295-329.

Rajaganapathi J, Thyagarajam SP, Edward JK. 2007. Study on cephalopod's ink for anti-retroviral activity. *Indian Journal of Experimental Biology*. 38: 519-520.

Sahalan AZ, Aziz AHA, Lian H & Ghani MKA. 2013. Divalent cations (Mg^{2+}, Ca^{2+}) protect bacterial outer membrane damage by Polymyxin B. *Sains Malaysiana*. 42(3): 301–306.

Vasantharaja D, Ravitchandirane V and Anandan V. 2014. Anti-microbial activity and spectro-chemical investigation of ink extracts of *Sepiella inermis* (Van Hasselt 1835). *Notulae Scientia Biologicae*. 6(3):273-275

Wang FR, XieZG, YeXQ, Deng SG, Hu YQ, Guo X, Chen SG. 2014. Effectiveness of treatment of iron deficiency anemia in rats with squid ink melanin-Fe. *Food Funct*. 5: 123–128.

Yuvaraj D, Suvasini B, Chellathai T, Fouziya R, Ivo Romauld S and Chandran M. 2015. Anti-bacterial studies on the different body parts of *Loligo* duvauceli. *Journal of Chemical and Pharmaceutical Research*. 7(6):406-408.

Zhong JP, Wang G, Shang JH, Pan JQ, Li K, Huang Y, Liu HZ. 2009. Protective effects of squid ink extract towards hemopoieticinjuries induced by cyclophosphamine. *Marine Drugs*. 7: 9-18.

Available online: journal.ipb.ac.id/index.php/jphpi DOI: http://dx.doi.org/10.17844/jphpi.v20i2.17907

AKTIVITAS ANTIBAKTERI DARI MELANIN TINTA SOTONG DAN CUMI-CUMI

Yuspihana Fitrial*, Iin Khusnul Khotimah
Program Studi Teknologi Hasil Perikanan, Fakultas Perikanan dan Kelautan, Universitas Lambung Mangkurat. Jl. A.Yani Km 36 Kotak Pos 6 Banjarbaru 70714, Kalimantan Selatan
Telepon/Fax: 0511-4772124
*Korespodensi: *yuspihana@gmail.com*
Diterima: 30 Mei 2017/ Disetujui: 6 Agustus 2017

Cara sitasi: Fitrial Y, Khotimah IK. 2017. Aktivitas antibakteri dari melanin tinta sotong dan cumi-cumi. *Jurnal Pengolahan Hasil Perikanan Indonesia*. 20(2): 266-274.

Abstrak

Kelas Cephalopoda (seperti cumi-cumi dan sotong) memiliki tinta sebagai pertahanan dirinya. Penelitian ini bertujuan untuk membandingkan aktivitas antibakteri melanin dari tinta sotong (*Sepia* sp.) dengan tinta cumi-cumi (*Loligo* sp.) terhadap *Escherichia coli*. Ekstraksi dan pemurnian terhadap tinta sotong dan cumi-cumi dilakukan untuk mendapatkan melanin dengan menggunakan HCl 0,5 M secara mekanik. Melanin yang diperoleh diuji aktivitasnya terhadap *E. coli* dengan metode kontak langsung antara melanin dan *E. coli* di dalam *nutrient broth*. Total mikroba dihitung dengan metode hitungan cawan. Tinta yang berasal dari *Sepia* sp. ataupun *Loligo* sp. juga diuji aktivitasnya. Hasil penelitian menunjukkan bahwa melanin dari tinta sotong dan cumi-cumi memiliki aktivitas penghambatan pada konsentrasi 10 mg/mL dan 20 mg/mL, secara berturut-turut mencapai 99,99% terhadap *E. coli*. Tinta dari kedua jenis Cephalopoda tersebut pada konsentrasi yang sama dengan melanin,tidak menunjukkan adanya aktivitas penghambatan terhadap *E. coli*. Melanin dari *Sepia* sp. memiliki aktivitas antibakteri terhadap *E. coli* lebih tinggi dibandingkan melanin dari *Loligo* sp.

Kata kunci: aktivitas antibakteri, *E. coli*, *Loligo* sp., melanin, tinta, *Sepia* sp.

Antibacterial Activity of Melanin from Cuttlefish and Squid Ink

Abstract

Class Cephalopods (such as squid and cuttlefish) have ink as are notable for their defences. This study aims to compare the antibacterial activity of melanin from cuttlefish ink (*Sepia* sp.) with squid ink (*Loligo* sp.) against *E. coli*. Extraction and purification studies were carried out on *Sepia* and *Loligo* melanin using a hydrochloric acid 0,5M treatment under mechanical.The melanins were obtained and further evaluated their activity by direct contact methods between melanin and *E. coli* in nutrient broth.Total microbes was counted by total plate count.Both inks also was tested their activity against *E. coli*. The results showed that melanin from cuttlefish and squid inks had inhibitory activity at concentrations of 10 mg/ml and 20 mg/ml, respectively reaching 99.99% against *E. coli*.The inks of both Cephalopods at the same concentration as melanin, did not show any inhibitory activity against *E. coli*. The melanin of *Sepia* sp. have a higher antibacterial activity than the melanin of *Loligo* sp.

Keywords: antibacterial activity, cuttlefish, *E. coli*, *Loligo* sp., melanin, *Sepia* sp.

PENDAHULUAN

Kelas Cephalopoda seperti sotong (*Sepia* sp.) dan cumi-cumi (*Loligo* sp.) merupakan komoditi hasil tangkapan perikanan laut yang pemanfaatannya masih sangat terbatas, sementara untuk sotong hanya dikonsumsi dalam bentuk segar. Tinta cumi-cumi ataupun sotong di daerah Kalimantan Selatan yang menjadi daerah pengambilan sampel biasanya dibuang atau tidak dimanfaatkan sebagai bagian dari olahan cumi-cumi.

Tinta cumi-cumi maupun tinta sotong mengandung melanin, protein, lemak dan

glikosaminoglikan. Tinta cumi-cumi dapat berperan sebagai obat pelindung sel pada pengobatan kanker dengan cara kemoterapi, melalui peningkatan jumlah sel leukosit dan sel nucleat sumsum tulang, yang jumlahnya menurun akibat penggunaan obat pembunuh sel tumor tersebut. Melanin dari tinta cumi-cumi mempunyai aktivitas anti-tumor dengan menghambat aktivitas plasmin untuk meningkatkan thromboxan dan meningkatkan sistem imun untuk membunuh sel kanker (Zhong *et al.* 2009). Melanin juga berperan sebagai antioksidan (Lei *et al.* 2007[a]), anti-radiasi (Lei *et al.* 2007[b]), dan anti-rotavirus (Rajaganapathi *et al.* 2007).

Hasil penelitian menyebutkan bahwa tinta sotong dan atau cumi-cumi memiliki aktivitas antibakteri (Nair *et al.* 2011). Aktivitas melanin sendiri sebagai antibakteri belum banyak diungkap. Beberapa peneliti telah melakukan pengujian aktivitas antibakteri hanya terhadap ekstrak dari tinta sotong dan atau cumi-cumi. Nithya *et al.* (2011) meneliti aktivitas antibakteri ekstrak heksan tinta sotong (*Sepia* pharaonis) yang dipurifikasi dengan dietil eter. Hasil penelitian ini menunjukkan ekstrak tersebut memiliki aktivitas penghambatan terhadap *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae* dan *E. coli*. Yuvaraj *et al.* (2015) membuktikan bahwa tinta cumi-cumi (*Loligo* duvauceli) tidak memiliki aktivitas penghambatan terhadap *E. coli*. Hasil penelitian tersebut, dilakukan pengujian aktivitas antibakteri dengan melakukan pendekatan terhadap kemampuan komponen tinta yaitu melanin dalam mengkelat logam. Hasil penelitian Chen *et al.* (2009) menunjukkan bahwa melanin dari tinta cumi-cumi (*Ommastrephes bartrami*) memiliki kemampuan menyerap Cd(II) dan Pb(II) oleh gugus fungsi yang terdapat di molekul melanin. Gugus fungsi tersebut adalah fenolik hidroksil (OH), karboksil (COOH) dan amina (NH). Kemampuan melanin menyerap ion logam inilah yang akan diamati melalui pengujian aktivitasnya terhadap pertumbuhan sel bakteri terutama bakteri Gram negarif seperti *E. coli*. Bakteri Gram negatif pada membran terluar selnya mengandung ion Mg^{2+} dan Ca^{2+} yang berperan penting dalam melindungi kestabilan struktur luar sel.

E. coli merupakan bakteri Gram negatif, bersifat patogen bagi manusia dan umumnya bukan merupakan bakteri indigenous pada ikan (Arias 2009). Adanya *E. coli* pada daging ikan akibat kontaminasi selama pemanenan, pengolahan ataupun penyimpanan. Meskipun demikian beberapa ahli menggolongkannya sebagai salah satu bakteri yang menyebabkan pembusukan pada bahan pangan (Dave dan Ghaly 2011). Penelitian terkait aktivitas antibakteri melanin dari tinta sotong dan cumi-cumi terhadap *E. coli* penting dilakukan untuk mendapatkan informasi tentang potensi melanin jika akan dikembangkan sebagai pengawet alami untuk produk perikanan.

BAHAN DAN METODE

Bahan dan Alat

Bahan yang digunakan adalah (tinta) cumi-cumi dan sotong yang diperoleh dari Pelabuhan Perikanan Muara Kintap Kabupaten Tanah Laut, Kalimantan Selatan. Bahan lain yang digunakan antara lain HCl 0,5M (Merck), aseton (Merck), akuades, Nutrient Broth (NB) (Merck), Nutrient Agar (NA) (Merck), EMBA (Merck), *Syringe filter sterile*-EO (Sartorius Minisart pore size 0,20 µm), Microbact TM GNB12A/B/E, 24E Identification Kits (oxoid) dan alkohol 70%.

Alat yang digunakan pada penelitian ini adalah *refrigerated centrifuge* (Labogene Scanspeed 1580R), *Freeze dryer* (Model Christ alpha 2-4 LD Plus), autoklaf (Pressure Steam Sterilizer Electric Model No.25X-2), *laminar flow* (Biobase), inkubator (Memmert), *coloni counter* (Quebec), *incubator shaker* (Wisd), spektrofotometer (Genesys 10uv) dan peralatan gelas lainnya.

Metode Penelitian

Tahapan penelitian yang dilakukan meliputi isolasi *E. coli* dari daging ikan busuk, ekstraksi dan purifikasi melanin dari tinta sotong dan cumi-cumi. Analisis rendemen dilakukan terhadap ekstrak kasar tinta dan pengujian aktivitas melanin dilakukan terhadap pertumbuhan bakteri uji yaitu *E. coli* serta uji kebocoran sel bakteri uji.

Isolasi *E. coli* dari daging ikan yang busuk

Daging ikan busuk dilarutkan dalam larutan garam fisiologis 0,85% dengan perbandingan 1: 10. Larutan daging sebanyak 1 mL di tumbuhkan dalam media EMB (*Eosin Methylene Blue*) Agar, kemudian diinkubasi selama 24 jam pada suhu 37 °C. Media EMBA merupakan media selektif dan diferensiasi. Eosin akan membedakan antara dua koliform utama, yaitu *E. coli* (koloni kecil dan hijau metalik) dan *Enterbacter aerogenes* (koloni berukuran besar, berwarna merah jambu). *Methylene Blue* secara selektif menghambat Gram positif, sehingga yang dapat tumbuh di media tersebut hanya Gram negatif.

Ekstraksi dan purifikasi melanin dari tinta cumi-cumi

Ekstraksi dan purifikasi melanin pada tinta cumi-cumi dan sotong dilakukan menurut metode Magarelli *et al.* (2010). Tahapan ekstraksi dan purifikasi dilakukan dalam media asam. Preparasi tinta dilakukan dengan mengambil habis tinta dari kantong tinta segar. Tinta sebanyak 50 g ditambahkan 100 mL HCl 0,5M dalam kondisi kedap cahaya. Larutan diaduk dengan menggunakan *magnetic stirrer* selama 30 menit, selanjutnya disimpan selama 24 jam pada suhu 10 °C. Endapan dipisahkan dari supernatan dengan menggunakan sentrifius (10.000 rpm pada suhu 5 °C selama 15 menit). Endapan (padatan) dicuci atau disuspensikan kembali dengan larutan HCl 0,5M sebanyak 3 kali, dilanjutkan dengan akuades, aseton dan terakhir dengan akuades. Tahap selanjutnya dilakukan liofilisasi selama kurang lebih 24 jam untuk memisahkan pelarut hingga diperoleh melanin kering dan di simpan dalam *freezer* sebelum dilakukan pengujian lebih lanjut. Perlakuan tinta (kontrol), tinta diambil dari kantong tinta, lalu dilakukan liofilisasi dengan *freeze dryer* seperti sampel melanin.

Pengujian aktivitas melanin terhadap bakteri uji

Pengujian aktivitas melanin dilakukan dengan metode kontak langsung antara melanin dengan bakteri uji dalam media cair nutrient broth (NB) (modifikasi dari Murhadi (2002)). Pengujian dilakukan dengan membuat seri pengujian di dalam tabung kecil berisi 2,970 mL NB steril ditambah 0,030 mL suspensi bakteri uji sehingga total larutan dalam tabung uji 3,000 mL. Melanin (dalam bentuk serbuk) ditambahkan ke dalam tabung uji sehingga konsentrasi melanin dalam tabung 0,000; 0,002; 0,006; 0,010 g/mL. Pembuatan seri tabung uji ke-1 (konsentrasi melanin 0,000 g/mL), digunakan 2,970 mL NB steril + 0,000 g melanin. Tabung seri ke-2 (konsentrasi melanin 0,002 g/mL), dibuat dengan cara menambahkan 2,964 mL NB steril + 0,006 g melanin, dan seterusnya.

Bakteri uji yang telah disegarkan kemudian disiapkan dan diinkubasi 24 jam (10^8-10^9 CFU/mL) pada 37 °C, lalu diencerkan 10 kali. Tabung uji tersebut diinokulasikan dengan 0,030 mL suspensi bakteri uji, dikocok dengan alat vortex selama 1-2 menit, kemudian diinkubasi pada *incubator shaker* suhu 37 °C selama 24 jam. Perhitungan jumlah bakteri dilakukan dengan metode hitungan cawan (TPC, *Total Plate Count*).

Persentase penghambatan bakteri ditentukan dengan modifikasi metode Cappaso *et al.* (1995) yang dinyatakan: 100 – (Nt x 100/No), Nt adalah jumlah bakteri CFU/mL dalam perlakuan penambahan melanin, sedangkan No adalah jumlah bakteri CFU/mL dalam kontrol (inokulum awal).

Pengujian aktivitas melanin terhadap pertumbuhan *E. coli*

Pengujian penghambatan melanin terhadap pertumbuhan *E. coli* dilakukan dengan cara yang sama dengan pengujian aktivitas melanin di atas, yaitu dengan konsentrasi 0,010 g/mL (yaitu konsentrasi melanin dimana persen penghambatan relatif nya terhadap jumlah mikroba awal mendekati 100%). Pengamatan dilakukan per tiga jam selama 24 jam.

Pengujian kebocoran sel bakteri uji

Pengujian ini untuk melihat akibat dari aktivitas melanin tinta sotong terhadap mikroba uji mengacu pada Bunduki *et al.* (1995). Analisis kebocoran sel dilakukan dengan menggunakan Spektrofotometer *Double Beam* pada panjang gelombang 280

dan 260 nm. Panjang gelombang 280 nm digunakan untuk mengukur kadar nitrogen dari protein sel, sedangkan panjang gelombang 260 nm untuk mengukur kadar nitrogen asam nukleat sel.

Kultur murni sebanyak 10 mL disentrifus pada 10.000 rpm selama 10 menit. Filtrat dibuang lalu ditambahkan 5 mL larutan garam fisiologis (0,85% NaCl) dalam endapan sel pada tabung reaksi, kemudian divorteks agar sel homogen dalam larutan fisiologis. Selanjutnya ditambahkan melanin dengan konsentrasi 0; 0,005; 0,010; 0,015; 0,020 g/mL dan dibiarkan pada suhu kamar selama 24 jam. Suspensi kemudian disentrifus pada 10.000 rpm selama 10 menit dan supernatan disaring dengan kertas saring (*Syringe filter sterile* 0,20 μm) untuk memisahkan selnya. Analisis dilakukan dengan mengamati OD (*Optical Density*) dari supernatan bebas sel.

HASIL DAN PEMBAHASAN

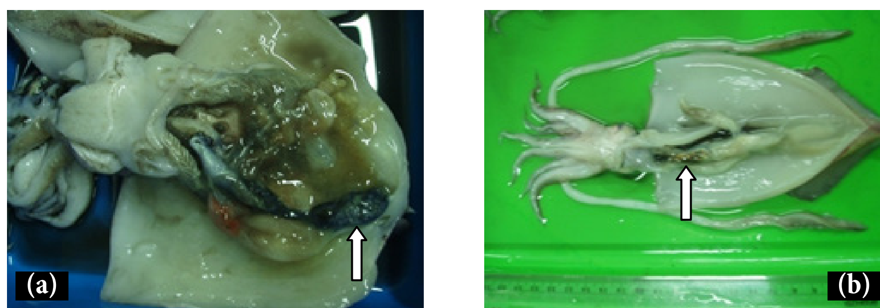
Isolasi *E. coli*

Koloni yang diduga *E. coli* yaitu yang berwarna hijau metalik diuji sifat biokimianya dengan menggunakan *microbact identification kits* (oxoid). Hasil pengujian dengan *microbact kit* menunjukkan lisin (+), ornitin (+), H_2S (-), glukosa (+), manitol (+), xilosa (+), ONPG (+), Indol (+), urease (-), V-P (-), citrate (-), dan TDA (-). Hasil identifikasi menunjukkan *Escherichia coli* (96,39%).

Rendemen Tinta dari *Sepia* sp. dan *Loligo* sp.

Sepia sp. memiliki kantong tinta yang panjang dan besar, sementara kantong tinta *Loligo* sp. berukuran kecil sehingga tinta yang dihasilkan juga lebih sedikit (Gambar 1). Tinta yang terdapat dalam kantong sangat ditentukan oleh kondisi terakhir sebelum ditangkap, jika sebelum ditangkap sudah banyak tinta yang dikeluarkan maka hanya sedikit yang tersisa di kantong. Analisis rendemen dari tinta berdasarkan perbandingan berat kantong (berisi tinta) terhadap berat badan per ekor yang dihasilkan oleh kedua sampel. Berat utuh *Loligo* sp. yaitu 116,6±40,36 g dan berat kantong tinta 0,6±0,1 g, sedangkan *Sepia* sp. memiliki berat utuh 173,0±19,6 g dan berat kantong tinta 4,0±1,4 g. Hasil analisis rendemen terlihat bahwa jenis *Sepia* memiliki rendemen tinta yang lebih besar (2,3%) dibandingkan tinta dari jenis *Loligo* (0,5%).

Nair *et al.* (2011) menyatakan bahwa tinta *Sepia* terdiri atas granula melanin dalam media yang kental tidak berwarna. Pigmen melanin diolah dalam sel mature kelenjar tinta, terutama pada bagian dasar kantong tinta yang terus-menerus memproduksi tinta. Akhir proses pematangan, sel-sel kelenjar tinta menyimpannya dalam kantong tinta yang berperan sebagai penampung. Setiap kantung tinta *Sepia* mengandung ~ 1 g melanin (Derby 2014), dan banyaknya melanin ~ 15% dari berat basah total tinta (Wang *et al.* 2014). Melanin *Sepia* terbentuk oleh banyak kelompok agregat. Agregat-agregat ini terbentuk juga oleh butiran bola kecil dengan distribusi ukuran yang berbeda. Diameter butiran kecil berkisar 100-200 nm (Mbonyiryivuze *et al.*2015). Ukuran butiran bola melanin pada cumi-cumi, berkisar antara



Gambar 1 Jenis chepalopoda dan kantong tinta yang digunakan pada penelitian ini. (a) jenis sotong (*Sepia* sp.), kantong tinta dari jenis *Sepia* sp., (b) jenis cumi-cumi (*Loligo* sp.), kantong tinta dari jenis *Loligo* sp.

50–150 nm (Chen *et al.* 2009). Berdasarkan hasil pengamatan, tinta *Loligo* sp. memiliki tekstur yang halus, sedangkan tinta *Sepia* sp. memiliki tekstur yang kasar.

Aktivitas Melanin Tinta *Sepia* sp. dan *Loligo* sp.

Pengaruh tinta dan melanin dari *Sepia* sp. dan *Loligo* sp. pada beberapa konsentrasi terhadap pertumbuhan *E. coli* dapat dilihat pada Tabel 1. Konsentrasi melanin semakin tinggi menghasillkan aktivitas penghambatan terhadap *E. coli* juga semakin besar. Melanin *Sepia* sp. pada konsentrasi 0,002 g/mL terlihat tidak ada aktivitas penghambatan, sementara pada konsentrasi yang lebih tinggi yaitu 0,006 g/mL terlihat jumlah koloni setelah inkubasi 24 jam tidak berbeda dengan jumlah awal (sebelum inkubasi). Hal ini menunjukkan pada konsentrasi 0,006 g/mL sudah terjadi penghambatan terhadap pertumbuhan sel bakteri, hingga 24 jam inkubasi tidak terjadi peningkatan yang berarti pada jumlah sel bakteri.

Konsentrasi yang lebih tinggi yaitu 0,010 g/mL, melanin mampu membunuh bakteri sehingga setelah inkubasi 24 jam hanya ada 1 sel yang hidup (penghambatan mencapai 99,99%). Melanin dari *Sepia* sp. memiliki aktivitas penghambatan yang lebih besar dibandingkan dengan melanin dari *Loligo* sp. Pada konsentrasi yang lebih rendah (1/2 dari konsentrasi melanin *Loligo* sp), melanin dari *Sepia* sp. mampu menghambat hampir 100%. Tabel 1 menunjukkan *E. coli* lebih sensitif terhadap melanin dari *Sepia* sp. dibandingkan dengan dari *Loligo* sp.

Tinta *Sepia* sp. dengan konsentrasi yang lebih tinggi dibandingkan dengan melaninnya tidak memiliki aktivitas penghambatan, sementra tinta *Loligo* sp. pada konsentrasi yang sama dengan melanin tidak memiliki aktivitas penghambatan terhadap *E. coli*. Hal ini disebabkan karena yang terkandung di dalam tinta tidak hanya melanin, melainkan ada komponen lain misalnya protein, lemak dan glikosaminoglikan yang diduga tidak memiliki aktivitas penghambatan terhadap *E. coli*, akan tetapi sebaliknya dapat meningkatkan pertumbuhan *E. coli*.

Tabel 1 Pertumbuhan *E. coli* pada media NB yang mengandung tinta dan melanin

Sumber		Konsentrasi (g/mL)	Jumlah *E. coli* (CFU/mL)		% Penghambatan relatif terhadap jumlah mikroba awal [100-(Ntx100/No)]	Log Peng-hambatan
			Inkubasi 0 jam (Nol)	Inkubasi 24 jam (Nt)		
Sepia sp.	Tinta	0	$8,6 \times 10^5$	$1,6 \times 10^{10}$	-	-4,27
		0,013	$8,6 \times 10^5$	$6,2 \times 10^9$	-	-3,86
		0,017	$8,6 \times 10^5$	$5,6 \times 10^9$	-	-3,81
		0,020	$8,6 \times 10^5$	$4,4 \times 10^9$	-	-3,71
	Melanin	0,002	$8,6 \times 10^5$	$1,2 \times 10^{10}$	-	-5,14
		0,006	$8,6 \times 10^5$	$6,0 \times 10^5$	30,23	0,16
		0,010	$8,6 \times 10^5$	$1,0 \times 10^0$	99,99	5,93
Loligo sp.	Tinta	0	$8,6 \times 10^5$	$3,1 \times 10^{10}$	-	-4,56
		0,013	$8,6 \times 10^5$	$8,6 \times 10^9$	-	-4,00
		0,017	$8,6 \times 10^5$	$2,3 \times 10^9$	-	-3,43
		0,020	$8,6 \times 10^5$	$1,0 \times 10^9$	-	-3,06
	Melanin	0,013	$8,6 \times 10^5$	$2,1 \times 10^4$	97,56	1,61
		0,017	$8,6 \times 10^5$	$2,1 \times 10^3$	99,76	2,61
		0,020	$8,6 \times 10^5$	$1,4 \times 10^2$	99.87	3,79

Keterangan: (-) nilai negatif artinya tidak ada penghambatan dan terjadi peningkatan

Aktivitas Melanin *Sepia* sp. terhadap Pertumbuhan *E. coli*

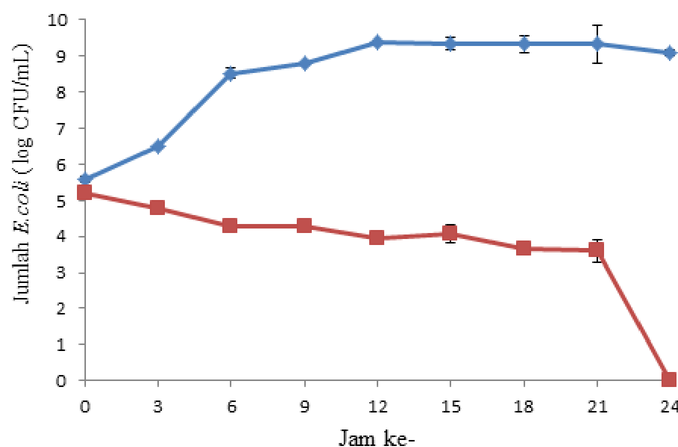
Aktivitas melanin dari *Sepia* terhadap pertumbuhan *E. coli* dapat dilihat pada Gambar 2. Pertumbuhan *E. coli* yang diberi perlakuan melanin dari *Sepia* sp. sebanyak 10 mg/mL terlihat jumlah koloninya yang hidup mengalami penurunan lebih dari 1 log_{10} setelah 6 jam inkubasi dan penurunan tersebut terus berlanjut hingga 2 log_{10} setelah 21 jam inkubasi. Akibat aktivitas melanin, terjadi perpanjangan fase adaptasi dan menyebabkan terjadinya penurunan jumlah koloni yang hidup, setelah 24 jam inkubasi, tidak ada lagi koloni yang hidup (Gambar 2). Hal ini menunjukkan bahwa selain memperpanjang fase adaptasi, melanin juga mempercepat fase kematian pada sel bakteri.

Pertumbuhan *E. coli* tanpa perlakuan melanin terjadi peningkatan jumlah koloni yang hidup lebih dari 4 log_{10} (dari 10^5 menjadi 10^9). Pertumbuhan pada 3 jam pertama memasuki fase adaptasi dan pertumbuhan awal yang dilanjutkan dengan fase logaritmik setelah 6 jam inkubasi. Setelah 6 jam terjadi peningkatan jumlah koloni yang cepat hingga 2 log_{10}, dan terus meningkat hingga jam ke-12 walaupun hanya sedikit terjadi pertambahan populasi (kurang dari 1 log_{10}). Pertumbuhan memasuki fase stasioner setelah jam ke-12 hingga jam ke-24.

