UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIA ANIMAL

Influência de Campos Magnéticos Estáticos na Produção e Atividade de Proteases

WENDELL WAGNER CAMPOS ALBUQUERQUE

Recife, 2015

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Dissertação apresentada ao Programa de Pós-Graduação em Biociência Animal da Universidade Federal Rural de Pernambuco, como pré-requisito necessário para a obtenção do grau de **Mestre** em Biociência Animal.

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INFLUÊNCIA DE CAMPOS MAGNÉTICOS ESTÁTICOS NA PRODUÇÃO E ATIVIDADE DE PROTEASES

Dissertação de mestrado

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Aos meus pais,

pelo apoio incondicional.

“Não se espante com a altura do vôo. Quanto mais alto, mais longe do perigo. Quanto mais você se eleva, mais tempo há de reconhecer uma pane. É quando se está próximo do solo que se deve desconfiar”

Santos Dumont

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SUMÁRIO

LISTA DE FIGURAS

REVISÃO BIBLIOGRÁFICA

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

APÊNDICE

LISTA DE EQUAÇÕES

Revisão Bibliográfica

LISTA DE TABELAS

RESUMO

Muitos esforços para elucidar o caráter duvidoso da influência de campos magnéticos estáticos (CME) sobre as células vivas têm sido feitos, embora o tema ainda enfrente controvérsias devido aos relatos confusos na literatura científica. Uma vez que o CME é citado como modificador da expressão gênica, da atividade enzimática e do metabolismo do cálcio, ele pode influenciar o crescimento, ciclo e toxicidade celular, como também o transporte transmembrana e a estrutura do citoesqueleto, parâmetros nos quais se tenta encontrar pistas para a explicação dos fenômenos. As proteases têm sido largamente aplicadas na biotecnologia atuando desde a indústria alimentícia à farmacêutica. A manipulação dessas enzimas tem despertado perspectivas para novos produtos e novas aplicações na área médica, incluindo saúde humana e animal. As proteases fibrinolíticas são exemplos de enzimas aplicáveis na terapia trombolítica e no combate a doenças circulatórias. Esse trabalho objetiva testar a influência de CMEs sobre a produção de proteases por microorganismos bem como na atividade de proteases in vitro. A produção de proteases por *Streptomyces Malayensis* foi investigada sob a influência do campo magnético. Essas fermentações ocorreram em biorreator, com duração de 120 horas e expostas a um gradiente de CME (pico de 100 mT). Parâmetros como o consumo de proteínas, produção de proteases e biomassa foram analisados. Alterações no metabolismo bacteriano não foram identificadas, exceto um aumento na produção de enzima fibrinolítica. O CME foi também capaz de alterar a cinética enzimática do extrato fermentativo. A atividade proteolítica a partir de extratos obtidos de fermentação em estado sólido por *Mucor subtillissimus* foi também investigada sob um gradiente de exposição magnética de 0 a 140 mT, promovido por dois ímãs permanentes (liga de NdFeB). Nesses experimentos diferentes métodos de quantificação da atividade fibrinolítica foram estudados sob um campo magnético e alterações no grau de degradação dos géis de fibrina puderam ser observadas. Em geral, este estudo dá uma contribuição ao entendimento de como o CME pode ser utilizado como agente físico para induzir mudanças na atividade enzimática e ser utilizado como uma ferramenta biotecnológica.

Palavras-chave: campo magnético estático, proteases, fibrinólise, atividade fibrinolítica

ABSTRACT

Many efforts to elucidate the dubious character of the influence of static magnetic fields (SMF) on living cells have been made, although the issue still faces controversy due to confuse reports in the literature. Once the SMF is mentioned as a modifier of gene expression, enzyme activity, and calcium metabolism; it can influence the growth cycle and cell toxicity, as well as the trans-membrane transport and the cytoskeletal structure, parameters on which it tries to find clues for the explanation of phenomena. Proteases have been widely applied in biotechnology acting from the food industry to the pharmaceutical. The manipulation of these enzymes has raised prospects for new products and new applications in the medical field, including human and animal health. Fibrinolytic proteases are examples of relevant enzymes in thrombolytic therapy and in combating circulatory diseases. This current study investigates the influence of the CME for the production of proteases in microorganisms as well as in vitro protease activity. The production of proteases by *Streptomyces Malayensis* was investigated under the SMF influence. These fermentations were carried out in bioreactor, lasting 120 hours and exposed to a SMF gradient (peak of 100 mT). Parameters as the protein consumption, protease production and biomass were analyzed. Alterations in the metabolism of the microorganism were not identified, except for an increase in the production of the fibrinolytic enzyme. The SMF was also able to modify the enzymatic kinetic of the fermentative extract. The proteolytic activity from extracts obtained from solid state fermentations by *Mucor subtillissimus* was also investigated under a gradient of since 0 to 140 mT, promoted by 2 permanents magnets (NdFeB alloy). In these experiments 2 different methods to quantify the fibrinolytic activity were studied under a magnetic field and changes in the degree of fibrin dissolution were detected. In general, these studies objective to give contribution so the SMF can be used as an agent for inducing physical changes in enzymatic activity and as a biotechnological tool.