Vasantharaja *et al.* (2014) melaporkan bahwa ekstrak metanol tinta *Sepiella inermis* dengan menggunakan GC-MS menunjukkan adanya campuran dari struktur oligomer yang merupakan gabungan antara dihidroksi indol-2-asam karboksilat dan dihidroksiindol. Ekstrak metanol ini memiliki aktivitas penghambatan terutama terhadap bakteri Gram negatif misalnya *Proteus vulgaris*, *Pseudomonas aeruginosa* dan *E. coli*. Neifar *et al.* (2009) melaporkan bahwa dihidroksiindol dan asam dikarboksilat dari *Sepia officinalis* memiliki aktivitas penghambatan terhadap mikroba.

Melanin merupakan tirosinase yang telah diidentifikasi terdapat di dalam tinta cumi-cumi (Derby 2014). Tinta cumi-cumi terdiri atas suspensi granula eumelanin di dalam media yang viscous dan tidak berwarna. Eumelanin bersifat heterogen, umumnya polimer yang tidak larut yang berkembang melalui oksidasi enzimatis dari asam amino tirosin. Produksi eumelanin di dalam sel pigmen terjadi di dalam organel khusus yang disebut melanosome. Eumelanin tersusun dari unit 5,6-dihidroksiindol (DHI) sekitar 20% dan unit 5,6-dihidroksiindol-2-asam karboksilat (DHICA) (Magarelli *et al.* 2010). Eumelanin alami dilaporkan merupakan molekul pigmen yang dapat mengadsorpsi logam pada konsentrasi tinggi. Kemampuan berikatan eumelanin dengan sisi dari logam merupakan parameter penting untuk memahami komplek logam-melanin (Lei *et al.* 2008; Chen *et al.* 2009).

Dinding sel bakteri mengandung banyak jenis kation termasuk Mg^{2+}, Ca^{2+}, Na+, dan K^+. Ion-ion ini bertanggung jawab atas berbagai aktivitas bakteri, termasuk kerja enzim,



Gambar 2 Kurva pertumbuhan *E. coli* yang diinkubasi dengan melanin *Sepia* sp. ◆ = kontrol (tanpa melanin), ■ = ditambah melanin.

Tabel 2 Nilai OD dari supernatan bebas sel *E. coli* yang diinkubasi dengan melanin dari *Sepia* sp.

Konsentrasi melanin (g/ml)	OD pada 260 nm	OD pada 280 nm
0	0,015	0,018
0,005	0,212	0,234
0,010	0,277	0,307
0,015	0,314	0,384
0,020	0,398	0,464

pengaturan metabolik dan menjaga integritas lapisan luar. Mg^{2+} dan ion Ca^{2+} khususnya, berperan penting dalam melindungi kestabilan struktur luar (Ferrero *et al.* 2007; Peshenko *et al.* 2007). Lapisan paling luar dari membran luar pada bakteri Gram negatif adalah lipopolisakarida (LPS), secara individu, molekul ini bermuatan negatif. Kation divalen membantu menstabilkan dan menjaga integritas membran luar dengan mengikat molekul LPS yang berdekatan. Kation *divalent* ini berfungsi sebagai jembatan garam berikatan dengan molekul lipid yang bermuatan negatif (Raetz *et al.* 2007). Membran luar pada sel bakteri berfungsi sebagai penghalang masuknya senyawa-senyawa yang tidak diperlukan sel (seperti bakteriosin, enzim dan senyawa hidrofobik). Jika kation tersebut dapat diadsorpsi oleh gugus fungsi melanin, maka sistem metabolisme sel bakteri akan terganggu, akibatnya pertumbuhan sel bakteri juga terganggu.

Sahalan *et al.* (2013) menjelaskan ion Ca^{2+} dan Mg^{2+} berperan melindungi membran terluar pada sel bakteri terhadap Polymyxin B yang berinteraksi dengan kation divalent, dengan mengganti kation dari tempat pengikatannya di molekul lipopolisakarida (LPS). Hal ini menyebabkan disorganisasi komponen membran luar bakteri Gram negatif, akibat lepasnya komponen LPS dari permukaan bakteri yang menyebabkan kebocoran membran dan akhirnya menyebabkan kematian sel. Ca^{2+} telah terbukti lebih efektif dalam melindungi sel bakteri dibandingkan Mg^{2+}.

Pengikatan gugus fungsi dari melanin yaitu gugus hidroksil fenolik (OH), karboksil (COOH) dan grup amina (NH) (Chen *et al.* 2009) terhadap ion Ca^{2+} dan Mg^{2+} pada membran luar mengakibatkan kebocoran pada membran bakteri Gram negatif dalam hal ini diwakili oleh *E. coli*. Kebocoran yang disebabkan oleh melanin terhadap sel *E. coli* dapat dilihat dari dihasilkannya supernatan bebas sel isi sel bakteri setelah diinkubasi selama 24 jam dengan melanin tinta Sepia sp. (Tabel 2). Tabel 2 menunjukkan hubungan antara konsentrasi melanin dengan supernatan bebas sel dari sel *E. coli*. Analisis kebocoran sel dilakukan dengan menggunakan Spektrofotometer *Double Beam* pada panjang gelombang 280 dan 260 nm. Panjang gelombang 280 nm digunakan untuk mengukur kadar nitrogen dari protein sel, sedangkan panjang gelombang 260 nm untuk mengukur kadar nitrogen asam nukleat sel. Semakin tinggi nilai OD baik pada panjang gelombang 260 nm maupun 280 nm menunjukkan semakin besarnya kebocoran sel akibat melanin. Semakin tinggi konsentrasi melanin, semakin besar tingkat kebocoran sel.

Hasil pengamatan terhadap spektrum Infra Red (IR) menggunakan FTIR spektrofotometer dari melanin tinta *Sepia* sp. dan *Loligo* sp. (data tidak dipublikasikan) menunjukkan kedua melanin memiliki pola spektrum yang sama, mengandung gugus fenolik, amina dan karboksil. Intensitas dari masing-masing gugus aktif tersebut yang berbeda diantara keduanya. Gugus fenolik, amina dan karboksil dari melanin *Sepia* sp. memiliki intensitas yang lebih tinggi dibandingkan dengan melanin dari *Loligo* sp. Intensitas ini menunjukkan konsentrasi dari gugus aktif tersebut di dalam melanin. Hal inilah yang diduga mengakibatkan perbedaan aktivitas kedua melanin tersebut terhadap *E. coli*.

KESIMPULAN

Melanin dari tinta sotong (*Sepia* sp.) dan cumi-cumi (*Loligo* sp) memiliki aktivitas penghambatan terhadap *E. coli*. Aktivitas penghambatan terhadap *E. coli* dari melanin tinta sotong lebih tinggi dibandingkan melanin dari cumi-cumi. Tinta sotong dan cumi-cumi pada konsentrasi 0,013-0,020 g/mL tidak memiliki aktivitas penghambatan terhadap *E. coli*.

UCAPAN TERIMA KASIH

Ucapan terima kasih disampaikan kepada Direktorat Riset dan Pengabdian Masyarakat Direktorat Jenderal Penguatan Riset dan Pengembangan, Kementerian Riset, Teknologi dan Pendidikan Tinggi atas bantuan dana penelitian melalui hibah Fundamental (sesuai dengan Surat Perjanjian Penugasan Pelaksanaan Program Penelitian Nomor: 024/SP2H/LT/DRPM/II/2016 tanggal 17 Februari 2016).

DAFTAR PUSTAKA

Arias C. 2009. Chilled and Frozen Raw Fish. Di dalam: Fernandes R (editor) Microbiology Handbook Fish and Seafood. United Kingdom (UK): Leatherhead Food International Ltd.

Bunduki MMC, KJ Flanders and CW Donnelly. 1995. Metabolic and structural sites of damage in heat and sanitizer-injuried population of *Listeria monocytogenes*. *Journal Food Protect*. 58: 410-415.

Cappaso R, Evidente A, Schivo L, Orru G, Marcialis MA, Cristinzio G. 1995. Antibacterial polyphenols from olive oil mill waste waters. *Journal of Applied. Bacteriology*. 79: 393-398.

Chen S, Xue C, Wang J, Feng H, Wang Y, Ma Q, Wang D. 2009. Adsorption of Pb(II) and Cd(II) by squid *Ommastrephes bartrami* melanin. *Bioinorganic Chemistry and Applications*. 1-7

Dave D, Ghaly AE.2011. Meat spoilage mechanisms and preservation techniques: a critical review. *American Journal of Agricultural and Biological Sciences.* 6(4): 486-510.

Derby CD.2014.Cephalopod ink: production, chemistry, functions and applications. *Marine drugs*. 12: 2700-2730.

Ferrero MA, Martínez-Blanco H, Lopez-VelascoFF, Ezquerro-SáenzC, NavasaN, Lozano S & Rodríguez-Aparicio LB. 2007. Purification and characterization of GlcNAc-6-P 2-epimerase from *Escherichia coli* K92. *Acta Biochimica Polonica*. 54(2): 387-399.

Lei M, Wang JF, Pang L.Wang YM, Chen SG, Xue CH. 2007a. Effects of *Sepia* on the metabolization of blood lipid and antioxidant ability in hyperlipidemia rats. *The Chinese Journalof Marine Drugs.* 3: 30-33.

Lei M, Wang JF,Wang YM, Pang L, Wang Y, Xu W,Xue CH. 2007b. Study of theradio-protective effect of cuttlefish ink on hemopoietic injury. *Asia Pacific Journal of Clinical Nutrition*.16: 239-243.

Lei M, Xue CH, Wang YM, Li ZJ, Xue Y, Wang JF. 2008. Effect of squid ink melanin-Fe on iron deficiency anemia remission. *Journal of Food Science.* 73(8): 207-11.

Mbonyiryivuze A, Nuru ZY, Diop Ngom B, Mwakikunga B, Simon Mokhotjwa Dhlamini SM, Park E, Maaza M. 2015. Morphological and chemical composition characterization of commercial *Sepia* melanin. *American Journal of Nanomaterials*. 3(1): 22-27.

Murhadi. 2002. Isolasi dan Karakterisasi Komponen Antibakteri dari Biji Atung (Parinarium glaberrimum Hassk). [disertasi]. Bogor (ID): Institut Pertanian Bogor.

Nair JR, Pillai D, Joseph SM, Gomathi P, Senan PV, and Sherief PM.2011. Cephalopod research and bioactive substances. *Indian Journal of Geo-Marine Sciences*. 40(1): 13-27.

Neifar A, Rebah FB, Gargouri AF, Abdelmouleh A. 2009. Physicochemical characterization of *Sepia officinalis*ink and the effects of storage conditions on the coagulation process. *Journal of the Marine Biological Association of the UK*. 89(04): 803-807.

Nithya M, Ambikapathy V, Panneerselvam A. 2011. Effect of pharaoh's cuttlefish ink

against bacterial pathogens. *Asian Journal of Plant Science and Research*. 1 (4):49-55

Peshenko IV & Dizhoor AM. 2007. Activation and inhibition of photoreceptor guanylyl cyclase by guanylyl cyclase activating protein 1 (GCAP-1): The functional role of Mg^{2+}/Ca^{2+} exchange in EF-hand domains. *The Journal of Biological Chemistry*. 82(30): 21645-21652.

Raetz C R, Reynolds CM, TrentMS & BishopRE. 2007. Lipid: A modification systems in gram-negative bacteria. *Annual Reviews Biochemistry*. 76: 295-329.

Rajaganapathi J, Thyagarajam SP, Edward JK. 2007. Study on cephalopod's ink for anti-retroviral activity. *Indian Journal of Experimental Biology*. 38: 519-520.

Sahalan AZ, Aziz AHA, Lian H & Ghani MKA. 2013. Divalent cations (Mg^{2+}, Ca^{2+}) protect bacterial outer membrane damage by Polymyxin B. *Sains Malaysiana*. 42(3): 301–306.

Vasantharaja D, Ravitchandirane V and Anandan V. 2014. Anti-microbial activity and spectro-chemical investigation of ink extracts of *Sepiella inermis* (Van Hasselt 1835). *Notulae Scientia Biologicae*. 6(3):273-275

Wang FR, XieZG, YeXQ, Deng SG, Hu YQ, Guo X, Chen SG. 2014. Effectiveness of treatment of iron deficiency anemia in rats with squid ink melanin-Fe. *Food Funct*. 5: 123–128.

Yuvaraj D, Suvasini B, Chellathai T, Fouziya R, Ivo Romauld S and Chandran M. 2015. Anti-bacterial studies on the different body parts of *Loligo* duvauceli. *Journal of Chemical and Pharmaceutical Research*. 7(6):406-408.

Zhong JP, Wang G, Shang JH, Pan JQ, Li K, Huang Y, Liu HZ. 2009. Protective effects of squid ink extract towards hemopoieticinjuries induced by cyclophosphamine. *Marine Drugs*. 7: 9-18.

Dear chief of editor

Egyptian Journal of Aquatic Biology and Fisheries,

We submit article entitle "*The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.*" for publish in Egyptian Journal of Aquatic Biology and Fisheries. All authors agree to publish in this journal and equally contribute to this study. All Authors declare that there is no conflict of interest. This paper is not recognized for publication in another journal and submitted for Egyptian Journal of Aquatic Biology and Fisheries only. We hope you accept our article for publish in Egyptian Journal of Aquatic Biology and Fisheries because this study analyze the the potential and bactericidal action of the cuttlefish ink melanin against *Aeromonas* sp.

Whats this study adds:

- The cuttlefish ink melanin inhibited the growth of *Aeromonas* sp. as indicated by the shrinkage of cell size and irregular cell shape.
- The results of this study are important information for dealing with *Aeromona*s sp. attack in cultured fish.

Thank you

Yuspihana Fitrial

Study Program of Fisheries Product Technology, Faculty of Fisheries and Marine, Lambung Mangkurat University, Banjarbaru 70712, South Kalimantan, Indonesia

*Corresponding Author: yuspihana.fitrial@ulm.ac.id

Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 24(--): … – … (2020)
www.ejabf.journals.ekb.eg

The antibacterial activity of melanin in cuttlefish (*Sepia* sp.) ink against *Aeromonas* sp.

ARTICLE INFO
Article History:
Received:
Accepted:
Online:

Keywords:
Cuttlefish,
Ink,
Melanin,
Antibacterial activity.

ABSTRACT

The marine environment consists of many organisms that are recognized to have bioactive compounds as a mechanism of self-defense or protection of eggs and embryos. One of them is cuttlefish (*Sepia* sp.). The aim of this study was to determine the potential and bactericidal action of the cuttlefish ink melanin against *Aeromonas* sp. Ink extraction and purification were carried out to obtain melanin mechanically using 0.5M HCl. The growth patterns of bacteria were studied by the Total Plate Count method, and the bactericidal mechanism of melanin was observed by Transmission Electron Microscopy (TEM). The results showed that cuttlefish ink melanin inhibited the growth of *Aeromonas* sp. as indicated by the shrinkage of cell size and irregular cell shape. The results of this study are important information for dealing with *Aeromonas* sp. attack in cultured fish.

INTRODUCTION

Cuttlefish are one of the fisheries commodities which utilization is still limited. So far, the use of cuttlefish has only been cooked with a mixture of spices, while the ink is thrown away or used as part of the cuttlefish processing in food. Several studies have shown that squid ink contains melanin, protein, fat and glycosaminoglycans **(Fiore *et al*., 2004)**. Squid ink can act as a cell protective drug in the treatment of cancer with chemotherapy, by increasing the number of leukocytes and bone marrow cell nucleates, which numbers are decreasing due to the use of drugs such as tumor cell killer **(Naraoka *et al*., 2000)**. In addition, melanin from squid ink has anti-tumor activity by inhibiting plasmin activity to increase thromboxane and increase the body's resistance to killing cancer cells **(Zhong *et al*., 2009)**. Melanin also contributes as an antioxidant **(Fahmy, 2013; Guo *et al*., 2014)**, anti-radiation **(Solano, 2020; Pralea *et al*., 2019)**, antirotavirus **(Rajaganapathi *et al*., 2000)** and antibacterial **(Yuvaraj *et al*., 2015; Aruldhason *et al*., 2014; Zakaria *et al*., 2019).** In addition, melanin also has the ability to absorb metals, especially in the phenolic hydroxyl (OH), carboxyl (COOH) and amine (NH) functional groups of the melanin molecule **(Chen *et al*., 2009)**.

Nair *et al*. (2011) stated that Sepia ink consists of melanin granules on thick colorless media. The pigment melanin is the main element in the ink glands which continuously produce ink. At the end of the ripening process, the ink gland cells store ink sacs that function as reservoirs. Each Sepia ink bag contains ± 1 g melanin **(Derby, 2014)**, and the amount of melanin is ± 15% of the total ink wet weight **(Wang *et al*., 2014)**. Melanin Sepia is formed by many aggregates. This aggregate is also formed by small round grains with different size distributions. Small grain diameter ranges from 100-200 nm **(Mbonyiryivuze *et al*., 2015)**.

The squid and or cuttlefish ink had antibacterial activity **(Nair *et al*., 2011).** Melanin's action as an antibacterial has not been much revealed. Some researchers have tested antibacterial activity against only extracts from cuttlefish and or squid ink. **Nithya *et al*. (2011)** examined the antibacterial activity of cuttlefish hexane extract (Sepia pharaonis) purified with diethyl ether. The results of this study indicate that the extract has inhibitory activity against *Pseudomonas aeruginosa*, *Staphylococcus epidermidis, Klebsiella pneumoniae*, and *E. coli*. **Yuvaraj *et al.* (2015)** proved that squid ink (*Loligo duvauceli*) does not have inhibitory activity against *E. coli*. The results of this study tested the antibacterial activity by approximating the ability of the ink component, that is melanin, to chelate metals. The cytotoxic effect of melanin squid ink is thought to also affect the growth of fish microbes, such as *Aeromona*s sp.

Aeromonas sp. are gram-negative, facultative anaerobes, which cause disease in both terrestrial and aquatic animals, as well as in humans. *Aeromonas* sp. considered as one of the important bacteria among disease-causing agents in fish **(Praveen *et al.,* 2016)**. It is necessary to conduct research to see the antibacterial activity of cuttlefish melanin against *Aeromonas* sp. Therefore, the aim of this study was to determine the potential and bactericidal activity of the cuttlefish ink melanin against *Aeromonas* sp.

MATERIALS AND METHODS

2.1 Materials

The material used was cuttlefish (ink) obtained from the Muara Kintap Fishing Port, Tanah Laut Regency, South Kalimantan. Other ingredients used include 0.5M HCl (Merck), acetone (Merck), aquades, Nutrient Broth (NB) (Merck), Nutrient Agar (NA) (Merck), Sterile-EO Syringe Filter (Sartorius Minisart pore size 0.20 µm), Microbact TM GNB12A/B/E, 24E Identification Kits (Oxoid) and 70% alcohol.

The tools used in this study were a refrigerated centrifuge (Labogene Scanspeed 1580R), Freeze dryer (Model Christ alpha 2-4 LD Plus), autoclave (Pressure Steam Sterilizer Electric Model No.25X-2), laminar flow (Biobase), incubator (Memmert), colony counter (Quebec), incubator shaker (Wisd), spectrophotometer (Genesys 10uv), SEM with Energy Dispersive X-ray (SEM-EDX) EDAX.SL, TEM JEOL, JEM1400, and other glassware.

2.2 Methods

2.2.1 Preparation of melanin

Isolation and purification of cuttlefish ink melanin according to the method **(Magarelli *et al*., 2010)** as follows: 50 g of cuttlefish ink is added with 100 ml of HCl (0.5 M molarity) in a dark container. The solution was stirred for 30 minutes (in a magnetic stirrer) and then stored for 24 hours at 10C. The pellet was separated from the supernatant by centrifugation (10000 rpm at 5°C for 15 minutes). The suspension was rinsed three times each with 0.5M HCl, water, acetone and finally with water, sequentially. Lyophilization was then carried out to remove the solvent

using a freeze dryer. Melanin was obtained and then characterized using infrared (IR) spectroscopy and its morphology by SEM, TEM, and the percentage of melanin elements using EDX (Energy Dispersive X-ray Spectroscopy).

2.2.2 Culture of organism

The microbes were isolated from rotten meat and digestive fish (Euthynnus affinis). Isolates were grown on GSP agar medium (Pseudomonas-Aeromonas-Selective agar, Merck). Species were determined using the Microbact identification kit GNB 12A/B/E, 24E (Oxoid).

2.2.3. Analysis of cell leakage

The test to study the mechanism of action of cuttlefish melanin inhibition in microbes refers to **Bunduki *et al*. (1995)**. Analysis of cell leakage was carried out using a spectrophotometer at a wavelength of 280 and 260 nm. The 280 nm wavelength is used to measure the nitrogen content of cell proteins, while the 260 nm wavelength is used to measure the nucleic nitrogen content in cells. 10 mL cultures were centrifuged at 10,000 rpm for 10 minutes. The filtrate is removed and then 5 mL of physiological salt solution (0.85% NaCl) is added to the cell sediment in the test tube. The solution is vortexed so that the cells are homogeneous in a physiological solution. Furthermore, melanin is added with a concentration of 0; 0.005; 0.010; 0.015; 0.020 g / mL, and incubated at room temperature for 24 hours. The suspension was centrifuged at 10,000 rpm for 10 minutes, and the supernatant filtered with filter paper (0.20µm sterile syringe filter) to separate cells. The analysis was carried out by observing the OD (Optical Density) of the liquid supernatant.

2.2.4 Testing of melanin activity against test bacteria

Melanin activity testing was carried out by using the direct contact method between melanin and the test bacteria in liquid nutrient broth (NB). The test series is in a small tube containing 2,970 mL of sterile NB plus 0.030 mL of bacterial suspension so that the total solution is 3,000 mL. Melanin (in powder form) was added to the test tube so that the melanin concentration in the tube was 0.000; 0.002; 0.006; 0.010 g / mL. For the first series of test tubes (melanin concentration 0,000 g/mL), 2,970 NB sterile + 0,000 g melanin were used. A second series tube (melanin concentration 0.002 g/mL), was prepared by adding 2.964 mL of sterile NB + 0.006 g melanin, and so on.

The bacteria were prepared, which had been freshened and incubated for 24 hours (108-109 CFU/mL) at 37 ° C, then diluted ten times. The test tube was inoculated with 0.030 mL of tested bacterial suspension, shaken with vortex for 1-2 minutes, then incubated in a shaker incubator at 37°C for 24 hours. Total Plate Count performs a bacterial count. Petri dishes were incubated for 24 hours at 37°C, and the number of colonies was counted. The total colony is the result of the average count on the three petri dishes that match a particular sample.

The percentage of bacterial inhibition was determined by modification of the method **(Capasso *et al*., 1995)** with the formula: 100 - (Nt x 100/No), where Nt is the number of CFU bacteria/mL in the addition of melanin treatment, while No is the number of CFU bacteria/mL initial (initial inoculum).

2.2.5 Testing of melanin activity to *Aeromonas* sp growth

The test for the inhibition of melanin on the growth of *Aeromonas* sp. was carried out in the same way as the melanin activity test above, with a concentration of 0.010 g/mL (ie the melanin concentration where the percentage of inhibition

relative to the number of initial microbes was close to 100%. Observations were carried out every three hours for 24 hours.

2.2.6. Preparation of *Aeromonas* sp. for TEM analysis.

Sample preparation followed the method by **Kim *et al*. (2007)** with some modifications. Bacteria were inoculated in NB medium and 0.005 g / mL and 0.010 g/mL melanin were added, then incubated for 24 hours at 37°C. Controls were prepared in NB medium. After incubation, the suspension was centrifuged at 1200 rpm for 10 minutes. The supernatant was washed with a phosphate buffer solution pH 7.2, and centrifuged at 2600 rpm for 5 minutes (4°C). For the sample, cell pellets were fixed with 2% glutaraldehyde. Aeromonas sp. Cell images were observed by transmission electron microscopy (TEM, JEOL, JEM 1400).

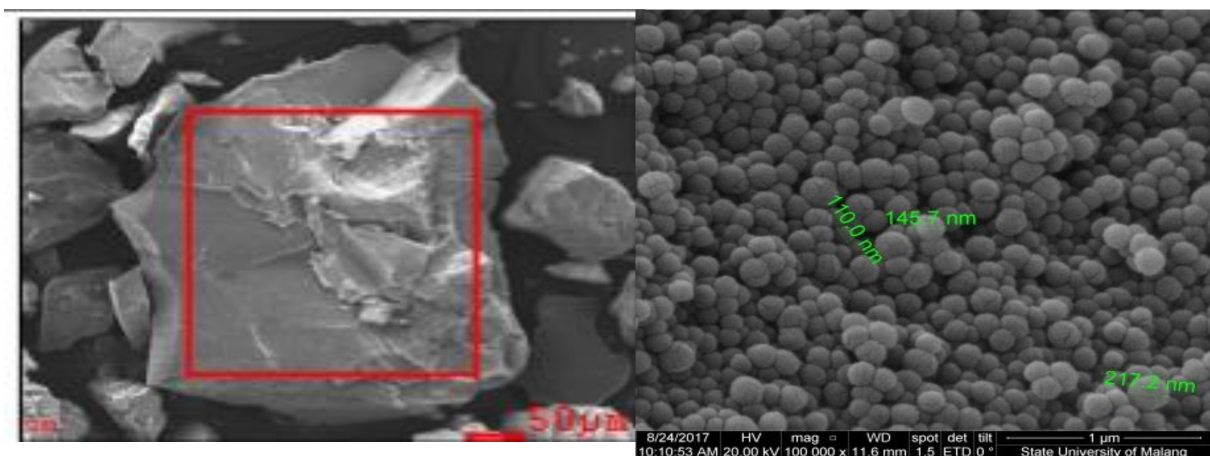
RESULTS

3.1 Isolation of Aeromonas sp.

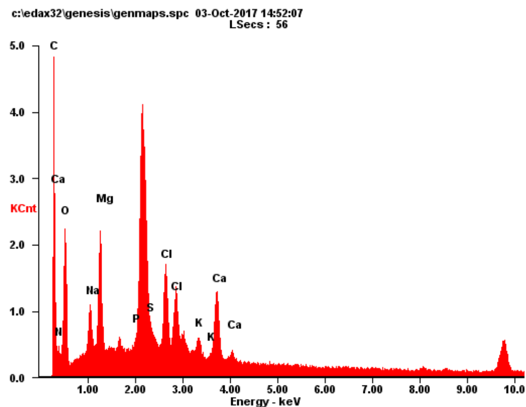
The colony presumably Aeromonas sp. is the yellow color on GSP agar, tested for biochemical properties using a microbact (oxoid) identification kit. The test results of the microbact kit showed oxidase (+), motility (+), nitrate (+), lysine (+), ornitine (+), H2S (+), glucose (+), mannitol (+), xylose (+) , ONPG (+), indole (+), urease (+), VP (+), citrate (+), TDA (-), gelatin (-), malonate (+), inositol (-), sorbitol (+) , rhamnose (+), sucrose (+), lactose (+), arabinose (+), adonitol (-), raffinose (+), salicin (-), arginine (-). The identification results showed Aeromonas hydrophilla (similarity reached 94.20%).

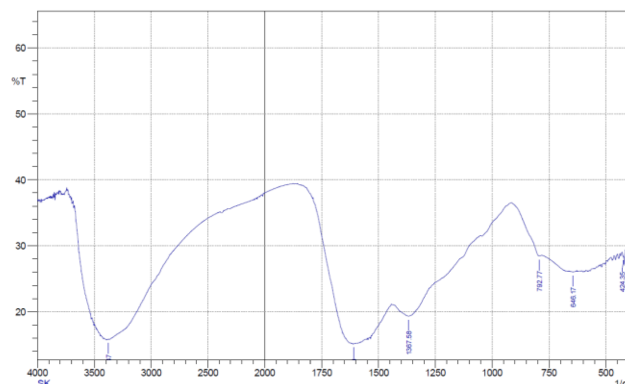
3.2 Purification of melanin

Cuttlefish melanin is an aggregate composed of many spherical grains. Small spherical grains also make these aggregates with different size distributions. The small grain diameter ranges from 100-200 nm (Figures 1 and 2). TEM micrograph showing spherical melanin granules (Figure 3).



(a) **(b)**

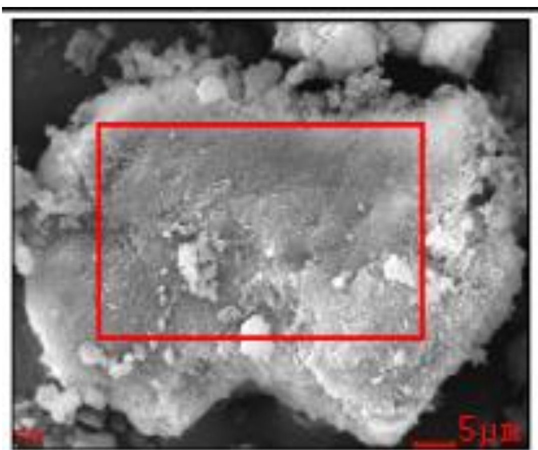


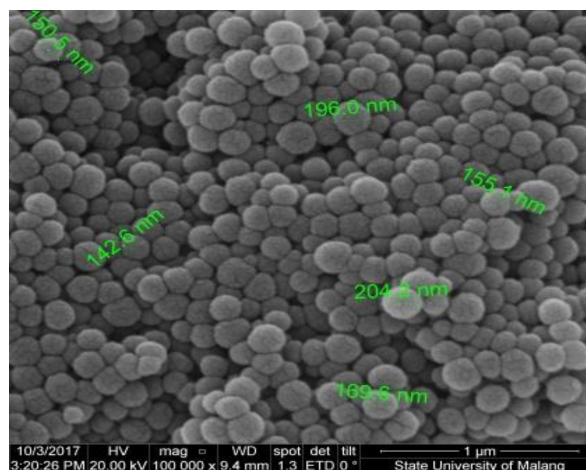


(c) **(d)**

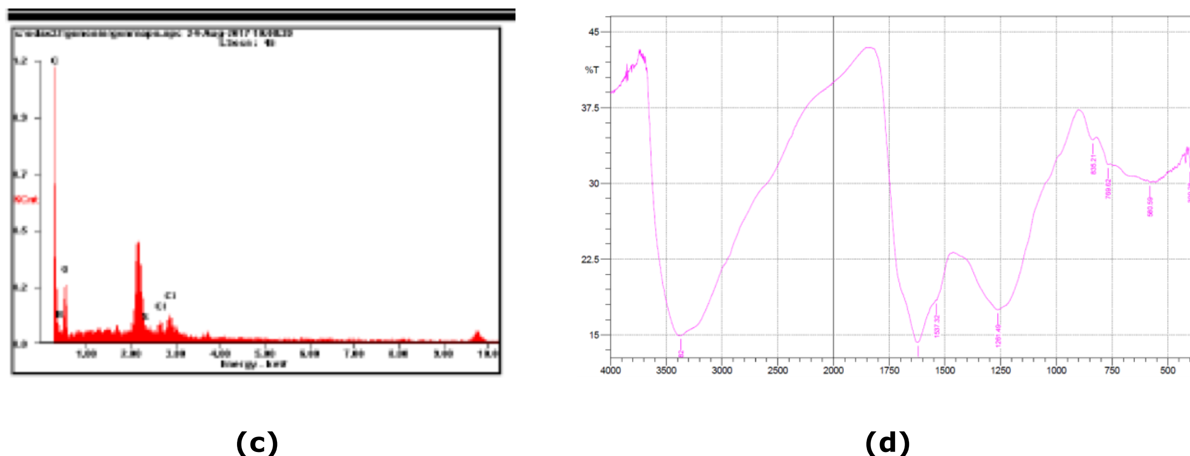
Figure 1. Scanning electron micrograph of cuttlefish ink melanin (a) and (b) aggregates melanin, (c) EDX pattern of melanin and (d) The FT-IR spectra of melanin before purified.

The FTIR spectrum showed that melanin had a strong Infrared absorption spectrum at 3300, 1620, and 1260 cm-1. It shows phenolic hydroxyl (OH), carboxyl (COOH), and amine (NH) groups (Figure 1d). The difference between before and after purification is mainly seen in the intensity of each functional group. Cuttlefish ink contains the most significant components, specifically C (50.22%), O (29.18%), and N (7.99%), as well as a small portion of Mg, Na, S, K, Ca, P and Cl. After the purification stage, several components of melanin were released, such as Ca, Na, Mg, P, and K (Table 1). There are differences in components after purification with commercial melanin *Sepia* studied by **Mbonyiryivuze *et al*. (2015)** which contains the main ingredients C, O, Na and Cl, as well as minor compounds Mg, Ca, K, S, N.





(a) **(b)**



(c) (d)

Figure 2. Scanning electron micrograph of cuttlefish ink melanin (a) and (b) aggregates melanin, (c) EDX pattern of melanin and (d) The FT-IR spectra of melanin after purified.

Table 1. Elemental percentages of melanin by EDX (Energy Dispersive X-ray Spectroscopy).

	C (%)	N (%)	O (%)	Na (%)	Mg (%)	P (%)	S (%)	Cl (%)	K (%)	Ca (%)
Before purified	50.22	7.99	29.18	2.38	3.98	0.83	0.98	1.99	0.52	1.93
After purified	62.76	16.02	20.28	-	-	-	0.49	0.45	-	-

Note: - = not detected

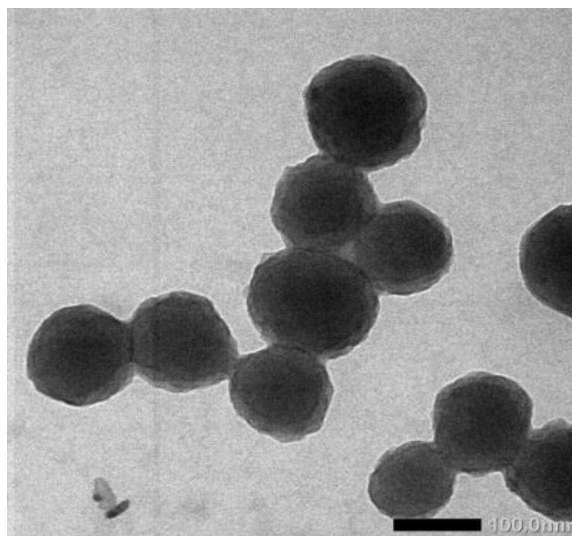


Figure 3. Granules melanin with TEM

3.3 Evaluation of antibacterial activity

Melanin activity from *Sepia* sp. to *Aeromonas* sp. growth is shown in Table 2. The concentration that causes mortality is close to 100% and then the action is

observed on Aeromonas growth. In this case, that is, at the level of 10 mg/ml. After Aeromonas was incubated with 10 mg/mL melanin, the number of live colonies was reduced by more than 1 log after 6 hours of incubation. The decline continued to 2 log10 after 21 hours of incubation. Melanin causes an extension of the adaptation phase and a decrease in the number of living colonies. After 24 hours of incubation, there were no more live colonies (Figure 4). This shows that in addition to prolonging the adaptation phase, melanin also accelerates the period of bacterial cell death. Melanin inhibits bacterial proliferation **(Mackintosh, 2001)**.

Table 2. The growth of bacteria in Nutrient Broth containing melanin.

Bacteria test	Melanin concentration (g/ml)	The number of bacteria (CFU/ml)		(%) inhibition relative to the initial microbial number [100-(Ntx100/No)]
		Incubation 0 hour (No)	Incubation 24 hour (Nt)	
Aeromonas sp.	0.002	$8.55x10^5$	$1.59x10^9$	0
	0.006	$8.55x10^5$	$3.6x10^5$	57.89
	0.010	$8.55x10^5$	$3.0x10^0$	99.99

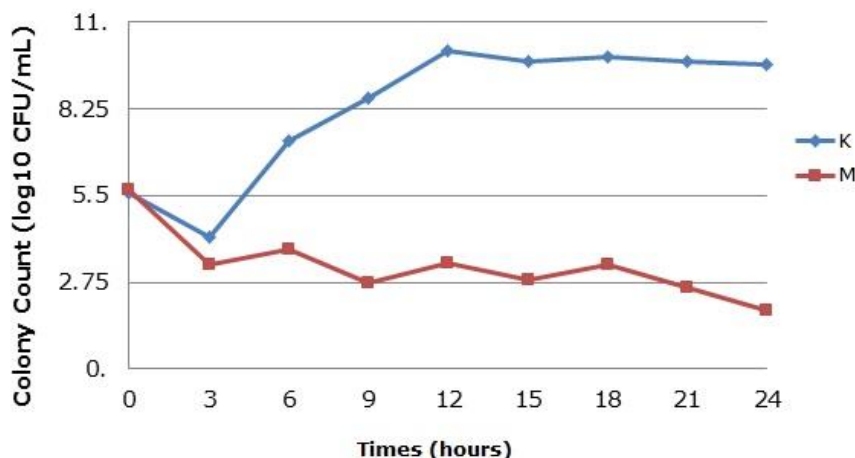
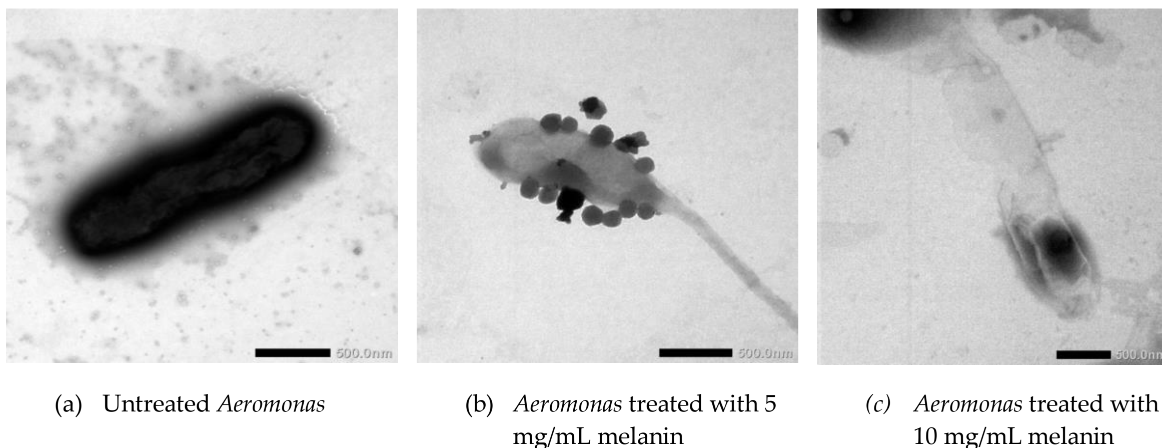


Figure 4. *Aeromonas* sp. growth curves (K) without melanin, and (M) with melanin.

Figure 5 showed that TEM from *Aeromonas* sp. without adding melanin (a) or incubated with 5 mg/mL melanin, and with 10 mg/ml melanin (arrows describe how melanin granules penetrate into cells). Melanin causes increased cell wall permeability so that melanin molecules can more easily enter cells due to damage to cell walls and membranes. This occured in the melanin treatment with a concentration of 5 mg/mL which caused the cell size to be small and the cell shape irregular.



(a) Untreated *Aeromonas* (b) *Aeromonas* treated with 5 mg/mL melanin (c) *Aeromonas* treated with 10 mg/mL melanin

Figure 5. TEM image of *Aeromonas* sp. morphology (a) before, (b) and (c) after treated with melanin.

Table 3 shows the relationship between melanin concentrations and the cell-free supernatant of *Aeromonas* cells. Analysis of cell leakage was carried out using a spectrophotometer at a wavelength of 280 and 260 nm. The 280 nm wavelength is used to measure the nitrogen content of cell proteins, while the 260 nm wavelength is to measure the nitrogen content of cell nucleic acids. Higher OD values at 260 nm and 280 nm wavelengths indicate more significant cell leakage due to melanin. The higher the melanin concentration, the greater the rate of cell leakage.

Table 3. OD values of *Aeromonas*-free supernatant after incubated with melanin from *Sepia* sp.

Melanin concentration (g/ml)	OD	
	260 nm	280 nm
0	0.038	0.037
0.005	0.179	0.198
0.010	0.297	0.321
0.015	0.359	0.395
0.020	0.451	0.499

DISCUSSION

Melanin is a tyrosinase that has been identified in squid ink (Derby, 2014). Squid ink consists of a suspension of eumelanin granules in a thick colorless medium. Eumelanin is heterogeneous, generally an insoluble polymer that develops through the enzymatic oxidation of the amino acid tyrosine. The production of eumelanin in pigment cells takes place in special organelles called melanosomes. Eumelanin consists of about 20% 5,6-dihidroxyindol (DHI) units and 5,6-dihydroxyindol-2-carboxylic acid (DHICA) units **(Magarelli *et al.*, 2010)**.

Natural melanin is reported as a pigment molecule capable of absorbing metals at high concentrations. The ability to bind eumelanin to the metal side is an

important parameter for understanding melanin metal complexes **(Lei *et al.,* 2008; Chen *et al*., 2009).** Phenolic hydroxyl (OH), carboxyl (COOH), and amine groups (NH) as functional groups that may be responsible for metal binding in melanin **(Chen *et al*., 2009)**.

Bacterial cell walls contain many types of cations including $Mg2^{+}$, $Ca2^{+}$, Na^{+}, and K^{+}. These ions are responsible for various bacterial activities, including enzyme work, metabolism regulation and maintaining the integrity of the outer layer. $Mg2^{+}$ and $Ca2^{+}$ ions in particular play an important role in protecting the stability of the external structure **(Chen *et al*., 2009; Ferrero *et al*., 2007; Peshenko and Dizhoor (2007)**. The outermost layer of the outer membrane in Gram negative bacteria is lipopolysaccharide (LPS), these molecules are individually negatively charged. The different cations help stabilize and maintain the integrity of the outer membrane by binding to adjacent LPS molecules. These divalent cations function as bridges that bind negatively charged lipid molecules **(Raetz *et al*., 2007)**. The outer membrane of bacterial cells serves as a barrier to the entry of unnecessary compounds into cells (such as bacteriocins, enzymes, and hydrophobic compounds). If the cation can be adsorbed by the melanin functional group **(Hong and Simon, 2007)**, the metabolic system where the bacterial cells will be disrupted, so that the growth of bacterial cells is also disturbed.

The outer membrane of bacterial cells is protected by $Ca2^{+}$ and $Mg2^{+}$ ions against Polymyxin B which has the ability to interact with divalent cations and replace them from their binding sites on lipopolysaccharides (LPS) **(Sahalan *et al*., 2013)**. This condition causes disorganization of the outer membrane of gram-negative bacteria. The LPS components detach from the surface of the bacteria, causing membrane leakage and ultimately cell death. $Ca2^{+}$ has been shown to be more effective in protecting bacterial cells than $Mg2^{+}$.

The binding of melanin functional groups, such as phenolic hydroxyl (OH), carboxyl (COOH), and amino (NH) groups **(Chen *et al*., 2009)** to $Ca2^{+}$ and $Mg2^{+}$ ions in the outer membrane results in leakage in the membrane of Gram negative bacteria. In this case represented by *Aeromonas*. Melanin leakage to *Aeromonas* cells can be seen from the production of bacterial cell-free supernatant after the cells were incubated for 24 hours with melanin ink (Table 3). This can lead to irregular hole formation in the outer membrane and alter membrane permeability, which is due to the gradual release of LPS molecules and membrane proteins. We can speculate that a similar mechanism causes degradation of the *Aeromonas* sp. membrane structure during addition of melanin. Extensive investigations aimed at a better understanding of the interactions between melanin and bacterial components should shed light on how this melanin material acts as a bacteriocidal material.

CONCLUSION

Melanin from *Sepia* sp. ink has inhibitory activity against the growth of Aeromonas sp., and causes reduced cell size and irregular cell shape. This is important information to overcome Aeromonas sp attack on cultured fish.

REFERENCES

Aruldhason, B. V.; Vairamani, S. and Annaian, S. (2014). Antimicrobial activity of methanolic extract and fractionated polysaccharide from Loligo duvauceli Orbingy 1848 and Doryteuthis sibogae Adam 1954 on human pathogenic microorganisms. Afr. J. Microbiol. Res., 8(3): 230–236.

Bunduki, M. M. C.; Flanders, K. J. and Donnelly, C. W. (1995). Metabolic and structural sites of damage in heat- and sanitizer-lnjured populations of listeria monocytogenes. J. Food Protect., 58(4): 410–415.

Capasso, R.; Evidente, A.; Schivo, L.; Orru, G.; Marcialis, M. A. and Cristinzio, G. (1995). Antibacterial polyphenols from olive oil mill waste waters. J. Appl. Bacteriol., 79(4): 393–398.

Derby, C. D. (2014). Cephalopod ink: Production, chemistry, functions and applications. Mar. Drugs., 12(5): 2700–2730.

Fahmy, R. (2013). In vitro antioxidant, analgesic and cytotoxic activities of Sepia officinalis ink and Coelatura aegyptiaca extracts. Afr. J. Pharm. Pharmacol., 7(22): 1512–1522.

Ferrero, M. Á.; Martínez-Blanco, H.; Lopez-Velasco, F. F.; Ezquerro-Sáenz, C.; Navasa, N.; Lozano, S. and Rodríguez-Aparicio, L. B. (2007). Purification and characterization of GlcNAc-6-P 2-epimerase from Escherichia coli K92. Acta Biochim. Pol., 54(2): 387–399.

Fiore, G.; Poli, A.; Di Cosmo, A.; d'Ischia, M. and Palumbo, A. (2004). Dopamine in the ink defence system of Sepia officinalis: Biosynthesis, vesicular compartmentation in mature ink gland cells, nitric oxide (NO)/cGMP-induced depletion and fate in secreted ink. Biochem. J., 378(3): 785–791.

Guo, X.; Chen, S.; Hu, Y.; Li, G.; Liao, N.; Ye, X.; Liu, D. and Xue, C. (2014). Preparation of water-soluble melanin from squid ink using ultrasound-assisted degradation and its anti-oxidant activity. J. Food Sci. Technol., 51(12): 3680–3690.

Hong, L. and Simon, J. D. (2007). Current Understanding of the Binding Sites, Capacity, Affinity, and Biological Significance of Metals in Melanin. J. Phys. Chem. B., 111(28): 7938–7947.

Kim, J. S.; Kuk, E.; Yu, K. N.; Kim, J. H.; Park, S. J.; Lee, H. J.; Kim, S. H.; Park, Y. K.; Park, Y. H.; Hwang, C. Y.; Kim, Y. K.; Lee, Y. S.; Jeong, D. H. and Cho, M. H. (2007). Antimicrobial effects of silver nanoparticles. Nanomedicine., 3(1): 95–101.

Lei, M.; Xue, C. H.; Wang, Y. M.; Li, Z. J.; Xue, Y. and Wang, J. F. (2008). Effect of squid ink melanin-Fe on iron deficiency anemia remission. J. Food Sci., 73(8): H207–H211.

Mackintosh, J. A. (2001). The antimicrobial properties of melanocytes, melanosomes and melanin and the evolution of black skin. J. Theor. Biol., 211(2): 101–113.

Magarelli, M.; Passamonti, P. and Renieri, C. (2010). Purification , characterization and analysis of sepia melanin from commercial sepia ink (Sepia Officinalis Purificación , caracterización y análisis de la melanina de sepia a partir de la tinta de sepia (Sepia Officinalis) Resumen. Ces. Med. Vet. Zootec., 5(2): 18–28.

Mbonyiryivuze, A.; Nuru, Z. Y.; Ngom, B. D.; Mwakikunga, B. W.; Dhlamini, M. S.; Park, E. and Maaza, M. (2015). Morphological and Chemical Composition Characterization of Commercial Sepia Melanin. Am. J. Nanomat., 3(1): 22–27.

Naraoka, T.; Chung, H-S.; Uchisawa, H.; Sasaki, J-I. and Matsue, H. (2000). Tyrosinase Activity in Antitumor Compounds of Squid Ink. Food Sci. Technol. Res., 6(3): 171–175.

Nithya, M.; Ambikapathy, V. and Panneerselvam, A. (2011). Effect of pharaoh' s cuttlefish ink against bacterial pathogens. Asian J. Plant Sci. Res., 1(4): 49–55.

Peshenko, I. V. and Dizhoor, A. M. (2007). Activation and inhibition of photoreceptor guanylyl cyclase by Guanylyl Cyclase Activating Protein 1 (GCAP-1): The functional role of Mg 2+/Ca2+ exchange in EF-hand domains. J. Biol. Chem., 282(30): 21645–21652.

Pralea, I. E.; Moldovan, R. C.; Petrache, A. M.; Ilieș, M.; Hegheș, S. C.; Ielciu, I.; Nicoară, R.; Moldovan, M.; Ene, M.; Radu, M.; Uifălean, A. and Iuga, C. A. (2019). From Extraction to Advanced Analytical Methods: The Challenges of Melanin Analysis. Int. J. Mol. Sci., 20(16): 3943.

Praveen, P. K.; Debnath, C.; Shekhar, S.; Dalai, N. and Ganguly, S. (2016). Incidence of Aeromonas spp. infection in fish and chicken meat and its related public health hazards: A review. Vet. World., 9(1): 6–11.

Raetz, C. R. H.; Reynolds, C. M.; Trent, M. S. and Bishop, R. E. (2007). Lipid a modification systems in gram-negative bacteria. Ann. Rev. Biochem., 76: 295–329.

Rajaganapathi, J.; Thyagarajan, S. P. and Patterson Edward, J. K. (2000). Study on cephalopod's ink for anti-retroviral activity. Indian J. Exp. Biol., 38(5): 519–520.

Nair, J.R.; Pillai, D.; Joseph, S.M.; Gomathi, P.; Senan, P. V. and Sherief, P.M. (2011). Cephalopod research and bioactive substances. Indian J. Mar. Sci., 40(1): 13–27.

Sahalan, A. Z.; Aziz, A. H. A.; Hing, H. and Ghani, M. K. A. (2013). Divalent CATIONS (Mg2+, Ca2+) protect bacterial outer membrane damage by polymyxin B. Sains Malaysiana, 42(3): 301–306.

Solano, F. (2020). Photoprotection and skin pigmentation: Melanin-related molecules and some other new agents obtained from natural sources. Molecules, 25(7): 1–18.

Wang, F. R.; Xie, Z. G.; Ye, X. Q.; Deng, S. G.; Hu, Y. Q.; Guo, X. and Chen, S. G. (2014). Effectiveness of treatment of iron deficiency anemia in rats with squid ink melanin-Fe. Food Funct., 5(1): 123–128.

Chen, S.; Xue, C.; Wang, J.; Feng, H.; Wang, Y.; Ma, Q. and Wang, D. (2009). Adsorption of Pb(II) and Cd(II) by Squid Ommastrephes bartrami Melanin. Bioinorg. Chem. Appl., 2009: 901563.

Yuvaraj, D.; Suvasini, B.; Chellathai, T.; Fouziya, R.; Ivo Romauld, S. and Chandran, M. (2015). Research Article Anti-bacterial studies on the different body parts of Loligo duvauceli. J. Chem. Pharm. Res., 7(6): 406–408.

Zakaria, F.; Fadjar, M. and Yanuhar, U. (2019). Cuttlefish (Sepia sp.) ink extract as antibacterial activity against aeromonas hydrophila. Int. J. Sci. Techn. Res., 8(11): 1175–1177.

Zhong, J. P.; Wang, G.; Shang, J. H.; Pan, J. Q.; Li, K.; Huang, Y. and Liu, H. Z. (2009). Protective effects of squid ink extract towards hemopoietic injuries induced by cyclophosphamine. Mar. Drugs., 7(1): 9–18.

Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 24(--): … – … (2020)
www.ejabf.journals.ekb.eg

The antibacterial activity of melanin in cuttlefish (*Sepia* sp.) ink against *Aeromonas* sp.

Yuspihana Fitrial [1,*] and Iin Khusnul Khotimah [1]

[1] Study Program of Fisheries Product Technology, Faculty of Fisheries and Marine, Lambung Mangkurat University, Banjarbaru 70712, South Kalimantan, Indonesia
[*]Corresponding Author: yuspihana.fitrial@ulm.ac.id

ARTICLE INFO
Article History:
Received:
Accepted:
Online:

Keywords:
Cuttlefish,
Ink,
Melanin,
Antibacterial activity.

ABSTRACT

The marine environment consists of many organisms recognized to have bioactive compounds as a mechanism of self-defence or protection of eggs and embryos. One of them is cuttlefish (*Sepia* sp.). This study aimed to determine the potential and bactericidal action of the cuttlefish ink melanin against *Aeromonas* sp. Ink extraction and purification were carried out to obtain melanin mechanically using 0.5M HCl. The growth patterns of bacteria were studied by the Total Plate Count method, and the bactericidal mechanism of melanin was observed by Transmission Electron Microscopy (TEM). The results showed that cuttlefish ink melanin inhibited *Aeromonas* sp. as indicated by the shrinkage of cell size and irregular cell shape. The results of this study are important information for dealing with *Aeromonas* sp. attack in cultured fish.

INTRODUCTION

Cuttlefish is one of the fisheries commodities which utilization is still limited. So far, the use of cuttlefish has only been cooked with a mixture of spices, while the ink is thrown away or used as part of the cuttlefish processing in food. Several studies have shown that squid ink contains melanin, protein, fat and glycosaminoglycans (**Fiore *et al.*, 2004**). Squid ink can act as a cell-protective drug in the treatment of cancer with chemotherapy by increasing the number of leukocytes and bone marrow cell nucleates, which numbers are decreasing due to the use of drugs such as tumor cell killer (**Naraoka *et al.*, 2000**). In addition, melanin from squid ink has anti-tumor activity by inhibiting plasmin activity from increasing thromboxane and increasing the body's resistance to killing cancer cells (**Zhong *et al.*, 2009**). Melanin also contributes as an antioxidant (**Fahmy, 2013; Guo *et al.*, 2014**), anti-radiation (**Solano, 2020; Pralea *et al.*, 2019**), antirotavirus (**Rajaganapathi *et al.*, 2000**) and antibacterial (**Aruldhason *et al.*, 2014; Zakaria *et al.*, 2019).** In addition, melanin also can absorb metals, especially in the phenolic hydroxyl (OH), carboxyl (COOH) and amine (NH) functional groups of the melanin molecule (**Chen *et al.*, 2009**).

Nair *et al*. (2011) stated that Sepia ink consists of melanin granules on thick colourless media. The pigment melanin is the main element in the ink glands that continuously produce ink. At the end of the ripening process, the ink gland cells store ink sacs that function as reservoirs. Each Sepia ink bag contains ± 1 g melanin **(Derby, 2014)**, and the amount of melanin is ± 15% of the total ink wet weight **(Wang *et al*., 2014)**. Melanin Sepia is formed by many aggregates. This aggregate is also formed by small round grains with different size distributions. Small grain diameter ranges from 100-200 nm **(Mbonyiryivuze *et al*., 2015)**.