Keywords: static magnetic field, proteases, fibrinolysis, fibrinolytic activity

1. INTRODUÇÃO

Campos Magnéticos Estáticos (CME) interagem com a matéria e causa mudanças em suas propriedades. Os efeitos do magnetismo sobre os sistemas biológicos tem sido de interesse da comunidade científica e mais recentemente ao público leigo, uma vez que os campos magnéticos têm sido relacionados a diversas aplicabilidades desde a indústria alcooleira (DA MOTTA et al., 2004) até ao tratamento do câncer (LI et al., 2013; RAYLMAN; CLAVO; WAHL, 1996).

Enzimas proteolíticas são catalizadores biológicos de grande importância para indústria e medicina (SUMANTHA; LARROCHE; PANDEY, 2006) e os campos magnéticos estáticos (CME), uma vez que oferecem novas alternativas na manipulação biotecnológica de proteínas, poderiam despontar como novo método para a tecnologia enzimática. Essa influência de campos magnéticos em enzimas é principalmente justificada por mecanismos de recombinação de pares de radicais (GRISSOM, 1995) e através da anisotropia diamagnética onde componentes susceptíveis em solução podem ser orientados por um campo magnético externo e isso alteraria as características catalíticas de algumas enzimas (VAJDA, 1980).

Relatos, desde o início do século XX (KIMBALL, 1938), têm tentado identificar a ação de CME nos mais diferentes componentes intracelulares. A literatura científica cita experimentos com diversas variações de intensidade (10^{-6} a 10^{1} T), tempo de exposição (observação em tempo real, ou minutos, horas e dias de duração), diferentes organismos: sistemas microbiais, plantas, mamíferos e células humanas (MCCANN et al., 1993; SATO et al., 1992) e também nos constituintes intracelulares: microtúbulos (CHIONNA et al., 2005), proteínas (TORBET; RONZIÈRE, 1984), atividade enzimática (MALING; WEISSBLUTH; JACOBS, 1965) a ácidos nucléicos (POTENZA et al., 2004a). Por esta razão os resultados são confusos e dispersos em uma gama de efeitos não tão clara.

Labes et al. (1966) citaram não haver um mecanismo satisfatório ou provas gerais para os efeitos dos campos magnéticos estáticos e, recentemente, Anton-Leberre et al. (2010) estudando leveduras, afirmaram que o magnetismo não afeta muitos dos processos celulares. Em contraste, Short e Goodwill (1992) encontraram evidências de degeneração celular, diminuição da viabilidade, diminuição na taxa de crescimento celular ou mesmo nenhum efeito e Zhang et al. (2003) comentaram que os efeitos relatados ainda não foram avaliados suficientemente. Essa crítica é baseada na falta de consenso sobre projetos experimentais, a

ausência de diretriz para faixa de exposição (HIGASHI et al., 1993) e até mesmo casos de fraude (DE CERTAINES, 1992). No entanto, apesar da confusão, um grande número de publicações propôs uma ação positiva de campos magnéticos estáticos.

Diante de um cenário bastante controverso em termos de pesquisas com campo magnético estático, nós procuramos estudar sua influência em enzimas proteolíticas, uma vez que o acervo de trabalhos teóricos é vasto, mas, no entanto existe uma deficiência de trabalhos experimentais que justifiquem ou embasem as teorias. Avanços na tecnologia do estudo da matéria à nível subatômico têm proporcionado novas ferramentas para discussão de fenômenos observados. A influência dos CMEs será aqui investigada tantos em termos de produção das enzimas, quanto das atividades enzimáticas In Vitro, e nossos experimentos serão discutidos baseados em trabalhos fundamentados em teorias já bem estabelecidas.

2.1 OBJETIVO GERAL

Objetiva-se verificar a influência de CMEs sobre produção e a atividade in Vitro de proteases gerais ou especificamente sobre proteases fibrinolíticas, obtidas a partir de diferentes fermentações por em meio líquido (em Biorreator) ou sólido (em Erlenmeyer).

2.2 OBJETIVOS ESPECÍFICOS

- Produzir proteases fibrinolíticas por *Streptomyces Malayensis* em Biorreator com capacidade de 1,5 litros.
- Aplicar o campo magnético estático nas culturas de *Streptomyces Malayensis* durante o processo de produção e avaliar a influência na produção enzimática em biorreator.
- Avaliar diferentes atividades proteolíticas sob influência do CME.
- Avaliar crescimento microbiano influenciado pelo magnetismo.
- Estudar a cinética enzimática de proteases sob CME.
- Avaliar a atividade fibrinolítica em diferentes métodos sob influência do CME.

3. REVISÃO BIBLIOGRÁFICA

3.1 Enzimas Proteolíticas

As proteases são citadas em aplicações diversas. Elas têm sido utilizadas desde há muito tempo na indústria de laticínios como agentes da coagulação do leite (coalho) para o fabrico de queijo (SUMANTHA; LARROCHE; PANDEY, 2006), além de terem sido essenciais para desenvolvimentos nas áreas médica e biotecnológica (RAWLINGS; BARRETT; BATEMAN, 2012). Essas enzimas também realizam modificações altamente específicas tais como a coagulação do sangue, a lise de coágulos de fibrina, processamento e transporte de proteínas secretoras através das membranas. A grande diversidade de proteases, em contraste com a especificidade da sua ação, tem atraído a atenção mundial também para tratamentos de doenças circulatórias e produção de agentes terapêuticos contra doenças fatais tais como câncer e AIDS (NAJAFI; DEOBAGKAR; DEOBAGKAR, 2005).