The squid and or cuttlefish ink had antibacterial activity **(Nair *et al*., 2011).** Melanin's action as an antibacterial has not been much revealed. Some researchers have tested antibacterial activity against only extracts from cuttlefish and or squid ink. **Nithya *et al*. (2011)** examined the antibacterial activity of cuttlefish hexane extract (*Sepia pharaonis*) purified with diethyl ether. The results of this study indicate that the extract has inhibitory activity against *Pseudomonas aeruginosa*, *Staphylococcus epidermidis, Klebsiella pneumoniae*, and *E. coli*. **Sari *et al.* (2019)** revealed that melanin from squid ink contains steroid and triterpenoids, which has a weak to medium antibacterial activity against *L. monocytogenes* and *E. coli*. The results of our study tested the antibacterial activity by approximating the ability of the ink component, that is melanin, to chelate metals. The cytotoxic effect of melanin squid ink is thought to also affect the growth of fish microbes, such as *Aeromona*s sp.

Aeromonas sp. are gram-negative, facultative anaerobes, which cause disease in both terrestrial and aquatic animals, as well as in humans. *Aeromonas* sp. is considered an important bacterium among disease-causing agents in fish **(Praveen *et al.,* 2016; Olga *et al.,* 2020)**. It is necessary to study the antibacterial activity of cuttlefish melanin against *Aeromonas* sp. Therefore, this study aimed to determine the potential and bactericidal activity of the cuttlefish ink melanin against *Aeromonas* sp.

MATERIALS AND METHODS

2.1 Materials

The material used was cuttlefish (ink) obtained from the Muara Kintap Fishing Port, Tanah Laut Regency, South Kalimantan. Other ingredients used include 0.5M HCl (Merck), acetone (Merck), aquades, Nutrient Broth (NB) (Merck), Nutrient Agar (NA) (Merck), Sterile-EO Syringe Filter (Sartorius Minisart pore size 0.20 µm), Microbact TM GNB12A/B/E, 24E Identification Kits (Oxoid) and 70% alcohol.

The tools used in this study were a refrigerated centrifuge (Labogene Scanspeed 1580R), Freeze dryer (Model Christ alpha 2-4 LD Plus), autoclave (Pressure Steam Sterilizer Electric Model No.25X-2), laminar flow (Biobase), incubator (Memmert), colony counter (Quebec), incubator shaker (Wisd), spectrophotometer (Genesys 10uv), SEM with Energy Dispersive X-ray (SEM-EDX) EDAX.SL, TEM JEOL, JEM1400, and beaker glass.

2.2 Methods

2.2.1 Preparation of melanin

Isolation and purification of cuttlefish ink melanin according to the method **(Magarelli *et al*., 2010)** as follows: 50 g of cuttlefish ink is added with 100 ml of HCl (0.5

M) in the dark. The solution was stirred for 30 minutes (in a magnetic stirrer) and then stored for 24 hours at 10ºC. The pellet was separated from the supernatant by centrifugation (10000 rpm at 5ºC for 15 minutes). The suspension was rinsed three times each with 0.5M HCl, sterile water, acetone and finally with water, sequentially. Lyophilization was then carried out to remove the solvent using a freeze dryer. Melanin was obtained and then characterized using infrared (IR) spectroscopy and its morphology by SEM, TEM, and the percentage of melanin elements using EDX (Energy Dispersive X-ray Spectroscopy).

2.2.2 Culture of the organism

The microbes were isolated from rotten meat and digestive fish (*Euthynnus affinis*). Isolates were grown on GSP agar medium (*Pseudomonas-Aeromonas*-Selective agar, Merck). Species were determined using the manual protocol of Microbact identification kit GNB 12A/B/E, 24E (Oxoid).

2.2.3. Analysis of Aeromonas cell leakage

The methods of cell leakage analysis were referred to **Bunduki *et al.* (1995)**. Analysis of *Aeromonas* cell leakage was carried out using a spectrophotometer at 280 and 260 nm. The 280 nm wavelength is used to measure the nitrogen content of cell proteins, while the 260 nm wavelength is used to measure the nucleic nitrogen content in cells. 10 mL of *Aeromonas* cultures were centrifuged at 10,000 rpm for 10 minutes. The filtrate was removed and added with 5 mL of physiological salt solution (0.85% NaCl) to the cell sediment. The solution was then vortexed. Furthermore, melanin was added in a concentration of 0; 5; 10; 15; 20 mg/mL, and incubated at room temperature for 24 hours. The suspension was centrifuged at 10,000 rpm for 10 minutes, and the supernatant filtered with filter paper (0.20 µm sterile syringe filter) to separate cells. The analysis was carried out by observing the OD (Optical Density) of the liquid supernatant.

2.2.4 Analysis of melanin activity as antibacterial

Melanin activity was analyzed using the direct contact method between melanin and bacteria in liquid nutrient broth. The test tube consists of 2,970 mL of sterile NB and 0.030 mL of bacterial suspension (3,000 mL of final solution). Powdered melanin was added to the test tube so that the melanin concentration in the tube was 0; 2; 6; 10 mg/mL. For the first series of test tubes (melanin concentration 0 mg/mL), 2,970 NB sterile + 0 mg melanin was used. A second series tube (melanin concentration 2 mg/mL), was prepared by adding 2.964 mL of sterile NB + 6 mg melanin, and so on.

The bacteria (10^8-10^9 CFU/mL) were freshened and incubated for 24 hours at 37 °C, then diluted ten times. The test tube was inoculated with 0.030 mL of bacterial suspension, vortexed for 1-2 minutes, and then incubated in a shaker incubator at 37°C for 24 hours. Total Plate Count was used to count the bacteria by incubating the bacteria for 24 hours at 37°C, the number of colonies was counted. The total colony is the average count on the three Petri dishes that match a particular sample.

The percentage of bacterial inhibition was determined by a modification of the method **(Capasso *et al.*, 1995)** with the formula:

$$Bacterial\ inhibition = 100 - (N_t \times 100/N_o) \quad (1)$$

where Nt is the number of CFU bacteria/mL in the addition of melanin treatment, while No is the number of CFU bacteria/mL initial (initial inoculum). Total Plate Count was used to count the bacteria by incubating the bacteria for 24 hours at 37°C, and then the number of colonies was counted. The total colony evaluation was carried out every 3 hours with repetitions.

2.2.5 Analysis of melanin activity to Aeromonas sp. growth

The inhibition of melanin on the growth of *Aeromonas* sp. was carried out in the same method as the melanin activity test above, with a concentration of 10,000 mg/mL. This concentration is defined by the percentage of inhibition relative to the number of initial microbes close to 100%. Observations were carried out every three hours for 24 hours.

2.2.6. Preparation of Aeromonas sp. for TEM analysis

Sample preparation followed the method by **Kim *et al.* (2007)** with some modifications. Bacteria were inoculated in NB medium, and 5,000 mg/mL and 10,000 mg/mL melanin were added, then incubated for 24 hours at 37°C. Negative controls (without melanin) were prepared in NB medium. After incubation, the suspension was centrifuged at 1200 rpm for 10 minutes. The supernatant was washed with a phosphate buffer solution pH 7.2 and centrifuged at 2,600 rpm for 5 minutes (4°C). For the sample, cell pellets were fixed with 2% glutaraldehyde. *Aeromonas* sp. Cell images were observed by transmission electron microscopy (TEM, JEOL, JEM 1400).

RESULTS

3.1 Isolation of *Aeromonas* sp.

The colony, presumably *Aeromonas* sp. is the yellow color on GSP agar, tested for biochemical properties using a microbact (oxoid) identification kit. The test results of the microbact kit showed in Table 1. The identification results showed *Aeromonas hydrophilla* (similarity reached 94.20%).

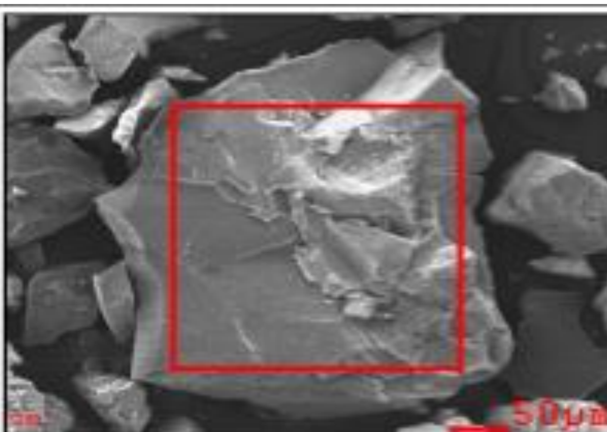
Table 1. The results of the microbact kit

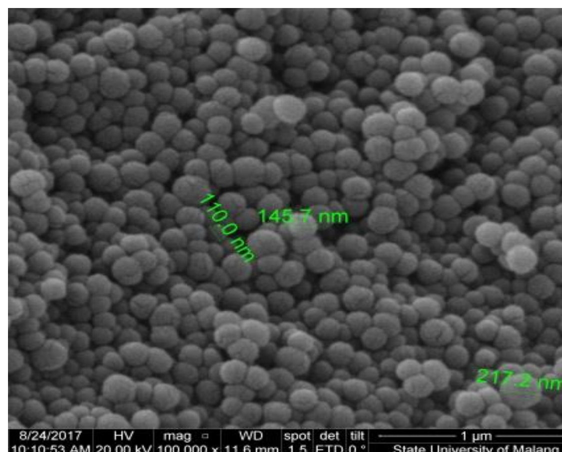
Microbact (oxoid) identification	Result
Oxidase	(+)
Motility	(+)
Nitrate	(+)
Lysine	(+)
Ornitine	(+)
H_2s	(+)
Glucose	(+)
Mannitol	(+)
Xylose	(+)
Onpg	(+)
Indole	(+)
Urease	(+)
Vp	(+)
Citrate	(+)

TDA	(-)
Gelatin	(-)
Malonate	(+)
Inositol	(-)
Sorbitol	(+)
Rhamnose	(+)
Sucrose	(+)
Lactose	(+)
Arabinose	(+)
Adonitol	(-)
Raffinose	(+)
Salicin	(-)
Arginine	(-)

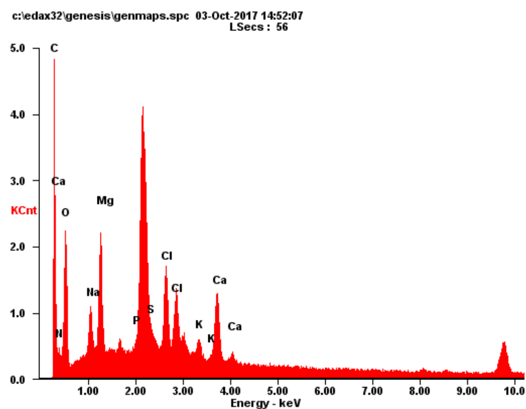
3.2 Purification of melanin

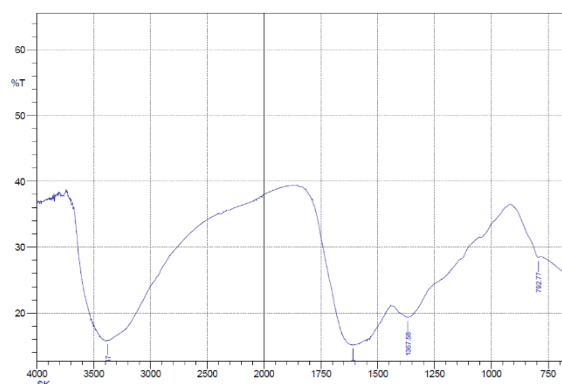
Cuttlefish melanin is an aggregate composed of many spherical grains. Small spherical grains also make these aggregates with different size distributions. The small grain diameter ranges from 100-200 nm (Figures 1 and 2). TEM micrograph showing spherical melanin granules (Figure 3).





(a) **(b)**

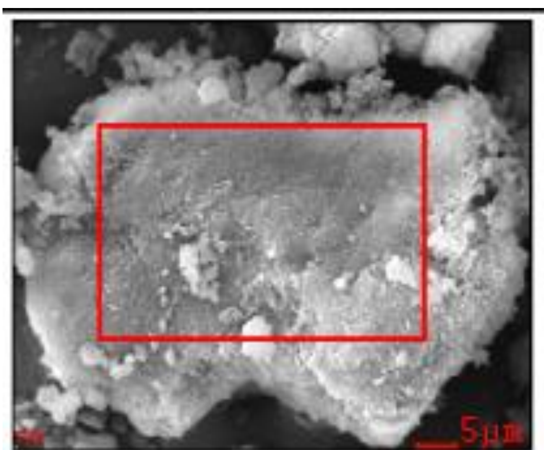


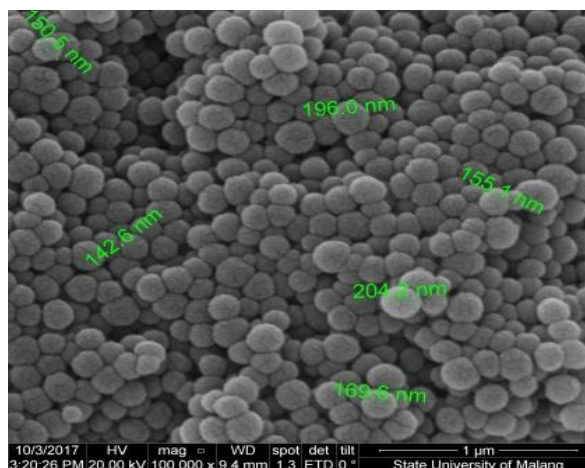


(c) (d)

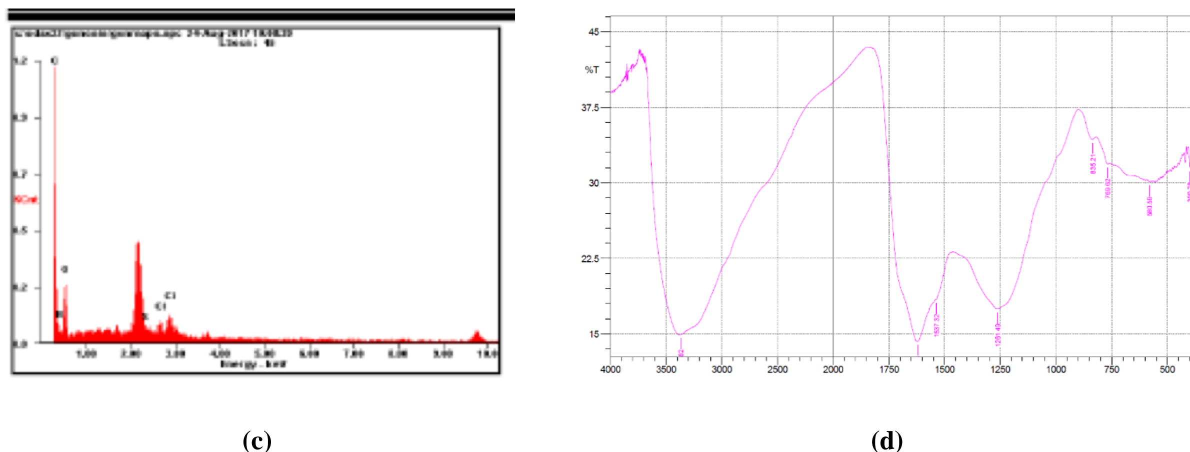
Figure 1. Scanning electron micrograph (SEM) of cuttlefish ink melanin (a) and (b) aggregates melanin, (c) EDX pattern of melanin and (d) The FT-IR spectra of melanin before purified.

The FTIR spectrum showed that melanin had a strong Infrared absorption spectrum at 3300, 1620, and 1260 cm-1. It shows phenolic hydroxyl (OH), carboxyl (COOH), and amine (NH) groups (Figure 1d). The difference between before and after purification is mainly seen in the intensity of each functional group. Cuttlefish ink contains the most significant components, specifically C (50.22%), O (29.18%), and N (7.99%), as well as a small portion of Mg, Na, S, K, Ca, P and Cl. After the purification stage, several components of melanin were released, such as Ca, Na, Mg, P, and K (Table 2).





(a) (b)



(c) (d)

Figure 2. Scanning electron micrograph of cuttlefish ink melanin (a) and (b) aggregates melanin, (c) EDX pattern of melanin and (d) The FT-IR spectra of melanin after purified.

Table 2. Elemental percentages of melanin by EDX (Energy Dispersive X-ray Spectroscopy).

	C (%)	N (%)	O (%)	Na (%)	Mg (%)	P (%)	S (%)	Cl (%)	K (%)	Ca (%)
Before purified	50.22	7.99	29.18	2.38	3.98	0.83	0.98	1.99	0.52	1.93
After purified	62.76	16.02	20.28	-	-	-	0.49	0.45	-	-

Note: - = not detected

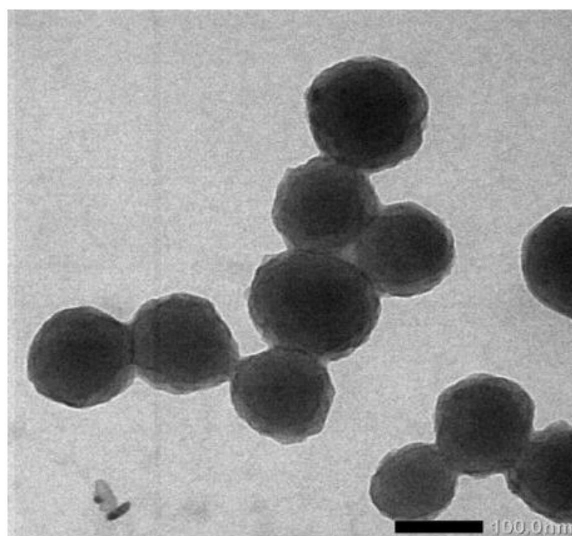


Figure 3. Granules melanin with TEM

3.3 Evaluation of antibacterial activity

Melanin activity from *Sepia* sp. to *Aeromonas* sp. growth is shown in Table 3. The concentration that causes mortality is close to 100% and then the action is observed on *Aeromonas* growth. 10 mg/ml of melanin cause a decrease in the number of live colonies

more than 1 log after 6 hours of incubation. The decline continued to 2 log10 after 21 hours of incubation. Melanin causes an extension of the adaptation phase and a decrease in the number of living colonies. After 24 hours of incubation, there were no more live colonies (Figure 4). Figure 4 shows the total bacteria measured every 3 hours. The existing method does not explain in detail, especially the method of measurement. It was indicates that in addition to prolonging the adaptation phase, melanin also accelerates the period of bacterial cell death.

Table 3. The growth of *Aeromonas* sp. in Nutrient Broth containing melanin.

Bacteria test	Melanin concentration after purification (mg/ml)	The number of bacteria (CFU/ml)		(%) inhibition relative to the initial microbial number [100-(Ntx100/No)]
		Incubation 0 hour (No)	Incubation 24 hours (Nt)	
Aeromonas sp.	2	$8.55x10^5$	$1.59x10^9$	0
	6	$8.55x10^5$	$3.6x10^5$	57.89
	10	$8.55x10^5$	$3.0x10^0$	99.99

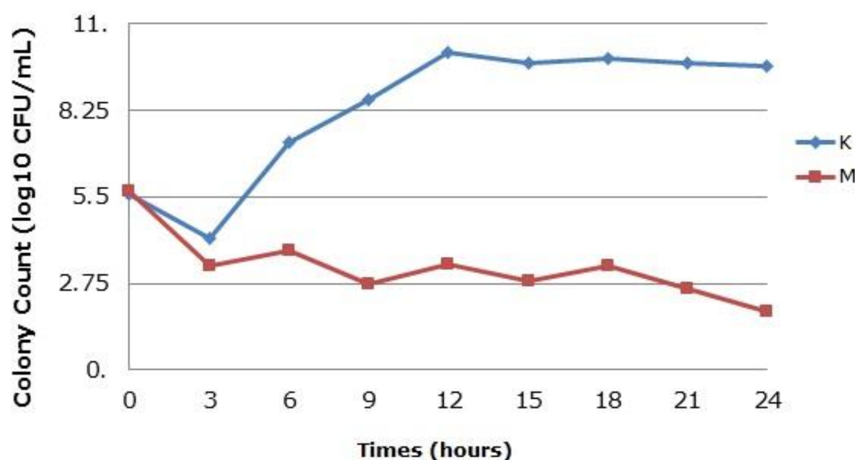
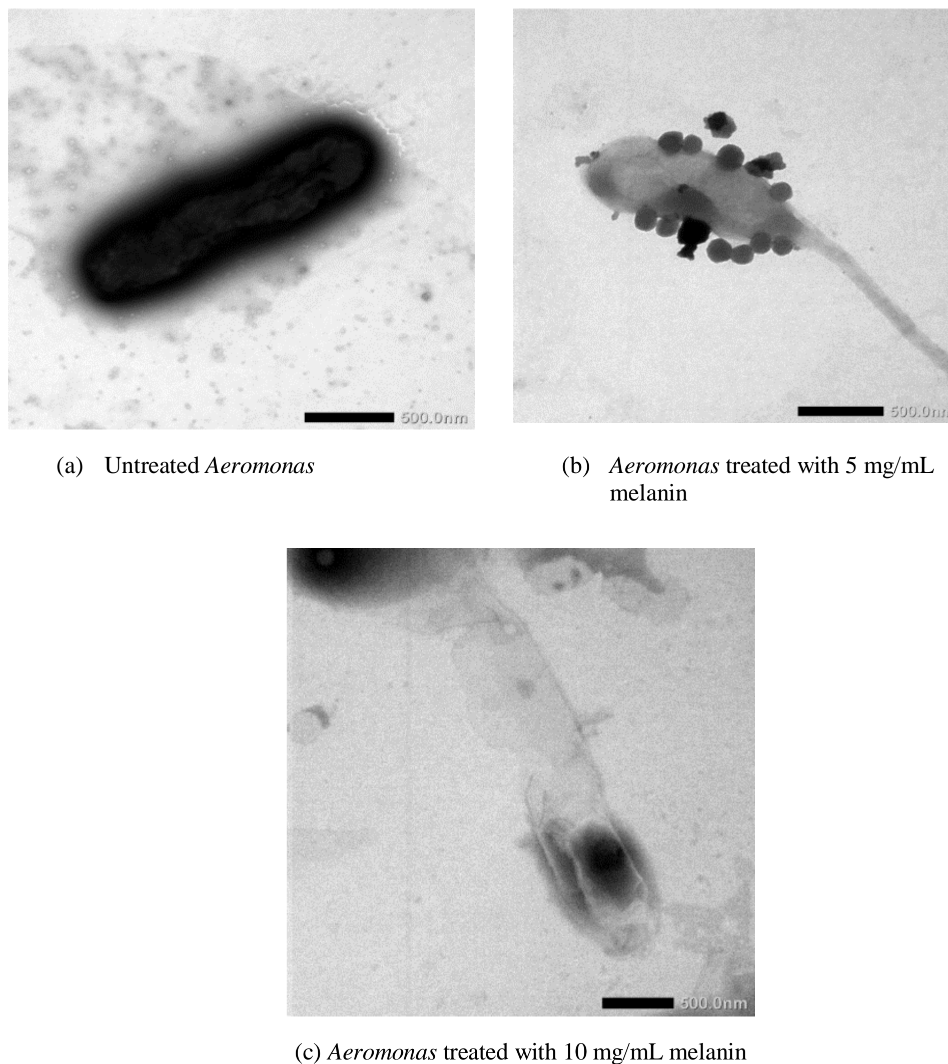


Figure 4. *Aeromonas* sp. growth curves (K) without melanin, and (M) with melanin.

Figure 5b showed that TEM from *Aeromonas* sp. without adding melanin (a) or incubated with 5 mg/mL melanin and with 10 mg/ml melanin (arrows describe how melanin granules penetrate cells). Melanin causes increased cell wall permeability and leads melanin molecules to penetrate the cells due to cell walls and membranes damage. This concentration of 5 mg/mL melanin caused the cell size to be small and irregular.



(a) Untreated *Aeromonas*

(b) *Aeromonas* treated with 5 mg/mL melanin

(c) *Aeromonas* treated with 10 mg/mL melanin

Figure 5. TEM image of *Aeromonas* sp. morphology (a) before, (b) and (c) after treated with melanin.

Table 4 showed the relationship between melanin concentrations and the cell-free supernatant of *Aeromonas* cells. Higher OD values at 260 nm and 280 nm indicated more significant cell leakage due to melanin. The higher the melanin concentration, the greater the rate of cell leakage.

Table 4. OD values of *Aeromonas*-free supernatant after incubated with melanin from *Sepia* sp.

Melanin concentration (mg/ml)	Optical Density	
	260 nm	280 nm
0	0.038	0.037
5	0.179	0.198
10	0.297	0.321

15	0.359	0.395
20	0.451	0.499

DISCUSSION

Melanin is a tyrosinase that has been identified in squid ink **(Derby, 2014)**. Squid ink consists of a suspension of eumelanin granules in a thick colorless medium. Eumelanin is heterogeneous, generally, an insoluble polymer that develops through the enzymatic oxidation of the amino acid tyrosine. The production of eumelanin in pigment cells takes place in special organelles called melanosomes. Eumelanin consists of about 20% 5,6-dihidroxyindol (DHI) and 5,6-dihydroxyindol-2-carboxylic acid (DHICA) **(Magarelli *et al*., 2010)**. Other sudy revelaed that melanin inhibits bacterial proliferation **(Mackintosh, 2001)**.

Natural melanin is reported as a pigment molecule capable of absorbing metals at high concentrations. The ability to bind eumelanin to the metal side is an important parameter for understanding melanin metal complexes **(Lei *et al.,* 2008; Chen *et al*., 2009).** Phenolic hydroxyl (OH), carboxyl (COOH), and amine groups (NH) as functional groups that may be responsible for metal binding in melanin **(Chen *et al*., 2009)**. **Zerrad *et al.* (2014)** showed that there is a tenuous vibration of NH and OH groups at a wavelength of 3365-2880 cm^{-1}. The band centred at 1637 cm^{-1} is associated with the vibration of the aromatic ring of the C = C boundary and/or the aromatic conjugated C = O group. The FTIR spectrum of the melanin pigment, centered at 1261 cm^{-1} corresponds to the bending of the carboxylic ion group (COOH). In comparison, other small peaks can be ascribed to the substitution of C-H alkenes in melanin pigments **(Sajjan *et al*., 2010; Surwase *et al*., 2012)**.

There are differences in components after purification with commercial melanin *Sepia* studied by **Mbonyiryivuze *et al.* (2015)** which contains the main ingredients C, O, Na and Cl, as well as minor compounds Mg, Ca, K, S, N. Melanin has the ability to bind various metal ions such as Ca, Fe, Cu, Zn, and Mg **(Zou *et al.,* 2015)**. One of the bacterial metals that plays a role in cell wall orders is Mg (Magnesium) **(Matthews *et al*., 1979)**. The antibacterial activity of melanin is thought to be by storing metal ions needed by bacteria for cell wall ordering **(Aisiah *et al*., 2020)**. The results were supported by **(Sari *et al*., 2019),** which showed the ability of melanin in the bacterial cell wall ordering event. C and N elements increase after purification, presumably because after purification, the release of ions makes C and N elements more concentrated. **Poernomo *et al.* (2020)** stated that carbon and nitrogen play a role in the strength of the antibacterial activity of a material.