Atualmente, algumas enzimas proteolíticas têm sido amplamente aplicadas na medicina humana e animal como antissépticos (FUGLSANG et al., 1995), anti-inflamatórios (VALDIVIA et al., 2005), anticoagulantes (VERHEIJ et al., 1980) e como agente terapêutico contra o câncer (MOHAMED; SLOANE, 2006; TALALAY et al., 1995), o que enfatiza ainda mais a relevância para a tentativa do melhoramento de suas características físico-químicas e de suas condições em termos de produção, empacotamento e atividade catalítica.

O mecanismo de ação é o de hidrólise de moléculas protéicas, degradadas em peptídeos e aminoácidos. Existe um grande complexo dessas enzimas, as quais diferem entre si em propriedades, tais como especificidade do substrato, mecanismo catalítico, pH e temperatura ótimos. A especificidade de enzimas proteolíticas é regulada pela natureza do aminoácido e outros grupos funcionais (aromáticos ou alifáticos ou que contenham enxofre) próximos à ligação a ser hidrolisada (SUMANTHA; LARROCHE; PANDEY, 2006).

3.2 Enzimas Fibrinolíticas e a Terapia Trombolítica

A fibrina é uma proteína filamentosa formadora de rede de coágulos e é oriunda da ativação do fibrinogênio pela ação proteolítica da enzima trombina. O processo de degradação de coágulos é feito por enzimas fibrinolíticas, que são representadas no sangue humano pela enzima plasmina (WANG; WU; LIANG, 2011). Muitas das disfunções patológicas provenientes de formação de trombos nos vasos sanguíneos advêm da disfunção na hidrólise

da fibrina, de onde decorre a trombose venosa, embolia, infarto do miocárdio, AVC e outras doenças (SIMKHADA et al., 2010).

Os distúrbios relacionados à formação de trombos no interior dos vasos sanguíneos levam a morte de milhões de pessoas no mundo segundo a Organização Mundial de Saúde (OMS) (PENG; YANG; ZHANG, 2005). A ação de agentes fibrinolíticos na terapia trombolítica tem se mostrado muito eficaz no tratamento das patologias mencionadas, e por muitos anos, diversas formas de administração dessas drogas via oral ou venosa têm sido investigadas com mais cautela (PENG; YANG; ZHANG, 2005).

Atualmente a terapia trombolítica é empregada principalmente a partir de agentes fibrinolíticos como a estreptoquinase (SK), a uroquinase (UK) e o ativador de plasminogênio tipo tecidual (TPA) (MURRAY et al., 2010). Uma vez que a UK e o TPA possuem uma relativa redução de meia vida in vivo, a SK tem sido escolhida como a principal droga para a terapia trombolítica (KUNAMNENI; ABDELGHANI; ELLAIAH, 2007).

3.3 Os Efeitos do Campo Magnético Estático (CME)

3.3.1. Efeitos do CME no DNA e na expressão gênica

A Expressão diferencial de genes desempenha um papel essencial na regulação do metabolismo, biossíntese e resposta ao estresse celular. O CME é citado como um agente de mudanças em tais processos, uma vez que atua sobre a integridade do DNA (AMARA et al., 2007), na mutação (ZHANG et al., 2003), e também nos processos de transcrição (PAUL; FERL; MEISEL, 2006) e tradução (GOTO et al., 2006).

Vários Estudos foram analisados com o objetivo de encontrar alguma relação entre o arranjo do magnetismo e os genes expressos ou aos danos no DNA. Experimentos que variam de baixa (15 mT) (JOUNI; ABDOLMALEKI; GHANATI, 2011) a alta intensidade de campo (37 T) (ANTON-LEBERRE et al., 2010) e duração de minutos, horas e dias de exposição, foram avaliados e não apresentam qualquer correlação linear com os efeitos. *Escherichia coli* foi freqüentemente citado nestes estudos (POTENZA et al., 2004a; ZHANG et al., 2003), por ser um organismo modelo e pioneiro com um genoma seqüenciado (BLATTNER, 1997), mas as observações foram feitas em muitos outros organismos tão diversos como *Salmonella*

entérica (MAY; SNOUSSI, 2009), *Drosophila melanogaster* (KALE; BAUM, 1980), células de plantas (PAUL; FERL; MEISEL, 2006) e células humanas (AMARA et al., 2007).

Os eventos complexos e os elementos que formam a maquinaria do metabolismo do DNA e RNA: complexo multi-enzimático, fatores de transcrição, mutagenicidade e elementos de transposição, são descritos abaixo segundo a literatura como sujeitos à ação do CME. Ikehata et al. (1999), estudando bactérias, mencionaram que o DNA de fita dupla tem, provavelmente, um caráter anisotrópico, mas, uma vez que o DNA genômico é geralmente compactado com proteínas chamadas de nucleóides, ela perderia sua susceptibilidade para o campo e, assim, o efeito magnetico-mecânico não conseguiria gerar os resultados observados. Os autores também sugerem que as interações eletrônicas, alteração na via de mutação de agentes químicos mutagênicos, mudanças na permeabilidade da membrana e processo de alquilação são possíveis mecanismos de ação sobre o DNA.

A variabilidade dos efeitos relatados é evidente nestes estudos. Autores como Anton-Leberre et al. (2010) e Kale and Baum (1980) verificaram pouca ou nenhuma sensibilidade do DNA sob um campo magnético estático. Mahdi et al. (1994) não encontraram nenhuma evidência de dano ao DNA ao expor *Escherichia coli* a um campo de 0,5-3 T. Amara et al. (2007) não conseguiram encontrar quebras significativas nas fitas de DNA em células humanas quando expostas a CME e Ikehata et al. (1999) não conseguiram demonstrar diferenças entre culturas de *Salmonella typhimurium* e *Escherichia coli* expostas e não expostas a fortes campos magnéticos. Em contraste, Goto et al. (2006) relataram um gene, Ntan1, que responde a um CME de 100 mT em neurônios do hipocampo de ratos e Jouni et al. (2011) observaram uma melhoria significativa de aberrações cromossômicas (ponte, fragmentos e cromossomos lagging) e número de cromossomos quando eles expuseram as células de *Vicia faba*, pré-cultivadas apenas expostas à radiação de fundo, a um CME de 15 mT. Zhang et al. (2003) demonstraram que um CME forte (até 9 T) pode aumentar significativamente a freqüência de mutação de genes soxR e sodAsodB.

Existe uma opinião consensual que a influência de um CME no DNA poderia estar relacionada com fatores de stress (diretamente gerado pelo CME ou ao stress oxidativo induzido dentro da célula). Potenza et al. (2004b) descobriram que um CME de 300 mT pode funcionar como indutor de estresse da atividade de transposição e May et al. (2009) relataram que a expressão do gene KatN poderia estar associada a uma resposta anti-oxidativa sob um CME de 200 mT por 10 horas de exposição.

Exemplos de genes citados com maior expressão são: rpoA, katN, dnaK, *Adh*/ GUS transgene (PAUL; FERL; MEISEL, 2006), soxR e sodAsodB. Vários artigos também mencionam campos magnéticos alternados que atuam sobre a expressão de genes de proteínas de choque térmico (GOODMAN; BLANK, 1998; LI; CHOW, 2001; LIN et al., 1998), mas campos alternados estão fora do escopo deste trabalho.

3.3.2 Efeitos do CME sobre o crescimento e a viabilidade celular

O ciclo celular, fatores de crescimento e sinais de transcrição do DNA são refletidos no número de células e na viabilidade e fornecem parâmetros úteis para detectar mudanças na maquinaria celular e estimar a influência de um agente extrínseco sobre o sistema intracelular. O magnetismo é suposto de promover alterações no crescimento e de provavelmente levar a célula a um estado de proliferação, a partir do qual se seguiria elevadas taxas de glicólise, biossíntese de lipídios, produção de lactato e outras macromoléculas (DEBERARDINIS et al., 2008). Estes difeririam marcadamente do metabolismo observado em células quiescentes dos mesmos tipos. A regulação enzimática também poderia promover mudanças no ciclo celular através da ligação de alguns íons específicos como o magnésio, manganês, cálcio, ferro e cofatores na estrutura enzimática sob campos magnéticos (BLANCHARD; BLACKMAN, 1994).

Trabalhos recentes têm especulado que alterações do ciclo celular podem ser responsáveis pela conexão entre a influência do CME e o crescimento celular. Onodera et al (2003) sugeriram que campos magnéticos fortes afetam o ciclo de divisão de linfócitos pela interação do campo com a replicação do DNA, mitose e equilíbrio de eletrólitos. Mas, ao mesmo tempo, Schiffer et al. (2003) expôs linhagens de células tumorais a campos estáticos de 1,5 T e 7,05 T e os resultados não mostraram nenhuma alteração no processo de transição do ciclo celular entre G_0/G_1, G_2/M e nem na fase S.

Independentemente das diferentes linhas de células (RAYLMAN; CLAVO; WAHL, 1996), intensidades (RUIZ-GÓMEZ et al., 2004) ou a duração da exposição (WISKIRCHEN et al., 1999) muitos estudos apresentaram os CMEs como influentes. Gamboa et al. (2007); May et al. (2009); Halpern e Greene (1964); Sato et al. (1992) e Malko e Constantinidis (1994) exemplificaram tais estudos com as mais diversas condições de exposição mas ainda sem respostas positivas. Em contraste, há relatos que mostram a eficácia do magnetismo em parâmetros, tais como: aumento da biomassa (MUNIZ et al., 2007), maior densidade óptica

(VANAGS et al., 2010), aumento na produção de CO_2 (MOTTA et al., 2001) e proliferação celular (GRUCHLIK et al., 2012).

Os efeitos positivos são contrastados por achados de inibição do crescimento ou pelo seu menor atraso. Iwasaka et al. (2004) observaram uma diminuição na taxa de proliferação de leveduras quando se aplica um forte campo de 14 Tesla por 16 horas. Eles propuseram um mecanismo pelo qual o gradiente de densidade de fluxo magnético funciona perturbando o transporte de O_2 e movendo a levedura para longe do gás através da flutuabilidade estimulada por forças diamagnéticas, impedindo a multiplicação celular mais rápido pela ação do oxigênio. Kimball (1938) relatou que mesmo um CME muito fraco de 4×10^{-4} T pode retardar a germinação da levedura, o que também seria o resultado do gradiente magnético que pode forçar o movimento dos componentes celulares sensíveis do mais fraco para o campo mais forte.