Bacterial cell walls contain many types of cations, including Mg^{2+}, Ca^{2+}, Na^{+}, and K^{+}. These ions are responsible for various bacterial activities, including enzyme work, metabolism regulation and maintaining the integrity of the outer layer. Mg^{2+} and Ca^{2+} ions, in particular, play an important role in protecting the stability of the external structure **(Chen *et al*., 2009; Ferrero *et al*., 2007; Peshenko and Dizhoor, 2007)**. The outermost layer of the outer membrane in Gram-negative bacteria is lipopolysaccharide (LPS). These molecules are individually negatively charged. The different cations help stabilize and maintain the integrity of the outer membrane by binding to adjacent LPS molecules. These divalent cations function as bridges that bind negatively charged lipid molecules **(Raetz *et***

***al*., 2007**). The outer membrane of bacterial cells serves as a barrier to the entry of unnecessary compounds into cells (such as bacteriocins, enzymes, and hydrophobic compounds). If the cation can be adsorbed by the melanin functional group **(Hong and Simon, 2007)**, the metabolic system where the bacterial cells will be disrupted so that the growth of bacterial cells is also disturbed. Figure 5 showed the disruption of the bacterial cell wall due to melanin's activity, which disrupts the metabolic system of the bacterial cell wall.

The outer membrane of bacterial cells is protected by Ca^{2+} and Mg^{2+} ions against Polymyxin B, which can interact with divalent cations and replace them from their binding sites on lipopolysaccharides (LPS) **(Sahalan *et al*., 2013)**. This condition causes disorganization of the outer membrane of gram-negative bacteria. The LPS components detach from the surface of the bacteria, causing membrane leakage and ultimately cell death. Ca^{2+} has been shown to be more effective in protecting bacterial cells than Mg^{2+}. This can be seen in Table 3 and Figure 4, where melanin has the property of chelating metal ions so that the formation of bacterial cell walls is not formed. The formation of a bacterial cell wall requires logomes such as Ca and Mg

The binding of melanin functional groups, such as phenolic hydroxyl (OH), carboxyl (COOH), and amino (NH) groups **(Chen *et al*., 2009)** to Ca^{2+} and Mg^{2+} ions in the outer membrane of *Aeromonas*. *Aeromonas* cells leakage can be seen from the production of bacterial cell-free supernatant after the cells were incubated for 24 hours with melanin ink (Table 3). This can lead to irregular whole formation in the outer membrane and alter membrane permeability due to the gradual release of LPS molecules and membrane proteins. The results of this study are supported by previous research, which shows an increase in the value of optical density (wavelengths of 260 nm and 280 nm), to the administration of melanin extract on the growth of *E. coli* **(Fitrial & Khotimah, 2017)**. The increase in optical density is closely related to the presence of bacteria, presumably due to the effect of melanin which inhibits bacterial growth by damaging cell walls **(Zhang *et al*., 2015)**. We indicated that a similar mechanism causes degradation of the *Aeromonas* sp. membrane structure during the addition of melanin. Extensive investigations aimed at a better understanding of the interactions between melanin and bacterial components should shed light on how this melanin material acts as a bacteriocidal material.

CONCLUSION

Melanin from *Sepia* sp. ink has inhibitory activity against the growth of *Aeromonas* sp., and causes reduced cell size and irregular cell shape. This is important information to overcome *Aeromonas* sp. attack on cultured fish.

REFERENCES

Yuvaraj, D.; Suvasini, B.; Chellathai, T.; Fouziya, R.; Ivo Romauld, S. and Chandran, M. (2015). Research Article Anti-bacterial studies on the different body parts of *Loligo duvauceli*. J. Chem. Pharm. Res., 7(6): 406–408.

Aisiah, S.; Olga, O.; Tanod, W. A.; Salosso, Y.; Bambang, B. and Riyadi, P. H. (2020). Computational analysis of ethyl acetate extract of Nauclea subdita (Korth.) Steud. leaves as peptidoglycan glycosyltransferase inhibitor in *Aeromonas hydrophila*. IOP Conf. Ser. Earth Environ. Sci., 012022.

Aruldhason, B. V.; Vairamani, S. and Annaian, S. (2014). Antimicrobial activity of methanolic extract and fractionated polysaccharide from *Loligo duvauceli* Orbingy 1848 and *Doryteuthis sibogae* Adam 1954 on human pathogenic microorganisms. Afr. J. Microbiol. Res., 8(3): 230–236.

Bunduki, M. M. C.; Flanders, K. J. and Donnelly, C. W. (1995). Metabolic and structural sites of damage in heat- and sanitizer-Injured populations of listeria monocytogenes. J. Food Protect., 58(4): 410–415.

Capasso, R.; Evidente, A.; Schivo, L.; Orru, G.; Marcialis, M. A. and Cristinzio, G. (1995). Antibacterial polyphenols from olive oil mill waste waters. J. Appl. Bacteriol., 79(4): 393–398.

Chen, S.; Xue, C.; Wang, J.; Feng, H.; Wang, Y.; Ma, Q. and Wang, D. (2009). Adsorption of Pb(II) and Cd(II) by Squid *Ommastrephes bartrami* Melanin. Bioinorg. Chem. Appl., 2009: 901563.

Derby, C. D. (2014). Cephalopod ink: Production, chemistry, functions and applications. Mar. Drugs., 12(5): 2700–2730.

Fahmy, R. (2013). In vitro antioxidant, analgesic and cytotoxic activities of *Sepia officinalis* ink and Coelatura aegyptiaca extracts. Afr. J. Pharm. Pharmacol., 7(22): 1512–1522.

Ferrero, M. Á.; Martínez-Blanco, H.; Lopez-Velasco, F. F.; Ezquerro-Sáenz, C.; Navasa, N.; Lozano, S. and Rodríguez-Aparicio, L. B. (2007). Purification and characterization of GlcNAc-6-P 2-epimerase from *Escherichia coli* K92. Acta Biochim. Pol., 54(2): 387–399.

Fiore, G.; Poli, A.; Di Cosmo, A.; d'Ischia, M. and Palumbo, A. (2004). Dopamine in the ink defence system of *Sepia officinalis*: Biosynthesis, vesicular compartmentation in mature ink gland cells, nitric oxide (NO)/cGMP-induced depletion and fate in secreted ink. Biochem. J., 378(3): 785–791.

Fitrial, Y. and Khotimah, I. K. (2017). Antibacterial Activity of Melanin from Cuttlefish and Squid Ink. Jurnal Pengolahan Hasil Perikanan Indonesia, 20(2): 266–274.

Guo, X.; Chen, S.; Hu, Y.; Li, G.; Liao, N.; Ye, X.; Liu, D. and Xue, C. (2014). Preparation of water-soluble melanin from squid ink using ultrasound-assisted degradation and its anti-oxidant activity. J. Food Sci. Technol., 51(12): 3680–3690.

Hong, L. and Simon, J. D. (2007). Current Understanding of the Binding Sites, Capacity, Affinity, and Biological Significance of Metals in Melanin. J. Phys. Chem. B., 111(28): 7938–7947.

Kim, J. S.; Kuk, E.; Yu, K. N.; Kim, J. H.; Park, S. J.; Lee, H. J.; Kim, S. H.; Park, Y. K.; Park, Y. H.; Hwang, C. Y.; Kim, Y. K.; Lee, Y. S.; Jeong, D. H. and Cho, M. H. (2007). Antimicrobial effects of silver nanoparticles. Nanomedicine., 3(1): 95–101.

Lei, M.; Xue, C. H.; Wang, Y. M.; Li, Z. J.; Xue, Y. and Wang, J. F. (2008). Effect of squid ink melanin-Fe on iron deficiency anemia remission. J. Food Sci., 73(8): H207–H211.

Mackintosh, J. A. (2001). The antimicrobial properties of melanocytes, melanosomes and melanin and the evolution of black skin. J. Theor. Biol., 211(2): 101–113.

Magarelli, M.; Passamonti, P. and Renieri, C. (2010). Purification, characterization and analysis of sepia melanin from commercial sepia ink (*Sepia officinalis* Purificación, caracterización y análisis de la melanina de sepia a partir de la tinta de sepia (*Sepia officinalis*) Resumen. Ces. Med. Vet. Zootec., 5(2): 18–28.

Matthews, T. H.; Doyle, R. J. and Streips, U. N. (1979). Contribution of peptidoglycan to the binding of metal ions by the cell wall of *Bacillus subtilis*. Cur. Microbiol., 3(1): 51–53.

Mbonyiryivuze, A.; Nuru, Z. Y.; Ngom, B. D.; Mwakikunga, B. W.; Dhlamini, M. S.; Park, E. and Maaza, M. (2015). Morphological and Chemical Composition Characterization of Commercial Sepia Melanin. Am. J. Nanomat., 3(1): 22–27.

Nair, J. R.; Pillai, D.; Joseph, S. M.; Gomathi, P.; Senan, P. V. and Sherief, P.M. (2011). Cephalopod research and bioactive substances. Indian J. Mar. Sci., 40(1): 13–27.

Naraoka, T.; Chung, H-S.; Uchisawa, H.; Sasaki, J-I. and Matsue, H. (2000). Tyrosinase Activity in Antitumor Compounds of Squid Ink. Food Sci. Technol. Res., 6(3): 171–175.

Nithya, M.; Ambikapathy, V. and Panneerselvam, A. (2011). Effect of pharaoh' s cuttlefish ink against bacterial pathogens. Asian J. Plant Sci. Res., 1(4): 49–55.

Olga, O.; Aisiah, S.; Tanod, W. A.; Risjani, Y.; Nursyam, H. and Maftuch, M. (2020). Immunogenization of Heat-Killed Vaccine Candidate from *Aeromonas hydrophila* in Catfish (*Pangasius hypophthalamus*) using Strain of Banjar, South Kalimantan, Indonesia. Egypt. J. Aquat. Biol. Fish., 24(4): 1–13.

Peshenko, I. V. and Dizhoor, A. M. (2007). Activation and inhibition of photoreceptor guanylyl cyclase by Guanylyl Cyclase Activating Protein 1 (GCAP-1): The functional role of Mg 2+/Ca2+ exchange in EF-hand domains. J. Biol. Chem., 282(30): 21645–21652.

Poernomo, A. T.; Nisa, S. K.; Aliyah, Z. S. and Isnaeni, I. (2020). Effects of carbon and nitrogen sources on the antibacterial activity of *Bacillus tequilensis* BSM-F symbiotic with *Halichondria panicea* sponge from the Cabbiya Coast, Madura, Indonesia. Pharmaciana, 10(2): 125–134.

Pralea, I. E.; Moldovan, R. C.; Petrache, A. M.; Ilieș, M.; Hegheș, S. C.; Ielciu, I.; Nicoară, R.; Moldovan, M.; Ene, M.; Radu, M.; Uifălean, A. and Iuga, C. A. (2019). From Extraction to Advanced Analytical Methods: The Challenges of Melanin Analysis. Int. J. Mol. Sci., 20(16): 3943.

Praveen, P. K.; Debnath, C.; Shekhar, S.; Dalai, N. and Ganguly, S. (2016). Incidence of *Aeromonas* spp. infection in fish and chicken meat and its related public health hazards: A review. Vet. World., 9(1): 6–11.

Raetz, C. R. H.; Reynolds, C. M.; Trent, M. S. and Bishop, R. E. (2007). Lipid a modification system in gram-negative bacteria. Ann. Rev. Biochem., 76: 295–329.

Rajaganapathi, J.; Thyagarajan, S. P. and Patterson Edward, J. K. (2000). Study on cephalopod's ink for anti-retroviral activity. Indian J. Exp. Biol., 38(5): 519–520.

Sahalan, A. Z.; Aziz, A. H. A.; Hing, H. and Ghani, M. K. A. (2013). Divalent CATIONS (Mg2+, $Ca^{2+)}$ protect bacterial outer membrane damage by polymyxin B. Sains Malaysiana, 42(3): 301–306.

Sajjan, S.; Kulkarni, G.; Yaligara, V.; Kyoung, L. and Karegoudar, T. B. (2010). Purification and physiochemical characterization of melanin pigment from *Klebsiella* sp. GSK. J. Microbiol. Biotechnol., 20(11): 1513–1520.

Sari, R. C.; Wijayanti, I. and Agustini, T. W. (2019). The Effectiveness of Melanin from Squid Ink (*Loligo* sp.) as Antibacterial Agent Against *Escherichia coli* and *Listeria monocytogenes*. IOP Conf. Ser. Earth Environ Sci, 246: 1-8.

Solano, F. (2020). Photoprotection and skin pigmentation: Melanin-related molecules and some other new agents obtained from natural sources. Molecules, 25(7): 1–18.

Surwase, S. N.; Patil, S. A.; Jadhav, S. B. and Jadhav, J. P. (2012). Optimization of l-DOPA production by Brevundimonas sp. SGJ using response surface methodology. Microbial Biotechnol., 5(6), 731–737. https://doi.org/10.1111/j.1751-7915.2012.00363.x

Wang, F. R.; Xie, Z. G.; Ye, X. Q.; Deng, S. G.; Hu, Y. Q.; Guo, X. and Chen, S. G. (2014). Effectiveness of treatment of iron deficiency anemia in rats with squid ink melanin-Fe. Food Funct., 5(1): 123–128.

Zakaria, F.; Fadjar, M. and Yanuhar, U. (2019). Cuttlefish (*Sepia* sp.) ink extract as antibacterial activity against aeromonas hydrophila. Int. J. Sci. Techn. Res., 8(11): 1175–1177.

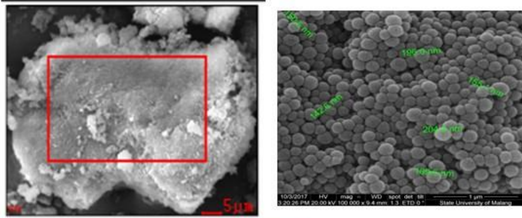
Zerrad, A.; Anissi, J.; Ghanam, J.; Sendide, K. and El Hassouni, M. (2014). Antioxidant and Antimicrobial Activities of Melanin Produced by a *Pseudomonas balearica* Strain. J. Biotechnol. Lett., 5(1): 87–94.

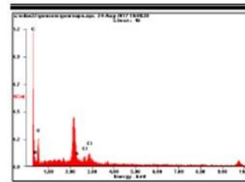
Zhang, X.; Wang, Y.; Guo, J.; Yu, Y.; Li, J.; Guo, Y. and Liu, C. (2015). Comparing two functions for optical density and cell numbers in bacterial exponential growth phase. J. Pure Appl. Microbiol., 9(1): 299–305.

Zhong, J. P.; Wang, G.; Shang, J. H.; Pan, J. Q.; Li, K.; Huang, Y. and Liu, H. Z. (2009). Protective effects of squid ink extract towards hemopoietic injuries induced by cyclophosphamine. Mar. Drugs., 7(1): 9–18.

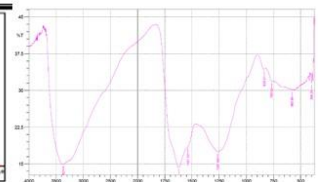
Zou, Y.; Zhao, Y. and Hu, W. (2015). Chemical composition and radical scavenging activity of melanin from *Auricularia auricula* fruiting bodies. Food Sci. Technol., 35(2): 253–258.

aggregates melanin

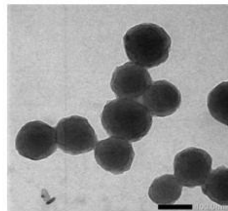




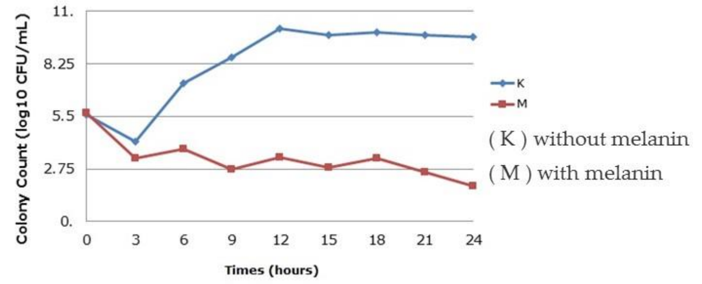
EDX pattern of melanin



The FT-IR spectra



Granules melanin of cuttlefish

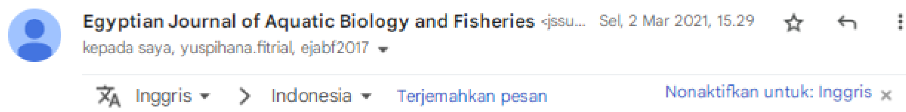


Aeromonas sp. growth curves

Korespondensi Artikel:

1. Submission

Acknowledgement of Submission (Manuscript #EJABF-2103-2098) Kotak Masuk ×



Manuscript ID: EJABF-2103-2098

Manuscript Title: **The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.**

Authors: Yuspihana Fitrial, Iin Khusnul Khotimah

Dear **Dr. Yuspihana Fitrial**

I wish to acknowledge receiving the of the above mentioned manuscript.

It should be noted that the manuscript will be reviewed for possible publication in the Scientific Journals Management System.

Please be sure that the submitted manuscript has not been published previously and will not be submitted elsewhere prior to our decision.

Our editorial decision will be brought to your attention once the paper has been reviewed due the referees consideration.

I wish to take this opportunity to thank you for sharing your work with us.

Truly yours,

Executive managing Editor of **Egyptian Journal of Aquatic Biology and Fisheries**

2. Revision

Manuscript Needs Major Revision (Manuscript #EJABF-2103-2098 (R1)) Kotak Masuk ×

Egyptian Journal of Aquatic Biology and Fisheries <jssu... Sel, 4 Mei 2021, 13.27

kepada saya, yuspihana.fitrial

Inggris > Indonesia Terjemahkan pesan Nonaktifkan untuk: Inggris ×

Manuscript ID: EJABF-2103-2098

Manuscript Title: **The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.**

Authors: Yuspihana Fitrial, Iin Khusnul Khotimah

Dear **Dr. Yuspihana Fitrial**

Your manuscript has obtained major revisions. In this case we normally treat it as unacceptable for publication. However, as numerous editorial errors have pointed out by the reviewers, the **Egyptian Journal of Aquatic Biology and Fisheries** editor believes that the manuscript could be rectified and prepare for possible publication.

Please let us know your views in this regard and in the case of positive response, reply us within 7 days time.

Truly yours,

Editorial Office of **Egyptian Journal of Aquatic Biology and Fisheries**

Reviewers Recommendation:

Reviewer 1:

File Sent by Reviewer:
https://ejabf.journals.ekb.eg/jufile?__file=nHdKMwDoJsdGmpWsn201u0T9HuJ6awUqmL6yA2H9orJDGb.G0sB8Lz7rpluGpTxelwoSlZfVn478CWfe8pdb5PhqghOVUbCxs2GJnGrBQ01zD.fvVeEfZHod3OHmJu_x8Epgrb6SBQHvyl2ioAf0UC9_dthCB_FpY3XY1Uz85Og-

Reviewer Comment For Author:

Introduction

The introduction should further highlight the literature study of the advantages of squid ink as an antibacterial. The compiled introduction does not yet describe the potential of squid ink as antibacterial, it is necessary to add more literature studies.

Methods

Methods should be described in more detail and structured.

Results

The results should be arranged coherently, according to the chronology of the study. So that the results of the research obtained can show a relationship with one another.

Disussion

The discussion has not described the relationship between the results obtained, which is supported by literature studies. The discussion should be built to discuss the results obtained. The discussion must be structured so that the results obtained can convince the reader.

Manuscript Evaluation Form:
https://ejabf.journals.ekb.eg/author?__au=4OmhHkPgrk_Rbl9sS_GEHFYmLqaP5p6KlHoonNL6.8M6BiAwaT6wC9GBsZ9Pq5c0

3. Submitting Revision

Acknowledgement of Revision (Manuscript #EJABF-2103-2098 (R1)) Kotak Masuk ×

Egyptian Journal of Aquatic Biology and Fisheries <jssu... Sel, 11 Mei 2021, 13.54

kepada saya, yuspihana.fitrial, ejabf2017

Inggris > Indonesia Terjemahkan pesan

Nonaktifkan untuk: Inggris ×

Manuscript ID: EJABF-2103-2098 (R1)

Manuscript Title: **The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.**

Authors: Yuspihana Fitrial, Iin Khusnul Khotimah

Date: 2021-03-02

Dear **Dr. Yuspihana Fitrial**

Thank you for submitting the revised file of your manuscript to the **Egyptian Journal of Aquatic Biology and Fisheries**

The Editorial Office will proceed on your manuscript and inform you in the earliest time.

If there is anything else, please do not hesitate to contact us.

Truly yours,

Executive Managing Director of **Egyptian Journal of Aquatic Biology and Fisheries**

Egyptian Journal of Aquatic Biology and Fisheries

Home | Browse | Journal Info | Guide for Authors | Submit Manuscript | Contact Us

Welcome: Yuspihana Fitrial
My Home Logout

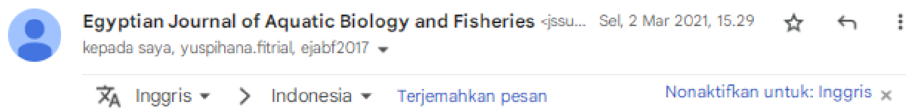
My Home > Author > Revisions Being Processed

#	Manuscript ID	Manuscript Type	Manuscript Title	Submit Date	Revise Date	Current Status	Modify Date	Manuscript Main File	Email	Withdraw
1	EJABF-2103-2098 (R1)	Original Article	The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.	2021-03-02	2021-05-11	Manuscript Revised by Author	2021-05-11			

Korespondensi Artikel:

1. Submission

Acknowledgement of Submission (Manuscript #EJABF-2103-2098) Kotak Masuk ×



Manuscript ID: EJABF-2103-2098

Manuscript Title: **The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.**

Authors: Yuspihana Fitrial, Iin Khusnul Khotimah

Dear **Dr. Yuspihana Fitrial**

I wish to acknowledge receiving the of the above mentioned manuscript.

It should be noted that the manuscript will be reviewed for possible publication in the Scientific Journals Management System.

Please be sure that the submitted manuscript has not been published previously and will not be submitted elsewhere prior to our decision.

Our editorial decision will be brought to your attention once the paper has been reviewed due the referees consideration.

I wish to take this opportunity to thank you for sharing your work with us.

Truly yours,

Executive managing Editor of **Egyptian Journal of Aquatic Biology and Fisheries**

2. Revision

Manuscript Needs Major Revision (Manuscript #EJABF-2103-2098 (R1)) Kotak Masuk ×

Egyptian Journal of Aquatic Biology and Fisheries <jssu... Sel, 4 Mei 2021, 13.27
kepada saya, yuspihana.fitrial

Inggris > Indonesia Terjemahkan pesan Nonaktifkan untuk: Inggris ×

Manuscript ID: EJABF-2103-2098

Manuscript Title: **The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.**

Authors: Yuspihana Fitrial, Iin Khusnul Khotimah

Dear **Dr. Yuspihana Fitrial**

Your manuscript has obtained major revisions. In this case we normally treat it as unacceptable for publication. However, as numerous editorial errors have pointed out by the reviewers, the **Egyptian Journal of Aquatic Biology and Fisheries** editor believes that the manuscript could be rectified and prepare for possible publication.

Please let us know your views in this regard and in the case of positive response, reply us within 7 days time.

Truly yours,

Editorial Office of **Egyptian Journal of Aquatic Biology and Fisheries**

Reviewers Recommendation:

Reviewer 1:

File Sent by Reviewer:
https://ejabf.journals.ekb.eg/jufile?_file=nHdKMwDoJsdGmpWsn201u0T9HuJ6awUqmL6yA2H9orJDGb.G0sB8Lz7rpluGpTxelwoSlZfVn478CWfe8pdb5PhqghOVUbCxs2GJnGrBQ01zD.fvVeEfZHod3OHmJu_x8Epgrb6SBQHvyl2ioAf0UC9_dthCB_FpY3XY1Uz85Og-

Reviewer Comment For Author:

Introduction
The introduction should further highlight the literature study of the advantages of squid ink as an antibacterial. The compiled introduction does not yet describe the potential of squid ink as antibacterial, it is necessary to add more literature studies.

Methods
Methods should be described in more detail and structured.

Results
The results should be arranged coherently, according to the chronology of the study. So that the results of the research obtained can show a relationship with one another.

Disussion
The discussion has not described the relationship between the results obtained, which is supported by literature studies. The discussion should be built to discuss the results obtained. The discussion must be structured so that the results obtained can convince the reader.

Manuscript Evaluation Form:
https://ejabf.journals.ekb.eg/author?_au=4OmhHkPgrk_Rbl9sS_GEHFYmLqaP5p6KIHoonNL6.8M6BiAwaT6wC9GBsZ9Pq5c0

3. Submitting Revision

Acknowledgement of Revision (Manuscript #EJABF-2103-2098 (R1)) Kotak Masuk ×

Egyptian Journal of Aquatic Biology and Fisheries <jssu... Sel, 11 Mei 2021, 13.54

kepada saya, yuspihana.fitrial, ejabf2017

Inggris > Indonesia Terjemahkan pesan Nonaktifkan untuk: Inggris ×

Manuscript ID: EJABF-2103-2098 (R1)

Manuscript Title: **The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.**

Authors: Yuspihana Fitrial, Iin Khusnul Khotimah

Date: 2021-03-02

Dear **Dr. Yuspihana Fitrial**

Thank you for submitting the revised file of your manuscript to the **Egyptian Journal of Aquatic Biology and Fisheries**

The Editorial Office will proceed on your manuscript and inform you in the earliest time.

If there is anything else, please do not hesitate to contact us.

Truly yours,

Executive Managing Director of **Egyptian Journal of Aquatic Biology and Fisheries**

ISSN 1110 - 6131

Egyptian Journal of Aquatic Biology and Fisheries

Home Browse Journal Info Guide for Authors Submit Manuscript Contact Us

Welcome: Yuspihana Fitrial
My Home Logout

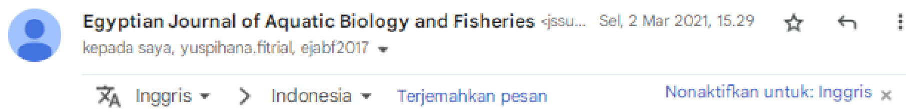
My Home > Author > Revisions Being Processed

#	Manuscript ID	Manuscript Type	Manuscript Title	Submit Date	Revise Date	Current Status	Modify Date	Manuscript Main File	Email	Withdraw
1	EJABF-2103-2098 (R1)	Original Article	The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.	2021-03-02	2021-05-11	Manuscript Revised by Author	2021-05-11			

Korespondensi Artikel:

1. Submission

Acknowledgement of Submission (Manuscript #EJABF-2103-2098) Kotak Masuk ×



Manuscript ID: EJABF-2103-2098

Manuscript Title: **The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.**

Authors: Yuspihana Fitrial, Iin Khusnul Khotimah

Dear **Dr. Yuspihana Fitrial**

I wish to acknowledge receiving the of the above mentioned manuscript.

It should be noted that the manuscript will be reviewed for possible publication in the Scientific Journals Management System.

Please be sure that the submitted manuscript has not been published previously and will not be submitted elsewhere prior to our decision.

Our editorial decision will be brought to your attention once the paper has been reviewed due the referees consideration.

I wish to take this opportunity to thank you for sharing your work with us.

Truly yours,

Executive managing Editor of **Egyptian Journal of Aquatic Biology and Fisheries**

2. Revision

Manuscript Needs Major Revision (Manuscript #EJABF-2103-2098 (R1)) Kotak Masuk ×

Egyptian Journal of Aquatic Biology and Fisheries <jssu... Sel, 4 Mei 2021, 13.27
kepada saya, yuspihana.fitrial

Inggris > Indonesia Terjemahkan pesan Nonaktifkan untuk: Inggris ×

Manuscript ID: EJABF-2103-2098

Manuscript Title: **The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.**

Authors: Yuspihana Fitrial, Iin Khusnul Khotimah

Dear **Dr. Yuspihana Fitrial**

Your manuscript has obtained major revisions. In this case we normally treat it as unacceptable for publication. However, as numerous editorial errors have pointed out by the reviewers, the **Egyptian Journal of Aquatic Biology and Fisheries** editor believes that the manuscript could be rectified and prepare for possible publication.