Uma sugestão interessante é que, em muitos estudos, foi proposto que os efeitos dependem apenas do tipo de célula ou organismo. Por exemplo, Aldinucci et al. (2003) não conseguiram encontrar nenhuma influência do CME em células mononucleares do sangue periférico (PBMC), mas encontraram uma diminuição da proliferação de células Jurkat com 4,75 T. Nagy e Fischl (2004) usaram CMEs fracos para obter crescimentos diferentes de conídios de *Alternaria alternata* e *Curvularia inaequalis* (aumento de 68-133%) e *Fusarium oxysporum* (diminuição de 79-83%). Sullivan et al. (2011) observaram uma inibição de 20% de crescimento de fibroblastos, em melanoma humano e ainda não apresentou efeitos em células-tronco, quando expostos a CMEs de 35-120 mT. Uma busca no banco de dados MEDLINE (acessado pelo PubMed), usando o termo de busca "campo magnético estático crescimento celular" resultou em 31 referências a estudos diretos relacionando CME ao crescimento celular entre 1976 e 2014 e os efeitos sobre a proliferação celular não foram representativos (Figura 1).

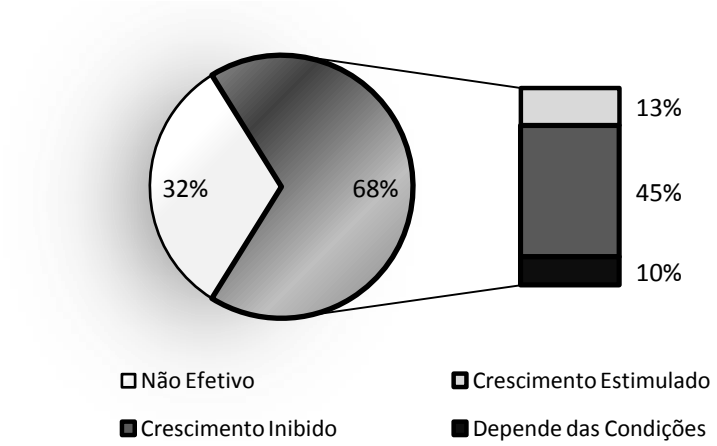

Figura 1. Distribuição percentual dos relatos de efeitos do CME no crescimento celular (base de dados MEDLINE). Alterações são descritas como: efeitos inibitórios (45%), estimuladores (13%) e mudanças dependendo das condições de exposição e/ou do tipo específico de célula (10%). Cerca de um terço (32%) dos estudos relataram ausência de efeitos.

A viabilidade e toxicidade da célula são relatadas como sujeitas ao CME através de mudanças no metabolismo do íon Ca^{+2}, na bicamada de fosfolipídios e ação enzimática (o qual cobre o sistema scavenger e a resposta anti-oxidante) (BÜYÜKUSLU; ÇELIK; ATAK, 2006; WANG; LI; LI, 2006). Buemi et al., (2001) aplicaram um CME de 0,5 mT em células renais e detectaram, após 2,4 e 6 dias, um aumento das células com morfologia necrótica e também uma diminuição da apoptose e proliferação. Valiron et al. (2005) descobriram que a adesão focal e o arranjo da actina são inibidos pelo CME (10 T) e resulta da anisotropia diamagnética de microtúbulos e filamentos de actina, com reflexos diretos na viabilidade celular, organização e diferenciação. Teodori et al. (2002), depois de aplicarem um fraco CME de 6 mT a células HL60 (linhagem celular de leucemia promielocítica humana), sugeriram que o campo magnético não pode induzir a apoptose ou necrose sozinho, mas age sinergicamente com outros agentes (Inibidor de DNA topoisomerase I CPT) através de mudanças nos fosfolipídios da membrana plasmática ou no metabolismo do Ca^{+2}. Gruchlik et al. (2012) e Khodarahmi et al. (2010) mencionaram total ausência de efeitos do CME na viabilidade celular. May e Snoussi (2009) concluíram que o crescimento e a viabilidade de células bacterianas dependem de variabilidade nas condições de exposição, e também o tipo de célula é um fator crucial para determinar se o CME exerce influência.

3.3.3 CMEs e a morfologia celular

Vários estudos têm indicado a anisotropia diamagnética de componentes intracelulares como o fator mais comum na alteração de estruturas do citoesqueleto responsáveis pela morfologia celular. Os artigos revisados concordam que membranas, microtúbulos, proteínas (principalmente aquelas com estrutura secundária α-hélice) recebem torques e, conseqüentemente, levam a célula ao movimento. Sabe-se que a ressonância eletrônica de ligações peptídicas planares (figura 2-a) dão propriedades diamagnéticas às α-hélices de proteínas (PAULING, 1979; WORCESTER, 1978), com as ligações planares orientadas paralelamente ao eixo da hélice. Por exemplo, os microtúbulos (Figure 2-b) se rearranjam na presença de um CME de modo que a sua unidade monomérica (tubulina), com α-hélices na sua estrutura, se alinha num arranjo tubular ao longo de um eixo comum (ROSEN; ROSEN, 1990) e túbulos de lipídios nas duplas camadas lipídicas se auto-organizam como cilindros e são também muitas vezes referidos como sendo orientado pelo CME (ROSENBLATT; YAGER; SCHOEN, 1987). Como os pares de bases em parte consistem em anéis aromáticos (MARET, 1990), os ácidos nucléicos são também referidos como tendo o caráter diamagnético.

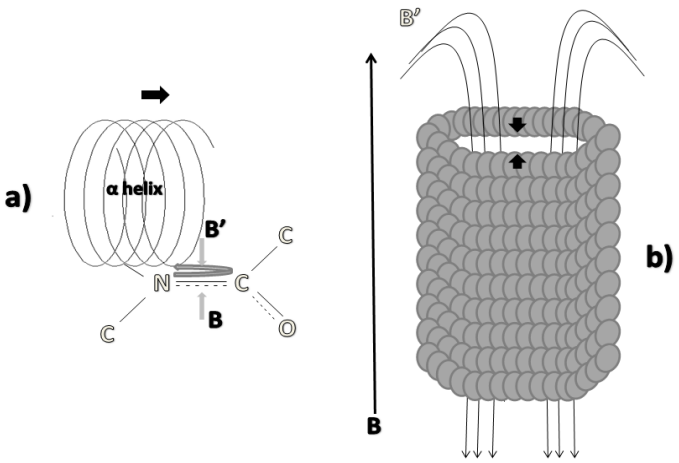

Figura 2. Anisotropia diamagnética em estruturas biológicas. Ligações peptídicas planares presentes na α-hélice proporcionam grande anisotropia diamagnética (a). Em microtúbulos (b), por exemplo, o alinhamento paralelo das ligações peptídicas com o eixo da α-hélice e seu arranjo como estrutura circular aumentam a magnitude da anisotropia magnética como uma soma de todos os campos secundário B'.

Componentes intracelulares podem ser submetidos a torques e alterar a sua conformação e seu alinhamento sob uma força magnética. Especula-se que as membranas, o citoesqueleto e distribuição de proteínas (como filamentos de actina) poderiam ser diretamente afetados pelo CME e que, conseqüentemente, promoveriam mudanças na morfologia celular (DINI; ABBRO, 2005). A relação entre o íon cálcio e o formato da célula é freqüentemente mencionada. Alterações no cálcio livre podem afetar interações do citoesqueleto ou ligação do Ca^{+2} a proteínas (MARHL et al., 2000). A mobilização de Ca^{2+} pelo campo magnético promove uma cascata de microfilamentos e reorganização de microtúbulos e conseqüentemente, a modificação da forma da célula (DINI; ABBRO, 2005). O estado alterado de fosforilação e desfosforilação de proteínas também pode interferir na organização dos elementos do citoesqueleto, quando expostos ao CME (POPOV et al., 1991).

Pacini et al. (1999) relataram que um CME de 0.2 T atuando em células neuronais humanas promoveu alterações visíveis na morfologia celular. Eles observaram vórtices de células, neurites ramificadas e neurites apresentando botões sinápticos após 15 minutos de exposição ao CME. Quando Albertini et al. (2003), expuseram o microrganismo *Fusarium culmorum* a 0,3 T de CME eles observaram, através de microscopia eletrônica, um encolhimento considerável das paredes das células. Além disso, a microscopia eletrônica de transmissão mostrou que o CME induziu um aumento de vacúolos e corpos lipídicos e desorganização de organelas. No entanto, não foram notificados efeitos quando Khodarahmi et al. (2010) aplicaram 2.1 T por 72 horas em astrócitos de ratos in situ e, particularmente, nenhuma protusão de membranas ou mudanças nas superfícies foram encontradas. A anisotropia diamagnética das estruturas do citoesqueleto varia de célula para célula e os componentes são sujeitos a forças lineares e torques que afetam diretamente a morfologia celular.

3.3.4 Atividade enzimática sob CME

A produção e a atividade enzimática são relatadas como influenciadas por campos magnéticos estáticos. No entanto, como muitos resultados contraditórios foram encontrados, não é possível ter a certeza sobre os fenômenos sem considerar as condições de exposição e os tipos de células que foram utilizados nos experimentos. A variedade de enzimas citadas inclui: tripsina (RABINOVITCH; MALING; WEISSBLUTH, 1967; VAJDA, 1980), endo-1,4-β-glucanase, celobiohidrolase, β-glucosidase (MANOLIU; OPRICK; CREANGA, 2006),

ribonuclease e RNA-succinato-citocromo c redutase (MALING; WEISSBLUTH; JACOBS, 1965); plasmina (IWASAKA; UENO; TSUDA, 1994), o malondialdeído (MDA)(AMARA et al., 2007), superóxido dismutase (SOD) e catalase (BÜYÜKUSLU; ÇELIK; ATAK, 2006; ÇELIK et al., 2009); transaminase glutâmico-pirúvica, transaminase glutâmica oxalacética e desidrogenase láctica (GORCZYNSKA; WEGRZYNOWICZ, 1989), α-amilase (YAN et al., 1997), a peroxidase (ATAK et al., 2007), endoglucanase (GEMISHEV et al., 2009), etanolaminaamônia-liase, metilmalonil-CoAmutase (TAOKA et al., 1997), carboxidismutase, glutamasedesidrogenase (HABERDITZL, 1967). As intensidades de campo variam nesses estudos de 10^{-4} a 10 T, sem relação dose-resposta.

Taoka et al., (1997) não encontraram nenhuma relação linear entre a intensidade variável do CME e a atividade enzimática. Alternativamente, alguns investigadores relataram fortes CMEs como sendo influente (UENO; IWASAKA; TSUDA, 1993; YAN et al., 1997), mas o mesmo não pôde ser reproduzido por Maling et al. (1965) e Vajda (1980). Contradições também são relatadas com campos mais baixos. Çelik et al. (2009) descobriram que CMEs na faixa de miliTesla pode aumentar a atividade de SOD e catalase e da mesma forma, Atak et al. (2007), observaram um aumento na atividade da peroxidase sob um fluxo magnético de 2,9-4,6 mT. Inversamente, Amara et al. (2007) mostraram que a 250 mT não ocorreu stress oxidativo e danos ao DNA de células THP1.