Please let us know your views in this regard and in the case of positive response, reply us within 7 days time.

Truly yours,

Editorial Office of **Egyptian Journal of Aquatic Biology and Fisheries**

Reviewers Recommendation:

Reviewer 1:

File Sent by Reviewer:
https://ejabf.journals.ekb.eg/jufile?__file=nHdKMwDoJsdGmpWsn201u0T9HuJ6aw
UqmL6yA2H9orJDGb.G0sB8Lz7rpluGpTxelwoSlZfVn478CWfe8pdb5PhqghOVUbCxs2GJnGrBQ01
zD.fvVeEfZHod3OHmJu_x8Epgrb6SBQHvyI2ioAf0UC9_dthCB_FpY3XY1Uz85Og-

Reviewer Comment For Author:

Introduction
The introduction should further highlight the literature study of the advantages of squid ink as an antibacterial. The compiled introduction does not yet describe the potential of squid ink as antibacterial, it is necessary to add more literature studies.

Methods
Methods should be described in more detail and structured.

Results
The results should be arranged coherently, according to the chronology of the study. So that the results of the research obtained can show a relationship with one another.

Disussion
The discussion has not described the relationship between the results obtained, which is supported by literature studies. The discussion should be built to discuss the results obtained. The discussion must be structured so that the results obtained can convince the reader.

Manuscript Evaluation Form:
https://ejabf.journals.ekb.eg/author?__au=4OmhHkPgrk_Rbl9sS_GEHFYmLqaP5p6KIHoonNL6.
8M6BiAwaT6wC9GBsZ9Pq5c0

3. Submitting Revision

Acknowledgement of Revision (Manuscript #EJABF-2103-2098 (R1)) Kotak Masuk ×

Egyptian Journal of Aquatic Biology and Fisheries <jssu... Sel, 11 Mei 2021, 13.54

kepada saya, yuspihana.fitrial, ejabf2017

Inggris > Indonesia Terjemahkan pesan Nonaktifkan untuk: Inggris ×

Manuscript ID: EJABF-2103-2098 (R1)

Manuscript Title: **The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.**

Authors: Yuspihana Fitrial, Iin Khusnul Khotimah

Date: 2021-03-02

Dear **Dr. Yuspihana Fitrial**

Thank you for submitting the revised file of your manuscript to the **Egyptian Journal of Aquatic Biology and Fisheries**

The Editorial Office will proceed on your manuscript and inform you in the earliest time.

If there is anything else, please do not hesitate to contact us.

Truly yours,

Executive Managing Director of **Egyptian Journal of Aquatic Biology and Fisheries**

Egyptian Journal of Aquatic Biology and Fisheries

Home Browse Journal Info Guide for Authors Submit Manuscript Contact Us

Welcome: Yuspihana Fitrial
My Home Logout

My Home > Author > Revisions Being Processed

#	Manuscript ID	Manuscript Type	Manuscript Title	Submit Date	Revise Date	Current Status	Modify Date	Manuscript Main File	Email	Withdraw
1	EJABF-2103-2098 (R1)	Original Article	The antibacterial activity of melanin in cuttlefish (Sepia sp.) ink against Aeromonas sp.	2021-03-02	2021-05-11	Manuscript Revised by Author	2021-05-11			

Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 24(--): … – … (2020)
www.ejabf.journals.ekb.eg

The antibacterial activity of melanin in cuttlefish (*Sepia* sp.) ink against *Aeromonas* sp.

ARTICLE INFO

Article History:
Received:
Accepted:
Online:

Keywords:
Cuttlefish,
Ink,
Melanin,
Antibacterial activity.

ABSTRACT

The marine environment consists of many organisms that are recognized to have bioactive compounds as a mechanism of self-defense or protection of eggs and embryos. One of them is cuttlefish (*Sepia* sp.). The aim of this study was to determine the potential and bactericidal action of the cuttlefish ink melanin against *Aeromonas* sp. Ink extraction and purification were carried out to obtain melanin mechanically using 0.5M HCl. The growth patterns of bacteria were studied by the Total Plate Count method, and the bactericidal mechanism of melanin was observed by Transmission Electron Microscopy (TEM). The results showed that cuttlefish ink melanin inhibited the growth of *Aeromonas* sp. as indicated by the shrinkage of cell size and irregular cell shape. The results of this study are important information for dealing with *Aeromonas* sp. attack in cultured fish.

INTRODUCTION

Cuttlefish are one of the fisheries commodities which utilization is still limited. So far, the use of cuttlefish has only been cooked with a mixture of spices, while the ink is thrown away or used as part of the cuttlefish processing in food. Several studies have shown that squid ink contains melanin, protein, fat and glycosaminoglycans **(Fiore *et al*., 2004)**. Squid ink can act as a cell protective drug in the treatment of cancer with chemotherapy, by increasing the number of leukocytes and bone marrow cell nucleates, which numbers are decreasing due to the use of drugs such as tumor cell killer **(Naraoka *et al*., 2000)**. In addition, melanin from squid ink has anti-tumor activity by inhibiting plasmin activity to increase thromboxane and increase the body's resistance to killing cancer cells **(Zhong *et al*., 2009)**. Melanin also contributes as an antioxidant **(Fahmy, 2013; Guo *et al*., 2014)**, anti-radiation **(Solano, 2020; Pralea *et al*., 2019)**, antirotavirus **(Rajaganapathi *et al*., 2000)** and antibacterial **(Yuvaraj *et al*., 2015; Aruldhason *et al*., 2014; Zakaria *et al*., 2019).** In addition, melanin also has the ability to absorb metals, especially in the phenolic hydroxyl (OH), carboxyl (COOH) and amine (NH) functional groups of the melanin molecule **(Chen *et al*., 2009)**.

Nair *et al*. (2011) stated that Sepia ink consists of melanin granules on thick colorless media. The pigment melanin is the main element in the ink glands which continuously produce ink. At the end of the ripening process, the ink gland cells store ink sacs that function as reservoirs. Each Sepia ink bag contains ± 1 g melanin **(Derby, 2014)**, and the amount of melanin is ± 15% of the total ink wet weight **(Wang *et al*., 2014)**. Melanin Sepia is formed by many aggregates. This aggregate is also formed by small round grains with different size distributions. Small grain diameter ranges from 100-200 nm **(Mbonyiryivuze *et al*., 2015)**.

The squid and or cuttlefish ink had antibacterial activity **(Nair *et al*., 2011).** Melanin's action as an antibacterial has not been much revealed. Some researchers have tested antibacterial activity against only extracts from cuttlefish and or squid ink. **Nithya *et al*. (2011)** examined the antibacterial activity of cuttlefish hexane extract (Sepia pharaonis) purified with diethyl ether. The results of this study indicate that the extract has inhibitory activity against *Pseudomonas aeruginosa*, *Staphylococcus epidermidis, Klebsiella pneumoniae*, and *E. coli*. **Yuvaraj *et al.* (2015)** proved that squid ink (*Loligo duvauceli*) does not have inhibitory activity against *E. coli*. The results of this study tested the antibacterial activity by approximating the ability of the ink component, that is melanin, to chelate metals. The cytotoxic effect of melanin squid ink is thought to also affect the growth of fish microbes, such as *Aeromona*s sp.

Aeromonas sp. are gram-negative, facultative anaerobes, which cause disease in both terrestrial and aquatic animals, as well as in humans. *Aeromonas* sp. considered as one of the important bacteria among disease-causing agents in fish **(Praveen *et al.,* 2016)**. It is necessary to conduct research to see the antibacterial activity of cuttlefish melanin against *Aeromonas* sp. Therefore, the aim of this study was to determine the potential and bactericidal activity of the cuttlefish ink melanin against *Aeromonas* sp.

MATERIALS AND METHODS

2.1 Materials

The material used was cuttlefish (ink) obtained from the Muara Kintap Fishing Port, Tanah Laut Regency, South Kalimantan. Other ingredients used include 0.5M HCl (Merck), acetone (Merck), aquades, Nutrient Broth (NB) (Merck), Nutrient Agar (NA) (Merck), Sterile-EO Syringe Filter (Sartorius Minisart pore size 0.20 μm), Microbact TM GNB12A/B/E, 24E Identification Kits (Oxoid) and 70% alcohol.

The tools used in this study were a refrigerated centrifuge (Labogene Scanspeed 1580R), Freeze dryer (Model Christ alpha 2-4 LD Plus), autoclave (Pressure Steam Sterilizer Electric Model No.25X-2), laminar flow (Biobase), incubator (Memmert), colony counter (Quebec), incubator shaker (Wisd), spectrophotometer (Genesys 10uv), SEM with Energy Dispersive X-ray (SEM-EDX) EDAX.SL, TEM JEOL, JEM1400, and other glassware.

2.2 Methods

2.2.1 Preparation of melanin

Isolation and purification of cuttlefish ink melanin according to the method **(Magarelli *et al*., 2010)** as follows: 50 g of cuttlefish ink is added with 100 ml of HCl (0.5 M molarity) in a dark container. The solution was stirred for 30 minutes (in a magnetic stirrer) and then stored for 24 hours at 10C. The pellet was separated from the supernatant by centrifugation (10000 rpm at 5°C for 15 minutes). The suspension was rinsed three times each with 0.5M HCl, water, acetone and finally with water, sequentially. Lyophilization was then carried out to remove the solvent

using a freeze dryer. Melanin was obtained and then characterized using infrared (IR) spectroscopy and its morphology by SEM, TEM, and the percentage of melanin elements using EDX (Energy Dispersive X-ray Spectroscopy).

2.2.2 Culture of organism

The microbes were isolated from rotten meat and digestive fish (Euthynnus affinis). Isolates were grown on GSP agar medium (Pseudomonas-Aeromonas-Selective agar, Merck). Species were determined using the Microbact identification kit GNB 12A/B/E, 24E (Oxoid).

2.2.3. Analysis of cell leakage

The test to study the mechanism of action of cuttlefish melanin inhibition in microbes refers to **Bunduki *et al*. (1995)**. Analysis of cell leakage was carried out using a spectrophotometer at a wavelength of 280 and 260 nm. The 280 nm wavelength is used to measure the nitrogen content of cell proteins, while the 260 nm wavelength is used to measure the nucleic nitrogen content in cells. 10 mL cultures were centrifuged at 10,000 rpm for 10 minutes. The filtrate is removed and then 5 mL of physiological salt solution (0.85% NaCl) is added to the cell sediment in the test tube. The solution is vortexed so that the cells are homogeneous in a physiological solution. Furthermore, melanin is added with a concentration of 0; 0.005; 0.010; 0.015; 0.020 g / mL, and incubated at room temperature for 24 hours. The suspension was centrifuged at 10,000 rpm for 10 minutes, and the supernatant filtered with filter paper (0.20μm sterile syringe filter) to separate cells. The analysis was carried out by observing the OD (Optical Density) of the liquid supernatant.

2.2.4 Testing of melanin activity against test bacteria

Melanin activity testing was carried out by using the direct contact method between melanin and the test bacteria in liquid nutrient broth (NB). The test series is in a small tube containing 2,970 mL of sterile NB plus 0.030 mL of bacterial suspension so that the total solution is 3,000 mL. Melanin (in powder form) was added to the test tube so that the melanin concentration in the tube was 0.000; 0.002; 0.006; 0.010 g / mL. For the first series of test tubes (melanin concentration 0,000 g/mL), 2,970 NB sterile + 0,000 g melanin were used. A second series tube (melanin concentration 0.002 g/mL), was prepared by adding 2.964 mL of sterile NB + 0.006 g melanin, and so on.

The bacteria were prepared, which had been freshened and incubated for 24 hours (108-109 CFU/mL) at 37 ° C, then diluted ten times. The test tube was inoculated with 0.030 mL of tested bacterial suspension, shaken with vortex for 1-2 minutes, then incubated in a shaker incubator at 37°C for 24 hours. Total Plate Count performs a bacterial count. Petri dishes were incubated for 24 hours at 37°C, and the number of colonies was counted. The total colony is the result of the average count on the three petri dishes that match a particular sample.

The percentage of bacterial inhibition was determined by modification of the method **(Capasso *et al*., 1995)** with the formula: 100 - (Nt x 100/No), where Nt is the number of CFU bacteria/mL in the addition of melanin treatment, while No is the number of CFU bacteria/mL initial (initial inoculum).

2.2.5 Testing of melanin activity to *Aeromonas* sp growth

The test for the inhibition of melanin on the growth of *Aeromonas* sp. was carried out in the same way as the melanin activity test above, with a concentration of 0.010 g/mL (ie the melanin concentration where the percentage of inhibition

relative to the number of initial microbes was close to 100%. Observations were carried out every three hours for 24 hours.

2.2.6. Preparation of *Aeromonas* sp. for TEM analysis.

Sample preparation followed the method by **Kim *et al*. (2007)** with some modifications. Bacteria were inoculated in NB medium and 0.005 g / mL and 0.010 g/mL melanin were added, then incubated for 24 hours at 37°C. Controls were prepared in NB medium. After incubation, the suspension was centrifuged at 1200 rpm for 10 minutes. The supernatant was washed with a phosphate buffer solution pH 7.2, and centrifuged at 2600 rpm for 5 minutes (4°C). For the sample, cell pellets were fixed with 2% glutaraldehyde. Aeromonas sp. Cell images were observed by transmission electron microscopy (TEM, JEOL, JEM 1400).

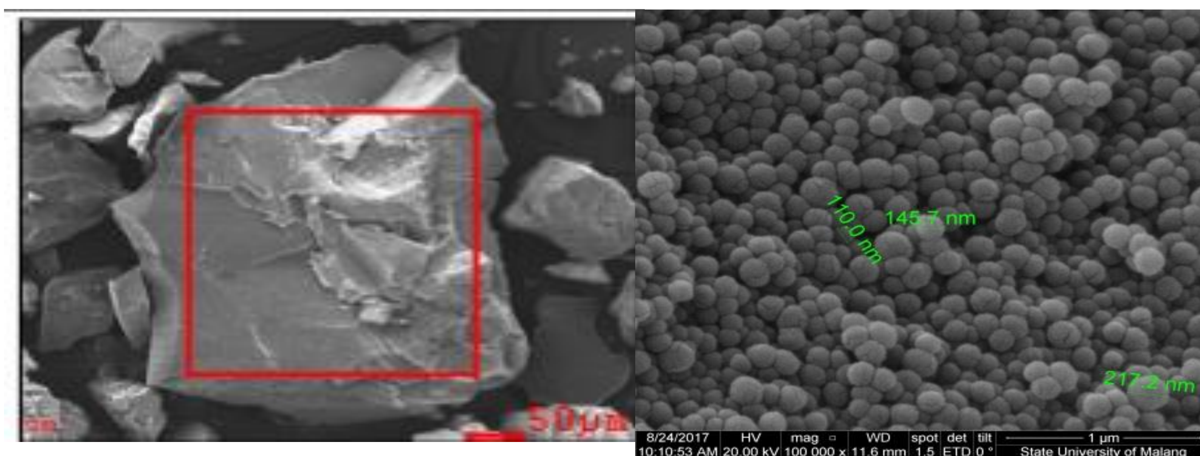
RESULTS

3.1 Isolation of Aeromonas sp.

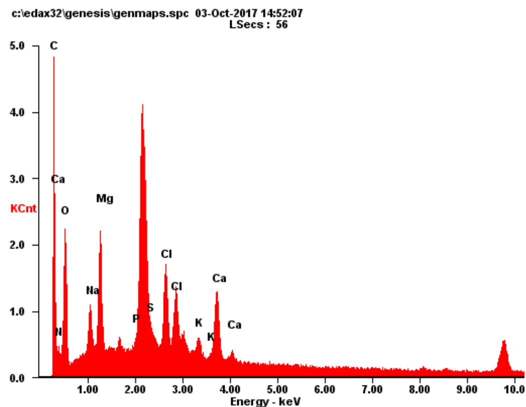
The colony presumably Aeromonas sp. is the yellow color on GSP agar, tested for biochemical properties using a microbact (oxoid) identification kit. The test results of the microbact kit showed oxidase (+), motility (+), nitrate (+), lysine (+), ornitine (+), H2S (+), glucose (+), mannitol (+), xylose (+) , ONPG (+), indole (+), urease (+), VP (+), citrate (+), TDA (-), gelatin (-), malonate (+), inositol (-), sorbitol (+) , rhamnose (+), sucrose (+), lactose (+), arabinose (+), adonitol (-), raffinose (+), salicin (-), arginine (-). The identification results showed Aeromonas hydrophilla (similarity reached 94.20%).

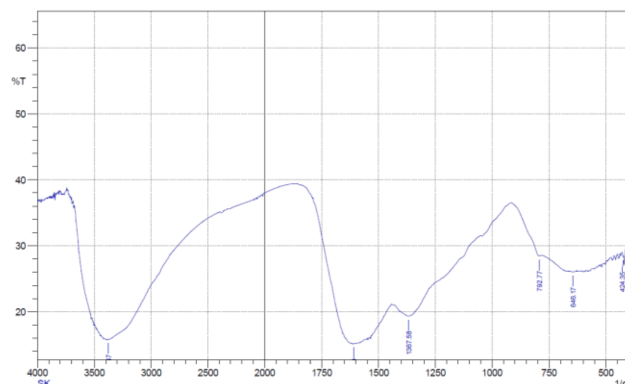
3.2 Purification of melanin

Cuttlefish melanin is an aggregate composed of many spherical grains. Small spherical grains also make these aggregates with different size distributions. The small grain diameter ranges from 100-200 nm (Figures 1 and 2). TEM micrograph showing spherical melanin granules (Figure 3).



(a) **(b)**

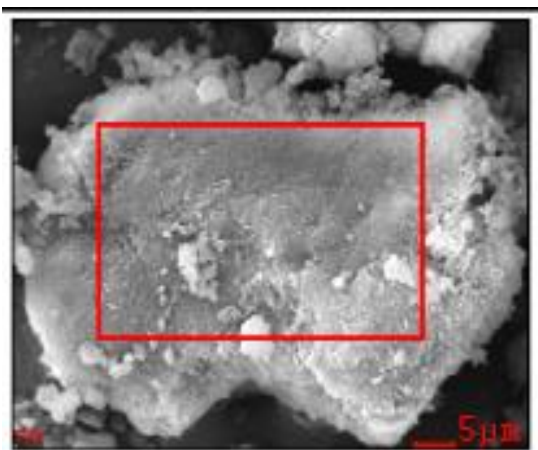


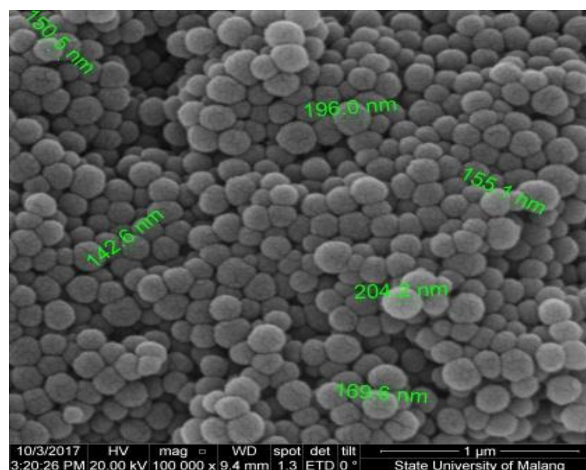


(c) **(d)**

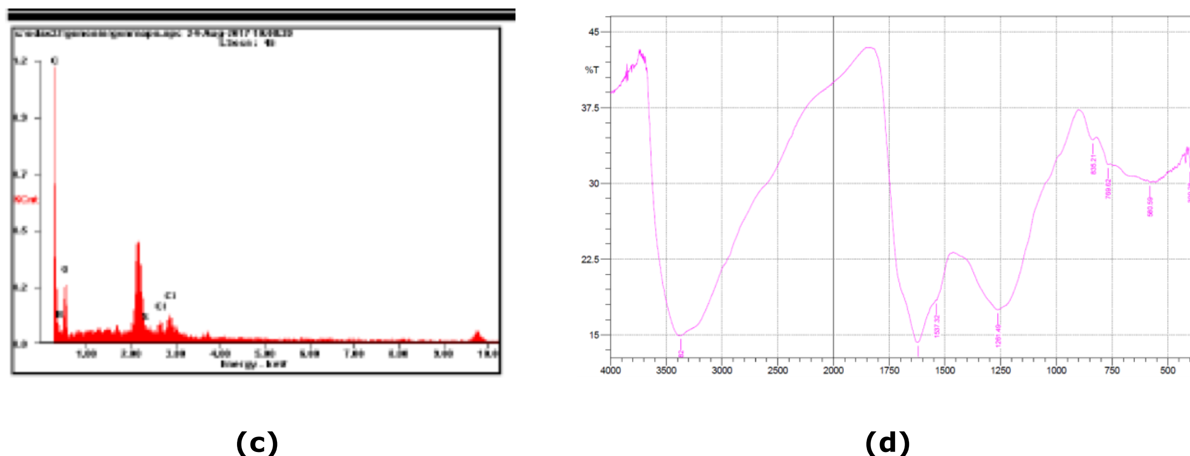
Figure 1. Scanning electron micrograph of cuttlefish ink melanin (a) and (b) aggregates melanin, (c) EDX pattern of melanin and (d) The FT-IR spectra of melanin before purified.

The FTIR spectrum showed that melanin had a strong Infrared absorption spectrum at 3300, 1620, and 1260 cm-1. It shows phenolic hydroxyl (OH), carboxyl (COOH), and amine (NH) groups (Figure 1d). The difference between before and after purification is mainly seen in the intensity of each functional group. Cuttlefish ink contains the most significant components, specifically C (50.22%), O (29.18%), and N (7.99%), as well as a small portion of Mg, Na, S, K, Ca, P and Cl. After the purification stage, several components of melanin were released, such as Ca, Na, Mg, P, and K (Table 1). There are differences in components after purification with commercial melanin *Sepia* studied by **Mbonyiryivuze *et al*. (2015)** which contains the main ingredients C, O, Na and Cl, as well as minor compounds Mg, Ca, K, S, N.





(a) **(b)**



(c) (d)

Figure 2. Scanning electron micrograph of cuttlefish ink melanin (a) and (b) aggregates melanin, (c) EDX pattern of melanin and (d) The FT-IR spectra of melanin after purified.

Table 1. Elemental percentages of melanin by EDX (Energy Dispersive X-ray Spectroscopy).

	C (%)	N (%)	O (%)	Na (%)	Mg (%)	P (%)	S (%)	Cl (%)	K (%)	Ca (%)
Before purified	50.22	7.99	29.18	2.38	3.98	0.83	0.98	1.99	0.52	1.93
After purified	62.76	16.02	20.28	-	-	-	0.49	0.45	-	-

Note: - = not detected

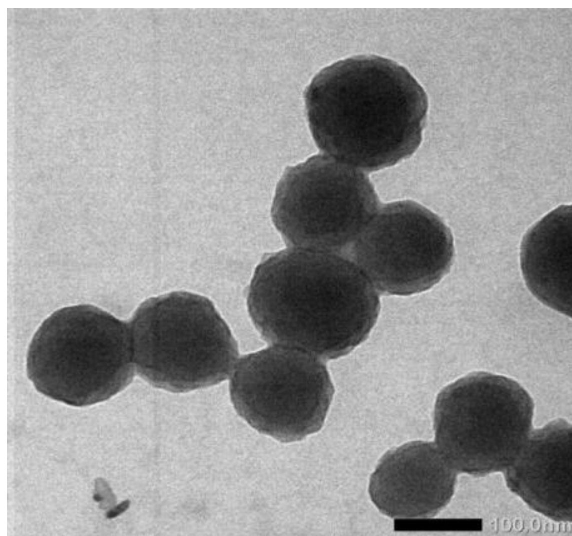


Figure 3. Granules melanin with TEM

3.3 Evaluation of antibacterial activity

Melanin activity from *Sepia* sp. to *Aeromonas* sp. growth is shown in Table 2. The concentration that causes mortality is close to 100% and then the action is

observed on Aeromonas growth. In this case, that is, at the level of 10 mg/ml. After Aeromonas was incubated with 10 mg/mL melanin, the number of live colonies was reduced by more than 1 log after 6 hours of incubation. The decline continued to 2 log10 after 21 hours of incubation. Melanin causes an extension of the adaptation phase and a decrease in the number of living colonies. After 24 hours of incubation, there were no more live colonies (Figure 4). This shows that in addition to prolonging the adaptation phase, melanin also accelerates the period of bacterial cell death. Melanin inhibits bacterial proliferation **(Mackintosh, 2001)**.

Table 2. The growth of bacteria in Nutrient Broth containing melanin.

Bacteria test	Melanin concentration (g/ml)	The number of bacteria (CFU/ml)		(%) inhibition relative to the initial microbial number [100-(Ntx100/No)]
		Incubation 0 hour (No)	Incubation 24 hour (Nt)	
Aeromonas sp.	0.002	8.55x10^5	1.59x10^9	0
	0.006	8.55x10^5	3.6x10^5	57.89
	0.010	8.55x10^5	3.0x10^0	99.99

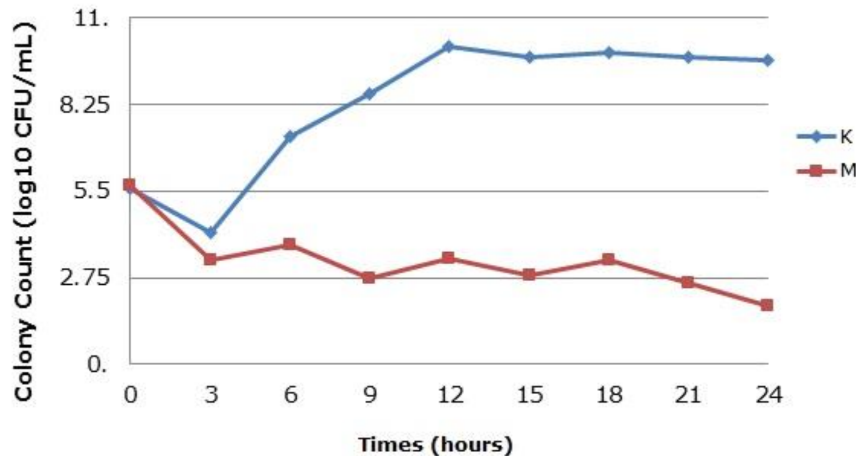
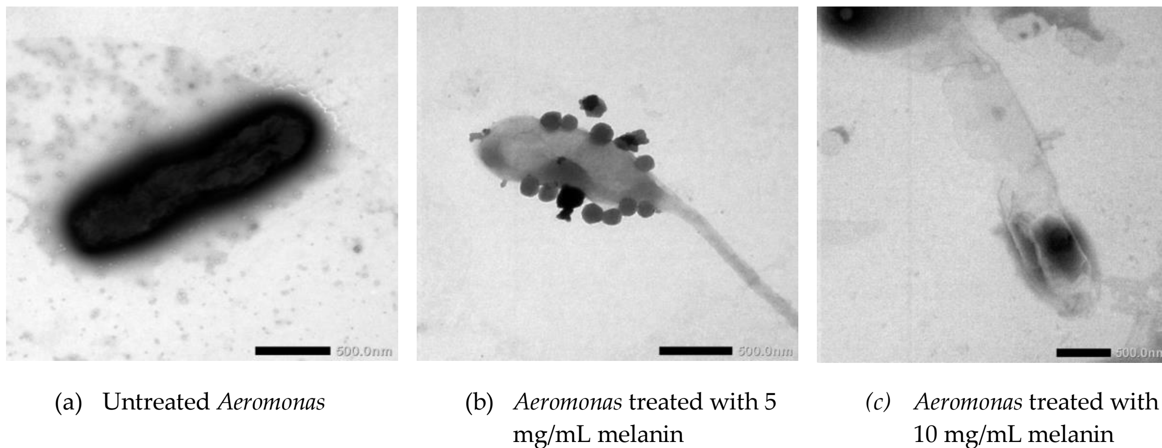


Figure 4. *Aeromonas* sp. growth curves (K) without melanin, and (M) with melanin.