Os resultados apresentados por Çelik et al. (2009), Büyükuslu et al. (2006) e Haberditzl (1967) mostram um aumento na atividade enzimática. A enzima proteolítica tripsina foi estudada por Rabinovitch et al. (1967) e Vajda (1980) e ambos os estudos não consideraram a enzima vulneráveis à ação de CMEs.

A Tabela 1 apresenta uma lista de enzimas, intensidades de exposição e seus diferentes efeitos sobre a atividade, produção e parâmetros cinéticos.

Tabela 1. Levantamento de dados da associação entre o CME com os efeitos sobre a atividade enzimática

Referência	Intensidade (T)	Enzima	Efeitos
(MANOLIU; OPRICK; CREANGA, 2006)	**0,08**	Celulases, Peroxidase e Catalase	- Atividade alternou entre a inibição e a estimulação de acordo com o tempo de exposição de 7 ou 14 dias.
(MALING; WEISSBLUTH; JACOBS, 1965)	**0 to 4,8**	Ribonuclease RNA e Succinato-citocromo c redutase	- As reações permaneceram constantes após a exposição.
(AMARA et al., 2007)	**0,25**	Malondialdeído, Glutationaperoxidase, catalase e SOD	- O CME não conseguiu causar estresse oxidativo e danos ao DNA.
(RABINOVITCH; MALING; WEISSBLUTH, 1967)	**22**	Tripsina	- Não foram observados efeitos sobre a reatividade.
(IWASAKA; UENO; TSUDA, 1994)	**8**	Plasmina	- Produtos de degradação da fibrina tiveram níveis mais elevados nas culturas expostas.
(VAJDA, 1980)	**1,4**	Tripsina	- Alterações detectáveis não foram observadas quando as culturas foram expostas ao campo.
(ÇELIK et al., 2009)	**2,9-4,6×10^{-3}**	Catalase e SOD	-SOD e catalase foram aumentados em 21,15% e 15,20%.
(YAN et al., 1997)	**0 to 0,20**	α-amilase	- A atividade foi aumentada em 27%, 34,1%, 37,8% em comparação com o controle.
(ATAK et al., 2007)	**2,9-4,6×10^{-3}**	Peroxidase	- A atividade da peroxidase aumentou significativamente em todos os tratamentos com campo magnético.
(GEMISHEV et al., 2009)	**5-70×10^{-3}**	Endoglucanase	- De acordo com o pré-tratamento aplicado do inóculo de esporos, foi observada uma maior atividade de endoglucanase.
(TAOKA et al., 1997)	**0 to 0,25**	Etanolamina Amônia-liase e Metilmalonil-CoA-mutase	- As enzimas não tiveram alterações na atividade maiores que 15% quando expostas ao CME.
(BÜYÜKUSLU; ÇELIK; ATAK, 2006)	**2,9-4,6×10^{-3}**	SOD	- A atividade da SOD aumentou significativamente.
(HABERDITZL, 1967)	**5 –7,8**	L-glutamico desidrogenase e catalase	- Os experimentos apresentaram aumentos na atividade das enzimas em campos não uniformes.

Os efeitos do CME sobre a atividade enzimática estão relacionados com a intensidade de campo para cada enzima observada em alguns estudos experimentais.

Iwasaka et al. (1994) defenderam a idéia de que os componentes diamagnéticos no meio intracelular podem ser afetados por um gradiente magnético e deriva através da flutuabilidade, assim, em fraca região de campos magnéticos, eles afetam a concentração local de substratos e enzimas, promovendo diferenças na sua atividade. Yan et al. (1997)

propuseram que a presença do íon Ca^{+2} na enzima α-amilase pode alterar a sua conformação secundária, quando exposto a um campo magnético e, por conseguinte, alterar a sua atividade e parâmetros cinéticos. Estudos realizados por Çelik et al. (2009) e Büyükuslu et al. (2006) sugerem que um campo magnético pode orientar elétrons desemparelhados em íons metálicos dando energia cinética para a molécula e transferindo energia para os outras, o que, por reação em cadeia, forma mais radicais.

3.3.5 O mecanismo de recombinação de pares de radicais

A orientação de spin no mecanismo de recombinação de pares é considerada um exemplo de como os campos magnéticos podem alterar a química de reações e causar efeitos biológicos. Na célula, as enzimas alteram as ligações químicas permitindo a formação de novas moléculas pós-reação com características diferentes do substrato inicial. Os elétrons de valência são responsáveis pela ligação em moléculas e quando a região de sobreposição de um orbital é quebrada, por causa da ação enzimática, os spins opostos de elétrons (nos radicais livres formados) passam do estado singlet (spin antiparalelo) ao estado triplet (spin paralelo), por causa da influência de núcleos próximos (interações chamadas acoplamentos hiperfinos) (BROCKLEHURST, 2002). A proporção das moléculas na conformação triplet e singlet indica a capacidade de re-reação dos radicais e que é responsável por causar lesões na célula. Os campos magnéticos são suspeitos de alterar o spin desses elétrons de valência (pós-reação) e interferir com a reatividade de radicais livres. Por respeito ao princípio de exclusão de Pauli, dois elétrons em estados triplet não ocupam o mesmo orbital e, portanto a ligação uma vez desfeita não é mais refeita, caso os radicais permaneçam nessa conformação.