Figure 5 showed that TEM from *Aeromonas* sp. without adding melanin (a) or incubated with 5 mg/mL melanin, and with 10 mg/ml melanin (arrows describe how melanin granules penetrate into cells). Melanin causes increased cell wall permeability so that melanin molecules can more easily enter cells due to damage to cell walls and membranes. This occured in the melanin treatment with a concentration of 5 mg/mL which caused the cell size to be small and the cell shape irregular.



(a) Untreated *Aeromonas* (b) *Aeromonas* treated with 5 mg/mL melanin (c) *Aeromonas* treated with 10 mg/mL melanin

Figure 5. TEM image of *Aeromonas* sp. morphology (a) before, (b) and (c) after treated with melanin.

Table 3 shows the relationship between melanin concentrations and the cell-free supernatant of *Aeromonas* cells. Analysis of cell leakage was carried out using a spectrophotometer at a wavelength of 280 and 260 nm. The 280 nm wavelength is used to measure the nitrogen content of cell proteins, while the 260 nm wavelength is to measure the nitrogen content of cell nucleic acids. Higher OD values at 260 nm and 280 nm wavelengths indicate more significant cell leakage due to melanin. The higher the melanin concentration, the greater the rate of cell leakage.

Table 3. OD values of *Aeromonas*-free supernatant after incubated with melanin from *Sepia* sp.

Melanin concentration (g/ml)	OD	
	260 nm	280 nm
0	0.038	0.037
0.005	0.179	0.198
0.010	0.297	0.321
0.015	0.359	0.395
0.020	0.451	0.499

DISCUSSION

Melanin is a tyrosinase that has been identified in squid ink (Derby, 2014). Squid ink consists of a suspension of eumelanin granules in a thick colorless medium. Eumelanin is heterogeneous, generally an insoluble polymer that develops through the enzymatic oxidation of the amino acid tyrosine. The production of eumelanin in pigment cells takes place in special organelles called melanosomes. Eumelanin consists of about 20% 5,6-dihidroxyindol (DHI) units and 5,6-dihydroxyindol-2-carboxylic acid (DHICA) units **(Magarelli *et al.*, 2010)**.

Natural melanin is reported as a pigment molecule capable of absorbing metals at high concentrations. The ability to bind eumelanin to the metal side is an

important parameter for understanding melanin metal complexes **(Lei *et al.,* 2008; Chen *et al*., 2009).** Phenolic hydroxyl (OH), carboxyl (COOH), and amine groups (NH) as functional groups that may be responsible for metal binding in melanin **(Chen *et al*., 2009)**.

Bacterial cell walls contain many types of cations including $Mg2^+$, $Ca2^+$, Na^+, and K^+. These ions are responsible for various bacterial activities, including enzyme work, metabolism regulation and maintaining the integrity of the outer layer. $Mg2^+$ and $Ca2^+$ ions in particular play an important role in protecting the stability of the external structure **(Chen *et al*., 2009; Ferrero *et al*., 2007; Peshenko and Dizhoor (2007)**. The outermost layer of the outer membrane in Gram negative bacteria is lipopolysaccharide (LPS), these molecules are individually negatively charged. The different cations help stabilize and maintain the integrity of the outer membrane by binding to adjacent LPS molecules. These divalent cations function as bridges that bind negatively charged lipid molecules **(Raetz *et al*., 2007)**. The outer membrane of bacterial cells serves as a barrier to the entry of unnecessary compounds into cells (such as bacteriocins, enzymes, and hydrophobic compounds). If the cation can be adsorbed by the melanin functional group **(Hong and Simon, 2007)**, the metabolic system where the bacterial cells will be disrupted, so that the growth of bacterial cells is also disturbed.

The outer membrane of bacterial cells is protected by $Ca2^+$ and $Mg2^+$ ions against Polymyxin B which has the ability to interact with divalent cations and replace them from their binding sites on lipopolysaccharides (LPS) **(Sahalan *et al*., 2013)**. This condition causes disorganization of the outer membrane of gram-negative bacteria. The LPS components detach from the surface of the bacteria, causing membrane leakage and ultimately cell death. $Ca2^+$ has been shown to be more effective in protecting bacterial cells than $Mg2^+$.

The binding of melanin functional groups, such as phenolic hydroxyl (OH), carboxyl (COOH), and amino (NH) groups **(Chen *et al*., 2009)** to $Ca2^+$ and $Mg2^+$ ions in the outer membrane results in leakage in the membrane of Gram negative bacteria. In this case represented by *Aeromonas*. Melanin leakage to *Aeromonas* cells can be seen from the production of bacterial cell-free supernatant after the cells were incubated for 24 hours with melanin ink (Table 3). This can lead to irregular hole formation in the outer membrane and alter membrane permeability, which is due to the gradual release of LPS molecules and membrane proteins. We can speculate that a similar mechanism causes degradation of the *Aeromonas* sp. membrane structure during addition of melanin. Extensive investigations aimed at a better understanding of the interactions between melanin and bacterial components should shed light on how this melanin material acts as a bacteriocidal material.

CONCLUSION

Melanin from *Sepia* sp. ink has inhibitory activity against the growth of Aeromonas sp., and causes reduced cell size and irregular cell shape. This is important information to overcome Aeromonas sp attack on cultured fish.

REFERENCES

Aruldhason, B. V.; Vairamani, S. and Annaian, S. (2014). Antimicrobial activity of methanolic extract and fractionated polysaccharide from Loligo duvauceli Orbingy 1848 and Doryteuthis sibogae Adam 1954 on human pathogenic microorganisms. Afr. J. Microbiol. Res., 8(3): 230–236.

Bunduki, M. M. C.; Flanders, K. J. and Donnelly, C. W. (1995). Metabolic and structural sites of damage in heat- and sanitizer-lnjured populations of listeria monocytogenes. J. Food Protect., 58(4): 410–415.

Capasso, R.; Evidente, A.; Schivo, L.; Orru, G.; Marcialis, M. A. and Cristinzio, G. (1995). Antibacterial polyphenols from olive oil mill waste waters. J. Appl. Bacteriol., 79(4): 393–398.

Derby, C. D. (2014). Cephalopod ink: Production, chemistry, functions and applications. Mar. Drugs., 12(5): 2700–2730.

Fahmy, R. (2013). In vitro antioxidant, analgesic and cytotoxic activities of Sepia officinalis ink and Coelatura aegyptiaca extracts. Afr. J. Pharm. Pharmacol., 7(22): 1512–1522.

Ferrero, M. Á.; Martínez-Blanco, H.; Lopez-Velasco, F. F.; Ezquerro-Sáenz, C.; Navasa, N.; Lozano, S. and Rodríguez-Aparicio, L. B. (2007). Purification and characterization of GlcNAc-6-P 2-epimerase from Escherichia coli K92. Acta Biochim. Pol., 54(2): 387–399.

Fiore, G.; Poli, A.; Di Cosmo, A.; d'Ischia, M. and Palumbo, A. (2004). Dopamine in the ink defence system of Sepia officinalis: Biosynthesis, vesicular compartmentation in mature ink gland cells, nitric oxide (NO)/cGMP-induced depletion and fate in secreted ink. Biochem. J., 378(3): 785–791.

Guo, X.; Chen, S.; Hu, Y.; Li, G.; Liao, N.; Ye, X.; Liu, D. and Xue, C. (2014). Preparation of water-soluble melanin from squid ink using ultrasound-assisted degradation and its anti-oxidant activity. J. Food Sci. Technol., 51(12): 3680–3690.

Hong, L. and Simon, J. D. (2007). Current Understanding of the Binding Sites, Capacity, Affinity, and Biological Significance of Metals in Melanin. J. Phys. Chem. B., 111(28): 7938–7947.

Kim, J. S.; Kuk, E.; Yu, K. N.; Kim, J. H.; Park, S. J.; Lee, H. J.; Kim, S. H.; Park, Y. K.; Park, Y. H.; Hwang, C. Y.; Kim, Y. K.; Lee, Y. S.; Jeong, D. H. and Cho, M. H. (2007). Antimicrobial effects of silver nanoparticles. Nanomedicine., 3(1): 95–101.

Lei, M.; Xue, C. H.; Wang, Y. M.; Li, Z. J.; Xue, Y. and Wang, J. F. (2008). Effect of squid ink melanin-Fe on iron deficiency anemia remission. J. Food Sci., 73(8): H207–H211.

Mackintosh, J. A. (2001). The antimicrobial properties of melanocytes, melanosomes and melanin and the evolution of black skin. J. Theor. Biol., 211(2): 101–113.

Magarelli, M.; Passamonti, P. and Renieri, C. (2010). Purification , characterization and analysis of sepia melanin from commercial sepia ink (Sepia Officinalis Purificación , caracterización y análisis de la melanina de sepia a partir de la tinta de sepia (Sepia Officinalis) Resumen. Ces. Med. Vet. Zootec., 5(2): 18–28.

Mbonyiryivuze, A.; Nuru, Z. Y.; Ngom, B. D.; Mwakikunga, B. W.; Dhlamini, M. S.; Park, E. and Maaza, M. (2015). Morphological and Chemical Composition Characterization of Commercial Sepia Melanin. Am. J. Nanomat., 3(1): 22–27.

Naraoka, T.; Chung, H-S.; Uchisawa, H.; Sasaki, J-I. and Matsue, H. (2000). Tyrosinase Activity in Antitumor Compounds of Squid Ink. Food Sci. Technol. Res., 6(3): 171–175.

Nithya, M.; Ambikapathy, V. and Panneerselvam, A. (2011). Effect of pharaoh' s cuttlefish ink against bacterial pathogens. Asian J. Plant Sci. Res., 1(4): 49–55.

Peshenko, I. V. and Dizhoor, A. M. (2007). Activation and inhibition of photoreceptor guanylyl cyclase by Guanylyl Cyclase Activating Protein 1 (GCAP-1): The functional role of Mg 2+/Ca2+ exchange in EF-hand domains. J. Biol. Chem., 282(30): 21645–21652.

Pralea, I. E.; Moldovan, R. C.; Petrache, A. M.; Ilieș, M.; Hegheș, S. C.; Ielciu, I.; Nicoară, R.; Moldovan, M.; Ene, M.; Radu, M.; Uifălean, A. and Iuga, C. A. (2019). From Extraction to Advanced Analytical Methods: The Challenges of Melanin Analysis. Int. J. Mol. Sci., 20(16): 3943.

Praveen, P. K.; Debnath, C.; Shekhar, S.; Dalai, N. and Ganguly, S. (2016). Incidence of Aeromonas spp. infection in fish and chicken meat and its related public health hazards: A review. Vet. World., 9(1): 6–11.

Raetz, C. R. H.; Reynolds, C. M.; Trent, M. S. and Bishop, R. E. (2007). Lipid a modification systems in gram-negative bacteria. Ann. Rev. Biochem., 76: 295–329.

Rajaganapathi, J.; Thyagarajan, S. P. and Patterson Edward, J. K. (2000). Study on cephalopod's ink for anti-retroviral activity. Indian J. Exp. Biol., 38(5): 519–520.

Nair, J.R.; Pillai, D.; Joseph, S.M.; Gomathi, P.; Senan, P. V. and Sherief, P.M. (2011). Cephalopod research and bioactive substances. Indian J. Mar. Sci., 40(1): 13–27.

Sahalan, A. Z.; Aziz, A. H. A.; Hing, H. and Ghani, M. K. A. (2013). Divalent CATIONS (Mg2+, Ca2+) protect bacterial outer membrane damage by polymyxin B. Sains Malaysiana, 42(3): 301–306.

Solano, F. (2020). Photoprotection and skin pigmentation: Melanin-related molecules and some other new agents obtained from natural sources. Molecules, 25(7): 1–18.

Wang, F. R.; Xie, Z. G.; Ye, X. Q.; Deng, S. G.; Hu, Y. Q.; Guo, X. and Chen, S. G. (2014). Effectiveness of treatment of iron deficiency anemia in rats with squid ink melanin-Fe. Food Funct., 5(1): 123–128.

Chen, S.; Xue, C.; Wang, J.; Feng, H.; Wang, Y.; Ma, Q. and Wang, D. (2009). Adsorption of Pb(II) and Cd(II) by Squid Ommastrephes bartrami Melanin. Bioinorg. Chem. Appl., 2009: 901563.

Yuvaraj, D.; Suvasini, B.; Chellathai, T.; Fouziya, R.; Ivo Romauld, S. and Chandran, M. (2015). Research Article Anti-bacterial studies on the different body parts of Loligo duvauceli. J. Chem. Pharm. Res., 7(6): 406–408.

Zakaria, F.; Fadjar, M. and Yanuhar, U. (2019). Cuttlefish (Sepia sp.) ink extract as antibacterial activity against aeromonas hydrophila. Int. J. Sci. Techn. Res., 8(11): 1175–1177.

Zhong, J. P.; Wang, G.; Shang, J. H.; Pan, J. Q.; Li, K.; Huang, Y. and Liu, H. Z. (2009). Protective effects of squid ink extract towards hemopoietic injuries induced by cyclophosphamine. Mar. Drugs., 7(1): 9–18.

ISSN 1110 - 6131

Egyptian Journal of Aquatic Biology and Fisheries

Home | Browse | Journal Info | Guide for Authors | Submit Manuscript | Contact Us

Welcome: Yuspihana Fitrial
My Home | Logout

My Home > Author > Finish Submission

Manuscript Approval

Thank you for submitting your manuscript to the journal.

You will receive a letter of confirmation through your email for your manuscript submission.

Author Dashboard

Research highlight:

- The cuttlefish ink melanin inhibited the growth of *Aeromonas* sp as indicated by the shrinkage of cell size and irregular cell shape.
- The results of this study are important information for dealing with *Aeromonas* sp attack in cultured fish.

Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 24(--): … – … (2020)
www.ejabf.journals.ekb.eg

The antibacterial activity of melanin in cuttlefish (*Sepia* sp.) ink against *Aeromonas* sp.

Yuspihana Fitrial [1,*] and Iin Khusnul Khotimah [1]

[1] Study Program of Fisheries Product Technology, Faculty of Fisheries and Marine, Lambung Mangkurat University, Banjarbaru 70712, South Kalimantan, Indonesia

[*]Corresponding Author: yuspihana.fitrial@ulm.ac.id

yuspihana fitrial <yuspihana.fitrial@ulm.ac.id>

Ask about manuscript status

1 message

yuspihana fitrial <yuspihana.fitrial@ulm.ac.id> Wed, Jul 28, 2021 at 1:53 PM
To: ejabf2017@gmail.com, mtkhalil52@hotmail.com

EJABF-2103-2098 (R1)

Dear Prof. Magdy Khalil,

Greetings,

I have submitted the revised manuscript on 2021-05-11. However, the status has remained unchanged ever since this date.
I would be grateful if you could let me know whether there has been any further progress on my submission because I need it for my doctoral examination. My institution needs to know the current status of my manuscript.
I hope you understand my circumstances.
For your reference, here I also attached the revised manuscript.
Thank you in advance

Best wishes,

Yuspihana Fitrial

EJABF-2103-2098-10-2 (revised 11 may 2021).docx
1030K

yuspihana fitrial <yuspihana.fitrial@ulm.ac.id>

Ask manuscript progress

1 message

yuspihana fitrial <yuspihana.fitrial@ulm.ac.id> Mon, Jul 19, 2021 at 7:52 PM
To: msalah61082@gmail.com

EJABF-2103-2098 (R1)

Dear Prof. Magdy Khalil,

Greetings,

I have submitted the revised manuscript on 2021-05-11. However, the status has remained unchanged ever since this date.
I would be grateful if you could let me know whether there has been any further progress on my submission because I need it for my doctoral examination. My institution needs to know the current status of my manuscript.
I hope you understand my circumstances.

Best wishes,

Yuspihana Fitrial

Available online: journal.ipb.ac.id/index.php/jphpi DOI: http://dx.doi.org/10.17844/jphpi.v20i2.17907

AKTIVITAS ANTIBAKTERI DARI MELANIN TINTA SOTONG DAN CUMI-CUMI

Yuspihana Fitrial*, Iin Khusnul Khotimah
Program Studi Teknologi Hasil Perikanan, Fakultas Perikanan dan Kelautan, Universitas Lambung Mangkurat. Jl. A.Yani Km 36 Kotak Pos 6 Banjarbaru 70714, Kalimantan Selatan
Telepon/Fax: 0511-4772124
*Korespodensi: *yuspihana@gmail.com*
Diterima: 30 Mei 2017/ Disetujui: 6 Agustus 2017

Cara sitasi: Fitrial Y, Khotimah IK. 2017. Aktivitas antibakteri dari melanin tinta sotong dan cumi-cumi. *Jurnal Pengolahan Hasil Perikanan Indonesia*. 20(2): 266-274.

Abstrak

Kelas Cephalopoda (seperti cumi-cumi dan sotong) memiliki tinta sebagai pertahanan dirinya. Penelitian ini bertujuan untuk membandingkan aktivitas antibakteri melanin dari tinta sotong (*Sepia* sp.) dengan tinta cumi-cumi (*Loligo* sp.) terhadap *Escherichia coli*. Ekstraksi dan pemurnian terhadap tinta sotong dan cumi-cumi dilakukan untuk mendapatkan melanin dengan menggunakan HCl 0,5 M secara mekanik. Melanin yang diperoleh diuji aktivitasnya terhadap *E. coli* dengan metode kontak langsung antara melanin dan *E. coli* di dalam *nutrient broth*. Total mikroba dihitung dengan metode hitungan cawan. Tinta yang berasal dari *Sepia* sp. ataupun *Loligo* sp. juga diuji aktivitasnya. Hasil penelitian menunjukkan bahwa melanin dari tinta sotong dan cumi-cumi memiliki aktivitas penghambatan pada konsentrasi 10 mg/mL dan 20 mg/mL, secara berturut-turut mencapai 99,99% terhadap *E. coli*. Tinta dari kedua jenis Cephalopoda tersebut pada konsentrasi yang sama dengan melanin,tidak menunjukkan adanya aktivitas penghambatan terhadap *E. coli*. Melanin dari *Sepia* sp. memiliki aktivitas antibakteri terhadap *E. coli* lebih tinggi dibandingkan melanin dari *Loligo* sp.

Kata kunci: aktivitas antibakteri, *E. coli*, *Loligo* sp., melanin, tinta, *Sepia* sp.

Antibacterial Activity of Melanin from Cuttlefish and Squid Ink

Abstract

Class Cephalopods (such as squid and cuttlefish) have ink as are notable for their defences. This study aims to compare the antibacterial activity of melanin from cuttlefish ink (*Sepia* sp.) with squid ink (*Loligo* sp.) against *E. coli*. Extraction and purification studies were carried out on *Sepia* and *Loligo* melanin using a hydrochloric acid 0,5M treatment under mechanical.The melanins were obtained and further evaluated their activity by direct contact methods between melanin and *E. coli* in nutrient broth.Total microbes was counted by total plate count.Both inks also was tested their activity against *E. coli*. The results showed that melanin from cuttlefish and squid inks had inhibitory activity at concentrations of 10 mg/ml and 20 mg/ml, respectively reaching 99.99% against *E. coli*.The inks of both Cephalopods at the same concentration as melanin, did not show any inhibitory activity against *E. coli*. The melanin of *Sepia* sp. have a higher antibacterial activity than the melanin of *Loligo* sp.

Keywords: antibacterial activity, cuttlefish, *E. coli*, *Loligo* sp., melanin, *Sepia* sp.

PENDAHULUAN

Kelas Cephalopoda seperti sotong (*Sepia* sp.) dan cumi-cumi (*Loligo* sp.) merupakan komoditi hasil tangkapan perikanan laut yang pemanfaatannya masih sangat terbatas, sementara untuk sotong hanya dikonsumsi dalam bentuk segar. Tinta cumi-cumi ataupun sotong di daerah Kalimantan Selatan yang menjadi daerah pengambilan sampel biasanya dibuang atau tidak dimanfaatkan sebagai bagian dari olahan cumi-cumi.

Tinta cumi-cumi maupun tinta sotong mengandung melanin, protein, lemak dan

glikosaminoglikan. Tinta cumi-cumi dapat berperan sebagai obat pelindung sel pada pengobatan kanker dengan cara kemoterapi, melalui peningkatan jumlah sel leukosit dan sel nucleat sumsum tulang, yang jumlahnya menurun akibat penggunaan obat pembunuh sel tumor tersebut. Melanin dari tinta cumi-cumi mempunyai aktivitas anti-tumor dengan menghambat aktivitas plasmin untuk meningkatkan thromboxan dan meningkatkan sistem imun untuk membunuh sel kanker (Zhong *et al.* 2009). Melanin juga berperan sebagai antioksidan (Lei *et al.* 2007[a]), anti-radiasi (Lei *et al.* 2007[b]), dan anti-rotavirus (Rajaganapathi *et al.* 2007).

Hasil penelitian menyebutkan bahwa tinta sotong dan atau cumi-cumi memiliki aktivitas antibakteri (Nair *et al.* 2011). Aktivitas melanin sendiri sebagai antibakteri belum banyak diungkap. Beberapa peneliti telah melakukan pengujian aktivitas antibakteri hanya terhadap ekstrak dari tinta sotong dan atau cumi-cumi. Nithya *et al.* (2011) meneliti aktivitas antibakteri ekstrak heksan tinta sotong (*Sepia* pharaonis) yang dipurifikasi dengan dietil eter. Hasil penelitian ini menunjukkan ekstrak tersebut memiliki aktivitas penghambatan terhadap *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae* dan *E. coli*. Yuvaraj *et al.* (2015) membuktikan bahwa tinta cumi-cumi (*Loligo* duvauceli) tidak memiliki aktivitas penghambatan terhadap *E. coli*. Hasil penelitian tersebut, dilakukan pengujian aktivitas antibakteri dengan melakukan pendekatan terhadap kemampuan komponen tinta yaitu melanin dalam mengkelat logam. Hasil penelitian Chen *et al.* (2009) menunjukkan bahwa melanin dari tinta cumi-cumi (*Ommastrephes bartrami*) memiliki kemampuan menyerap Cd(II) dan Pb(II) oleh gugus fungsi yang terdapat di molekul melanin. Gugus fungsi tersebut adalah fenolik hidroksil (OH), karboksil (COOH) dan amina (NH). Kemampuan melanin menyerap ion logam inilah yang akan diamati melalui pengujian aktivitasnya terhadap pertumbuhan sel bakteri terutama bakteri Gram negarif seperti *E. coli*. Bakteri Gram negatif pada membran terluar selnya mengandung ion Mg^{2+} dan Ca^{2+} yang berperan penting dalam melindungi kestabilan struktur luar sel.

E. coli merupakan bakteri Gram negatif, bersifat patogen bagi manusia dan umumnya bukan merupakan bakteri indigenous pada ikan (Arias 2009). Adanya *E. coli* pada daging ikan akibat kontaminasi selama pemanenan, pengolahan ataupun penyimpanan. Meskipun demikian beberapa ahli menggolongkannya sebagai salah satu bakteri yang menyebabkan pembusukan pada bahan pangan (Dave dan Ghaly 2011). Penelitian terkait aktivitas antibakteri melanin dari tinta sotong dan cumi-cumi terhadap *E. coli* penting dilakukan untuk mendapatkan informasi tentang potensi melanin jika akan dikembangkan sebagai pengawet alami untuk produk perikanan.

BAHAN DAN METODE

Bahan dan Alat

Bahan yang digunakan adalah (tinta) cumi-cumi dan sotong yang diperoleh dari Pelabuhan Perikanan Muara Kintap Kabupaten Tanah Laut, Kalimantan Selatan. Bahan lain yang digunakan antara lain HCl 0,5M (Merck), aseton (Merck), akuades, Nutrient Broth (NB) (Merck), Nutrient Agar (NA) (Merck), EMBA (Merck), *Syringe filter sterile*-EO (Sartorius Minisart pore size 0,20 μm), Microbact TM GNB12A/B/E, 24E Identification Kits (oxoid) dan alkohol 70%.

Alat yang digunakan pada penelitian ini adalah *refrigerated centrifuge* (Labogene Scanspeed 1580R), *Freeze dryer* (Model Christ alpha 2-4 LD Plus), autoklaf (Pressure Steam Sterilizer Electric Model No.25X-2), *laminar flow* (Biobase), inkubator (Memmert), *coloni counter* (Quebec), *incubator shaker* (Wisd), spektrofotometer (Genesys 10uv) dan peralatan gelas lainnya.

Metode Penelitian

Tahapan penelitian yang dilakukan meliputi isolasi *E. coli* dari daging ikan busuk, ekstraksi dan purifikasi melanin dari tinta sotong dan cumi-cumi. Analisis rendemen dilakukan terhadap ekstrak kasar tinta dan pengujian aktivitas melanin dilakukan terhadap pertumbuhan bakteri uji yaitu *E. coli* serta uji kebocoran sel bakteri uji.

Isolasi *E. coli* dari daging ikan yang busuk

Daging ikan busuk dilarutkan dalam larutan garam fisiologis 0,85% dengan perbandingan 1: 10. Larutan daging sebanyak 1 mL di tumbuhkan dalam media EMB (*Eosin Methylene Blue*) Agar, kemudian diinkubasi selama 24 jam pada suhu 37 °C. Media EMBA merupakan media selektif dan diferensiasi. Eosin akan membedakan antara dua koliform utama, yaitu *E. coli* (koloni kecil dan hijau metalik) dan *Enterbacter aerogenes* (koloni berukuran besar, berwarna merah jambu). *Methylene Blue* secara selektif menghambat Gram positif, sehingga yang dapat tumbuh di media tersebut hanya Gram negatif.

Ekstraksi dan purifikasi melanin dari tinta cumi-cumi

Ekstraksi dan purifikasi melanin pada tinta cumi-cumi dan sotong dilakukan menurut metode Magarelli *et al.* (2010). Tahapan ekstraksi dan purifikasi dilakukan dalam media asam. Preparasi tinta dilakukan dengan mengambil habis tinta dari kantong tinta segar. Tinta sebanyak 50 g ditambahkan 100 mL HCl 0,5M dalam kondisi kedap cahaya. Larutan diaduk dengan menggunakan *magnetic stirrer* selama 30 menit, selanjutnya disimpan selama 24 jam pada suhu 10 °C. Endapan dipisahkan dari supernatan dengan menggunakan sentrifius (10.000 rpm pada suhu 5 °C selama 15 menit). Endapan (padatan) dicuci atau disuspensikan kembali dengan larutan HCl 0,5M sebanyak 3 kali, dilanjutkan dengan akuades, aseton dan terakhir dengan akuades. Tahap selanjutnya dilakukan liofilisasi selama kurang lebih 24 jam untuk memisahkan pelarut hingga diperoleh melanin kering dan di simpan dalam *freezer* sebelum dilakukan pengujian lebih lanjut. Perlakuan tinta (kontrol), tinta diambil dari kantong tinta, lalu dilakukan liofilisasi dengan *freeze dryer* seperti sampel melanin.

Pengujian aktivitas melanin terhadap bakteri uji

Pengujian aktivitas melanin dilakukan dengan metode kontak langsung antara melanin dengan bakteri uji dalam media cair nutrient broth (NB) (modifikasi dari Murhadi (2002)). Pengujian dilakukan dengan membuat seri pengujian di dalam tabung kecil berisi 2,970 mL NB steril ditambah 0,030 mL suspensi bakteri uji sehingga total larutan dalam tabung uji 3,000 mL. Melanin (dalam bentuk serbuk) ditambahkan ke dalam tabung uji sehingga konsentrasi melanin dalam tabung 0,000; 0,002; 0,006; 0,010 g/mL. Pembuatan seri tabung uji ke-1 (konsentrasi melanin 0,000 g/mL), digunakan 2,970 mL NB steril + 0,000 g melanin. Tabung seri ke-2 (konsentrasi melanin 0,002 g/mL), dibuat dengan cara menambahkan 2,964 mL NB steril + 0,006 g melanin, dan seterusnya.

Bakteri uji yang telah disegarkan kemudian disiapkan dan diinkubasi 24 jam (10^8-10^9 CFU/mL) pada 37 °C, lalu diencerkan 10 kali. Tabung uji tersebut diinokulasikan dengan 0,030 mL suspensi bakteri uji, dikocok dengan alat vortex selama 1-2 menit, kemudian diinkubasi pada *incubator shaker* suhu 37 °C selama 24 jam. Perhitungan jumlah bakteri dilakukan dengan metode hitungan cawan (TPC, *Total Plate Count*).

Persentase penghambatan bakteri ditentukan dengan modifikasi metode Cappaso *et al.* (1995) yang dinyatakan: 100 – (Nt x 100/No), Nt adalah jumlah bakteri CFU/mL dalam perlakuan penambahan melanin, sedangkan No adalah jumlah bakteri CFU/mL dalam kontrol (inokulum awal).

Pengujian aktivitas melanin terhadap pertumbuhan *E. coli*

Pengujian penghambatan melanin terhadap pertumbuhan *E. coli* dilakukan dengan cara yang sama dengan pengujian aktivitas melanin di atas, yaitu dengan konsentrasi 0,010 g/mL (yaitu konsentrasi melanin dimana persen penghambatan relatif nya terhadap jumlah mikroba awal mendekati 100%). Pengamatan dilakukan per tiga jam selama 24 jam.

Pengujian kebocoran sel bakteri uji

Pengujian ini untuk melihat akibat dari aktivitas melanin tinta sotong terhadap mikroba uji mengacu pada Bunduki *et al.* (1995). Analisis kebocoran sel dilakukan dengan menggunakan Spektrofotometer *Double Beam* pada panjang gelombang 280

dan 260 nm. Panjang gelombang 280 nm digunakan untuk mengukur kadar nitrogen dari protein sel, sedangkan panjang gelombang 260 nm untuk mengukur kadar nitrogen asam nukleat sel.

Kultur murni sebanyak 10 mL disentrifus pada 10.000 rpm selama 10 menit. Filtrat dibuang lalu ditambahkan 5 mL larutan garam fisiologis (0,85% NaCl) dalam endapan sel pada tabung reaksi, kemudian divorteks agar sel homogen dalam larutan fisiologis. Selanjutnya ditambahkan melanin dengan konsentrasi 0; 0,005; 0,010; 0,015; 0,020 g/mL dan dibiarkan pada suhu kamar selama 24 jam. Suspensi kemudian disentrifus pada 10.000 rpm selama 10 menit dan supernatan disaring dengan kertas saring (*Syringe filter sterile* 0,20 µm) untuk memisahkan selnya. Analisis dilakukan dengan mengamati OD (*Optical Density*) dari supernatan bebas sel.

HASIL DAN PEMBAHASAN

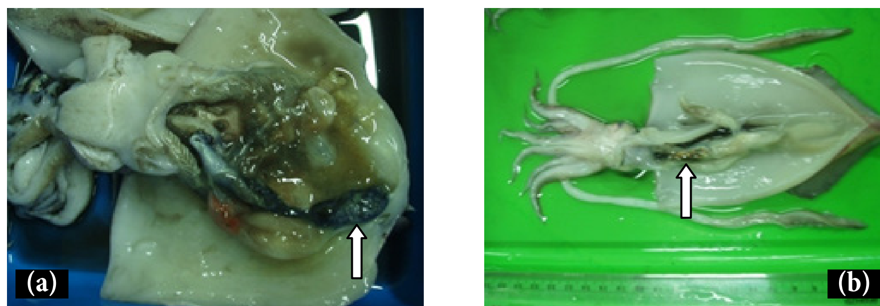
Isolasi *E. coli*

Koloni yang diduga *E. coli* yaitu yang berwarna hijau metalik diuji sifat biokimianya dengan menggunakan *microbact identification kits* (oxoid). Hasil pengujian dengan *microbact kit* menunjukkan lisin (+), ornitin (+), H_2S (-), glukosa (+), manitol (+), xilosa (+), ONPG (+), Indol (+), urease (-), V-P (-), citrate (-), dan TDA (-). Hasil identifikasi menunjukkan *Escherichia coli* (96,39%).

Rendemen Tinta dari *Sepia* sp. dan *Loligo* sp.

Sepia sp. memiliki kantong tinta yang panjang dan besar, sementara kantong tinta *Loligo* sp. berukuran kecil sehingga tinta yang dihasilkan juga lebih sedikit (Gambar 1). Tinta yang terdapat dalam kantong sangat ditentukan oleh kondisi terakhir sebelum ditangkap, jika sebelum ditangkap sudah banyak tinta yang dikeluarkan maka hanya sedikit yang tersisa di kantong. Analisis rendemen dari tinta berdasarkan perbandingan berat kantong (berisi tinta) terhadap berat badan per ekor yang dihasilkan oleh kedua sampel. Berat utuh *Loligo* sp. yaitu 116,6±40,36 g dan berat kantong tinta 0,6±0,1 g, sedangkan *Sepia* sp. memiliki berat utuh 173,0±19,6 g dan berat kantong tinta 4,0±1,4 g. Hasil analisis rendemen terlihat bahwa jenis *Sepia* memiliki rendemen tinta yang lebih besar (2,3%) dibandingkan tinta dari jenis *Loligo* (0,5%).

Nair *et al.* (2011) menyatakan bahwa tinta *Sepia* terdiri atas granula melanin dalam media yang kental tidak berwarna. Pigmen melanin diolah dalam sel mature kelenjar tinta, terutama pada bagian dasar kantong tinta yang terus-menerus memproduksi tinta. Akhir proses pematangan, sel-sel kelenjar tinta menyimpannya dalam kantong tinta yang berperan sebagai penampung. Setiap kantung tinta *Sepia* mengandung ~ 1 g melanin (Derby 2014), dan banyaknya melanin ~ 15% dari berat basah total tinta (Wang *et al.* 2014). Melanin *Sepia* terbentuk oleh banyak kelompok agregat. Agregat-agregat ini terbentuk juga oleh butiran bola kecil dengan distribusi ukuran yang berbeda. Diameter butiran kecil berkisar 100-200 nm (Mbonyiryivuze *et al.*2015). Ukuran butiran bola melanin pada cumi-cumi, berkisar antara



Gambar 1 Jenis chepalopoda dan kantong tinta yang digunakan pada penelitian ini. (a) jenis sotong (*Sepia* sp.), kantong tinta dari jenis *Sepia* sp., (b) jenis cumi-cumi (*Loligo* sp.), kantong tinta dari jenis *Loligo* sp.

50–150 nm (Chen *et al.* 2009). Berdasarkan hasil pengamatan, tinta *Loligo* sp. memiliki tekstur yang halus, sedangkan tinta *Sepia* sp. memiliki tekstur yang kasar.

Aktivitas Melanin Tinta *Sepia* sp. dan *Loligo* sp.

Pengaruh tinta dan melanin dari *Sepia* sp. dan *Loligo* sp. pada beberapa konsentrasi terhadap pertumbuhan *E. coli* dapat dilihat pada Tabel 1. Konsentrasi melanin semakin tinggi menghasillkan aktivitas penghambatan terhadap *E. coli* juga semakin besar. Melanin *Sepia* sp. pada konsentrasi 0,002 g/mL terlihat tidak ada aktivitas penghambatan, sementara pada konsentrasi yang lebih tinggi yaitu 0,006 g/mL terlihat jumlah koloni setelah inkubasi 24 jam tidak berbeda dengan jumlah awal (sebelum inkubasi). Hal ini menunjukkan pada konsentrasi 0,006 g/mL sudah terjadi penghambatan terhadap pertumbuhan sel bakteri, hingga 24 jam inkubasi tidak terjadi peningkatan yang berarti pada jumlah sel bakteri.

Konsentrasi yang lebih tinggi yaitu 0,010 g/mL, melanin mampu membunuh bakteri sehingga setelah inkubasi 24 jam hanya ada 1 sel yang hidup (penghambatan mencapai 99,99%). Melanin dari *Sepia* sp. memiliki aktivitas penghambatan yang lebih besar dibandingkan dengan melanin dari *Loligo* sp. Pada konsentrasi yang lebih rendah (1/2 dari konsentrasi melanin *Loligo* sp), melanin dari *Sepia* sp. mampu menghambat hampir 100%. Tabel 1 menunjukkan *E. coli* lebih sensitif terhadap melanin dari *Sepia* sp. dibandingkan dengan dari *Loligo* sp.

Tinta *Sepia* sp. dengan konsentrasi yang lebih tinggi dibandingkan dengan melaninnya tidak memiliki aktivitas penghambatan, sementra tinta *Loligo* sp. pada konsentrasi yang sama dengan melanin tidak memiliki aktivitas penghambatan terhadap *E. coli*. Hal ini disebabkan karena yang terkandung di dalam tinta tidak hanya melanin, melainkan ada komponen lain misalnya protein, lemak dan glikosaminoglikan yang diduga tidak memiliki aktivitas penghambatan terhadap *E. coli*, akan tetapi sebaliknya dapat meningkatkan pertumbuhan *E. coli*.

Tabel 1 Pertumbuhan *E. coli* pada media NB yang mengandung tinta dan melanin

Sumber		Konsentrasi (g/mL)	Jumlah *E. coli* (CFU/mL)		% Penghambatan relatif terhadap jumlah mikroba awal [100-(Ntx100/No)]	Log Peng-hambatan
			Inkubasi 0 jam (Nol)	Inkubasi 24 jam (Nt)		
Sepia sp.	Tinta	0	$8,6 \times 10^5$	$1,6 \times 10^{10}$	-	-4,27
		0,013	$8,6 \times 10^5$	$6,2 \times 10^9$	-	-3,86
		0,017	$8,6 \times 10^5$	$5,6 \times 10^9$	-	-3,81
		0,020	$8,6 \times 10^5$	$4,4 \times 10^9$	-	-3,71
	Melanin	0,002	$8,6 \times 10^5$	$1,2 \times 10^{10}$	-	-5,14
		0,006	$8,6 \times 10^5$	$6,0 \times 10^5$	30,23	0,16
		0,010	$8,6 \times 10^5$	$1,0 \times 10^0$	99,99	5,93
Loligo sp.	Tinta	0	$8,6 \times 10^5$	$3,1 \times 10^{10}$	-	-4,56
		0,013	$8,6 \times 10^5$	$8,6 \times 10^9$	-	-4,00
		0,017	$8,6 \times 10^5$	$2,3 \times 10^9$	-	-3,43
		0,020	$8,6 \times 10^5$	$1,0 \times 10^9$	-	-3,06
	Melanin	0,013	$8,6 \times 10^5$	$2,1 \times 10^4$	97,56	1,61
		0,017	$8,6 \times 10^5$	$2,1 \times 10^3$	99,76	2,61
		0,020	$8,6 \times 10^5$	$1,4 \times 10^2$	99.87	3,79

Keterangan: (-) nilai negatif artinya tidak ada penghambatan dan terjadi peningkatan

Aktivitas Melanin *Sepia* sp. terhadap Pertumbuhan *E. coli*

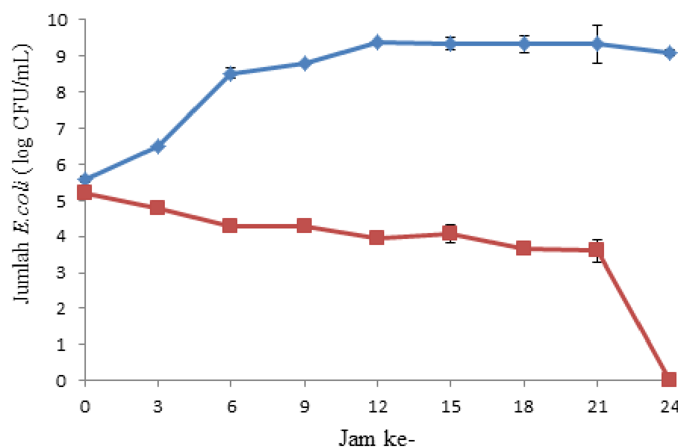
Aktivitas melanin dari *Sepia* terhadap pertumbuhan *E. coli* dapat dilihat pada Gambar 2. Pertumbuhan *E. coli* yang diberi perlakuan melanin dari *Sepia* sp. sebanyak 10 mg/mL terlihat jumlah koloninya yang hidup mengalami penurunan lebih dari 1 $\log_{10}$ setelah 6 jam inkubasi dan penurunan tersebut terus berlanjut hingga 2 $\log_{10}$ setelah 21 jam inkubasi. Akibat aktivitas melanin, terjadi perpanjangan fase adaptasi dan menyebabkan terjadinya penurunan jumlah koloni yang hidup, setelah 24 jam inkubasi, tidak ada lagi koloni yang hidup (Gambar 2). Hal ini menunjukkan bahwa selain memperpanjang fase adaptasi, melanin juga mempercepat fase kematian pada sel bakteri.

Pertumbuhan *E. coli* tanpa perlakuan melanin terjadi peningkatan jumlah koloni yang hidup lebih dari 4 $\log_{10}$ (dari 10^5 menjadi 10^9). Pertumbuhan pada 3 jam pertama memasuki fase adaptasi dan pertumbuhan awal yang dilanjutkan dengan fase logaritmik setelah 6 jam inkubasi. Setelah 6 jam terjadi peningkatan jumlah koloni yang cepat hingga 2 $\log_{10}$, dan terus meningkat hingga jam ke-12 walaupun hanya sedikit terjadi pertambahan populasi (kurang dari 1 $\log_{10}$). Pertumbuhan memasuki fase stasioner setelah jam ke-12 hingga jam ke-24.

Vasantharaja *et al.* (2014) melaporkan bahwa ekstrak metanol tinta *Sepiella inermis* dengan menggunakan GC-MS menunjukkan adanya campuran dari struktur oligomer yang merupakan gabungan antara dihidroksi indol-2-asam karboksilat dan dihidroksiindol. Ekstrak metanol ini memiliki aktivitas penghambatan terutama terhadap bakteri Gram negatif misalnya *Proteus vulgaris*, *Pseudomonas aeruginosa* dan *E. coli*. Neifar *et al.* (2009) melaporkan bahwa dihidroksiindol dan asam dikarboksilat dari *Sepia officinalis* memiliki aktivitas penghambatan terhadap mikroba.

Melanin merupakan tirosinase yang telah diidentifikasi terdapat di dalam tinta cumi-cumi (Derby 2014). Tinta cumi-cumi terdiri atas suspensi granula eumelanin di dalam media yang viscous dan tidak berwarna. Eumelanin bersifat heterogen, umumnya polimer yang tidak larut yang berkembang melalui oksidasi enzimatis dari asam amino tirosin. Produksi eumelanin di dalam sel pigmen terjadi di dalam organel khusus yang disebut melanosome. Eumelanin tersusun dari unit 5,6-dihidroksiindol (DHI) sekitar 20% dan unit 5,6-dihidroksiindol-2-asam karboksilat (DHICA) (Magarelli *et al.* 2010). Eumelanin alami dilaporkan merupakan molekul pigmen yang dapat mengadsorpsi logam pada konsentrasi tinggi. Kemampuan berikatan eumelanin dengan sisi dari logam merupakan parameter penting untuk memahami komplek logam-melanin (Lei *et al.* 2008; Chen *et al.* 2009).

Dinding sel bakteri mengandung banyak jenis kation termasuk Mg^{2+}, Ca^{2+}, Na+, dan K^{+}. Ion-ion ini bertanggung jawab atas berbagai aktivitas bakteri, termasuk kerja enzim,



Gambar 2 Kurva pertumbuhan *E. coli* yang diinkubasi dengan melanin *Sepia* sp. ◆ = kontrol (tanpa melanin), ■ = ditambah melanin.

Tabel 2 Nilai OD dari supernatan bebas sel *E. coli* yang diinkubasi dengan melanin dari *Sepia* sp.

Konsentrasi melanin (g/ml)	OD pada 260 nm	OD pada 280 nm
0	0,015	0,018
0,005	0,212	0,234
0,010	0,277	0,307
0,015	0,314	0,384
0,020	0,398	0,464

pengaturan metabolik dan menjaga integritas lapisan luar. Mg^{2+} dan ion Ca^{2+} khususnya, berperan penting dalam melindungi kestabilan struktur luar (Ferrero *et al.* 2007; Peshenko *et al.* 2007). Lapisan paling luar dari membran luar pada bakteri Gram negatif adalah lipopolisakarida (LPS), secara individu, molekul ini bermuatan negatif. Kation divalen membantu menstabilkan dan menjaga integritas membran luar dengan mengikat molekul LPS yang berdekatan. Kation *divalent* ini berfungsi sebagai jembatan garam berikatan dengan molekul lipid yang bermuatan negatif (Raetz *et al.* 2007). Membran luar pada sel bakteri berfungsi sebagai penghalang masuknya senyawa-senyawa yang tidak diperlukan sel (seperti bakteriosin, enzim dan senyawa hidrofobik). Jika kation tersebut dapat diadsorpsi oleh gugus fungsi melanin, maka sistem metabolisme sel bakteri akan terganggu, akibatnya pertumbuhan sel bakteri juga terganggu.

Sahalan *et al.* (2013) menjelaskan ion Ca^{2+} dan Mg^{2+} berperan melindungi membran terluar pada sel bakteri terhadap Polymyxin B yang berinteraksi dengan kation divalent, dengan mengganti kation dari tempat pengikatannya di molekul lipopolisakarida (LPS). Hal ini menyebabkan disorganisasi komponen membran luar bakteri Gram negatif, akibat lepasnya komponen LPS dari permukaan bakteri yang menyebabkan kebocoran membran dan akhirnya menyebabkan kematian sel. Ca^{2+} telah terbukti lebih efektif dalam melindungi sel bakteri dibandingkan Mg^{2+}.

Pengikatan gugus fungsi dari melanin yaitu gugus hidroksil fenolik (OH), karboksil (COOH) dan grup amina (NH) (Chen *et al.* 2009) terhadap ion Ca^{2+} dan Mg^{2+} pada membran luar mengakibatkan kebocoran pada membran bakteri Gram negatif dalam hal ini diwakili oleh *E. coli*. Kebocoran yang disebabkan oleh melanin terhadap sel *E. coli* dapat dilihat dari dihasilkannya supernatan bebas sel isi sel bakteri setelah diinkubasi selama 24 jam dengan melanin tinta Sepia sp. (Tabel 2). Tabel 2 menunjukkan hubungan antara konsentrasi melanin dengan supernatan bebas sel dari sel *E. coli*. Analisis kebocoran sel dilakukan dengan menggunakan Spektrofotometer *Double Beam* pada panjang gelombang 280 dan 260 nm. Panjang gelombang 280 nm digunakan untuk mengukur kadar nitrogen dari protein sel, sedangkan panjang gelombang 260 nm untuk mengukur kadar nitrogen asam nukleat sel. Semakin tinggi nilai OD baik pada panjang gelombang 260 nm maupun 280 nm menunjukkan semakin besarnya kebocoran sel akibat melanin. Semakin tinggi konsentrasi melanin, semakin besar tingkat kebocoran sel.

Hasil pengamatan terhadap spektrum Infra Red (IR) menggunakan FTIR spektrofotometer dari melanin tinta *Sepia* sp. dan *Loligo* sp. (data tidak dipublikasikan) menunjukkan kedua melanin memiliki pola spektrum yang sama, mengandung gugus fenolik, amina dan karboksil. Intensitas dari masing-masing gugus aktif tersebut yang berbeda diantara keduanya. Gugus fenolik, amina dan karboksil dari melanin *Sepia* sp. memiliki intensitas yang lebih tinggi dibandingkan dengan melanin dari *Loligo* sp. Intensitas ini menunjukkan konsentrasi dari gugus aktif tersebut di dalam melanin. Hal inilah yang diduga mengakibatkan perbedaan aktivitas kedua melanin tersebut terhadap *E. coli*.

KESIMPULAN

Melanin dari tinta sotong (*Sepia* sp.) dan cumi-cumi (*Loligo* sp) memiliki aktivitas penghambatan terhadap *E. coli*. Aktivitas penghambatan terhadap *E. coli* dari melanin tinta sotong lebih tinggi dibandingkan melanin dari cumi-cumi. Tinta sotong dan cumi-cumi pada konsentrasi 0,013-0,020 g/mL tidak memiliki aktivitas penghambatan terhadap *E. coli*.

UCAPAN TERIMA KASIH

Ucapan terima kasih disampaikan kepada Direktorat Riset dan Pengabdian Masyarakat Direktorat Jenderal Penguatan Riset dan Pengembangan, Kementerian Riset, Teknologi dan Pendidikan Tinggi atas bantuan dana penelitian melalui hibah Fundamental (sesuai dengan Surat Perjanjian Penugasan Pelaksanaan Program Penelitian Nomor: 024/SP2H/LT/DRPM/II/2016 tanggal 17 Februari 2016).

DAFTAR PUSTAKA

Arias C. 2009. Chilled and Frozen Raw Fish. Di dalam: Fernandes R (editor) Microbiology Handbook Fish and Seafood. United Kingdom (UK): Leatherhead Food International Ltd.

Bunduki MMC, KJ Flanders and CW Donnelly. 1995. Metabolic and structural sites of damage in heat and sanitizer-injuried population of *Listeria monocytogenes. Journal Food Protect*. 58: 410-415.

Cappaso R, Evidente A, Schivo L, Orru G, Marcialis MA, Cristinzio G. 1995. Antibacterial polyphenols from olive oil mill waste waters. *Journal of Applied. Bacteriology*. 79: 393-398.

Chen S, Xue C, Wang J, Feng H, Wang Y, Ma Q, Wang D. 2009. Adsorption of Pb(II) and Cd(II) by squid *Ommastrephes bartrami* melanin. *Bioinorganic Chemistry and Applications*. 1-7

Dave D, Ghaly AE.2011. Meat spoilage mechanisms and preservation techniques: a critical review. *American Journal of Agricultural and Biological Sciences.* 6(4): 486-510.

Derby CD.2014.Cephalopod ink: production, chemistry, functions and applications. *Marine drugs*. 12: 2700-2730.

Ferrero MA, Martínez-Blanco H, Lopez-VelascoFF, Ezquerro-SáenzC, NavasaN, Lozano S & Rodríguez-Aparicio LB. 2007. Purification and characterization of GlcNAc-6-P 2-epimerase from *Escherichia coli* K92. *Acta Biochimica Polonica*. 54(2): 387-399.

Lei M, Wang JF, Pang L.Wang YM, Chen SG, Xue CH. 2007a. Effects of *Sepia* on the metabolization of blood lipid and antioxidant ability in hyperlipidemia rats. *The Chinese Journalof Marine Drugs.* 3: 30-33.

Lei M, Wang JF,Wang YM, Pang L, Wang Y, Xu W,Xue CH. 2007b. Study of theradio-protective effect of cuttlefish ink on hemopoietic injury. *Asia Pacific Journal of Clinical Nutrition*.16: 239-243.

Lei M, Xue CH, Wang YM, Li ZJ, Xue Y, Wang JF. 2008. Effect of squid ink melanin-Fe on iron deficiency anemia remission. *Journal of Food Science.* 73(8): 207-11.

Mbonyiryivuze A, Nuru ZY, Diop Ngom B, Mwakikunga B, Simon Mokhotjwa Dhlamini SM, Park E, Maaza M. 2015. Morphological and chemical composition characterization of commercial *Sepia* melanin. *American Journal of Nanomaterials*. 3(1): 22-27.

Murhadi. 2002. Isolasi dan Karakterisasi Komponen Antibakteri dari Biji Atung (Parinarium glaberrimum Hassk). [disertasi]. Bogor (ID): Institut Pertanian Bogor.

Nair JR, Pillai D, Joseph SM, Gomathi P, Senan PV, and Sherief PM.2011. Cephalopod research and bioactive substances. *Indian Journal of Geo-Marine Sciences*. 40(1): 13-27.

Neifar A, Rebah FB, Gargouri AF, Abdelmouleh A. 2009. Physicochemical characterization of *Sepia officinalis*ink and the effects of storage conditions on the coagulation process. *Journal of the Marine Biological Association of the UK*. 89(04): 803-807.

Nithya M, Ambikapathy V, Panneerselvam A. 2011. Effect of pharaoh's cuttlefish ink

against bacterial pathogens. *Asian Journal of Plant Science and Research*. 1 (4):49-55

Peshenko IV & Dizhoor AM. 2007. Activation and inhibition of photoreceptor guanylyl cyclase by guanylyl cyclase activating protein 1 (GCAP-1): The functional role of Mg^{2+}/Ca^{2+} exchange in EF-hand domains. *The Journal of Biological Chemistry*. 82(30): 21645-21652.

Raetz C R, Reynolds CM, TrentMS & BishopRE. 2007. Lipid: A modification systems in gram-negative bacteria. *Annual Reviews Biochemistry*. 76: 295-329.

Rajaganapathi J, Thyagarajam SP, Edward JK. 2007. Study on cephalopod's ink for anti-retroviral activity. *Indian Journal of Experimental Biology*. 38: 519-520.

Sahalan AZ, Aziz AHA, Lian H & Ghani MKA. 2013. Divalent cations (Mg^{2+}, Ca^{2+}) protect bacterial outer membrane damage by Polymyxin B. *Sains Malaysiana*. 42(3): 301–306.

Vasantharaja D, Ravitchandirane V and Anandan V. 2014. Anti-microbial activity and spectro-chemical investigation of ink extracts of *Sepiella inermis* (Van Hasselt 1835). *Notulae Scientia Biologicae*. 6(3):273-275

Wang FR, XieZG, YeXQ, Deng SG, Hu YQ, Guo X, Chen SG. 2014. Effectiveness of treatment of iron deficiency anemia in rats with squid ink melanin-Fe. *Food Funct*. 5: 123–128.

Yuvaraj D, Suvasini B, Chellathai T, Fouziya R, Ivo Romauld S and Chandran M. 2015. Anti-bacterial studies on the different body parts of *Loligo* duvauceli. *Journal of Chemical and Pharmaceutical Research*. 7(6):406-408.

Zhong JP, Wang G, Shang JH, Pan JQ, Li K, Huang Y, Liu HZ. 2009. Protective effects of squid ink extract towards hemopoieticinjuries induced by cyclophosphamine. *Marine Drugs*. 7: 9-18.