3.3.6 Discussão sobre a interação entre o CME e radicais livres a nível quântico

A análise quântica das reações enzimática permite-nos entender os possíveis efeitos que um campo magnético estático externo gera no sistema. Um elétron desemparelhado num átomo é fonte de um pequeno momento magnético sujeito à modulação por qualquer fonte magnética externa. E é isso que ocorre nos radicais liberados pós a ação de uma enzima no substrato. Com a quebra da ligação química no substrato, os elétrons desemparelhados apresentam momento angular intrínseco, denominado spin, expostos. O spin é uma característica quântica de partículas subatômicas associada aos seus momentos angulares, mas sem qualquer comparativo ou descrição no mundo clássico. Sabe-se que o spin possui valores e orientações quantizados, i.e. possuem valores discretos e não contínuos. As diversas

contribuições experimentais e teóricas da física quântica permitiu descrever valores de ½ ħ para o Spin **S** de um elétron, tendo esse componentes cartesianos (S_z por exemplo) definidos como $S_z = m_s\hbar$ (ATKINS e FRIEDMAN, (2011).

Matematicamente a projeção linear de uma função em outra função num espaço de mesmas dimensões é tratada com operadores lineares. Portanto a matemática de operadores é a maneira mais comum de tratamento dos spins. A combinação de spins para um sistema de 2 elétrons é feita através da utilização de "vetores de operadores" e assim é possível definir algumas combinações de vetores para um sistema de 2 elétrons (KÖHLER; BÄSSLER, 2009)

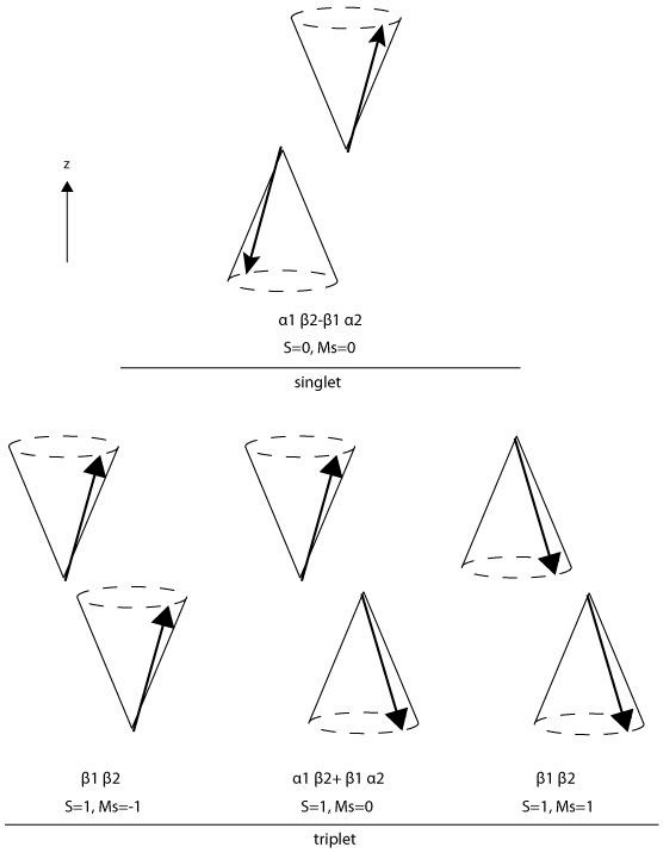

Figura 3. Diagrama de Vetores com as orientações dos dois spins dos elétrons para os estados de singlet e triplet. As funções de onda de spin e os autovalores de S e M_s também são apresentados.

Quando esses vetores de spin têm combinação de spins contrários e fora de fase, temos uma combinação que forma um campo magnético nulo e, portanto temos um valor físico nulo ou "observável nulo", essa combinação é denominada singlet. Para combinação de spins contrários e em fase temos a combinação de spin up e down contrárias gerando um número magnético de spin nulo, porém um vetor de momento angular resultante. Vetores resultantes

também aparecem nas combinações de spins de mesma orientação o que gera um sistema triplet para sistemas de 2 elétrons, com possibilidades de número quântico magnético de -1, 0 e 1 (figura 3) (KÖHLER; BÄSSLER, 2009) O spin do elétron em um orbital atômico preenchido (2 elétron) é probabilisticamente composto das duas possíveis orientações de spin, chamadas de up e down e então poderiam ser defletidos a partir de um campo magnético externo.

Quando um CME externo é aplicado, as configurações de orientações de spins sofrem alterações em termo de conversões inter estados singlet-triplet o que pode gerar conseqüências na proporção em que produtos são formados ou na taxa de recombinação de pares de radicais, um fenômeno já bastante discutido na literatura cientifica e comprovado experimentalmente (GRISSOM, 1995).

Qualquer efeito a nível quântico tem tendência a ser ignorado pela falta de projeção no mundo clássico macroscópico. A alteração na taxa de radicais livres pós-reação enzimática por campos magnéticos tem sido tratada por muitos autores, sendo inclusive comprovada em diversos experimentos (EVESON et al., 2000)

Para um elétron o momento de dipolo magnético intrínseco é chamado de Magnéton de Bohr $\boldsymbol{\mu_B}$ e a relação entre o caráter magnético e o momento angular é definida pela razão giromagnética:

$$\gamma = \text{□}/|\mathrm{L}| \qquad \textit{Equação 1}$$

ou classicamente:

$$\gamma = -e/(2\mathrm{m}_e) \quad \textit{Equação 2}$$

Uma vez que os momentos magnéticos e orbitais podem ser definidos em termos da carga do elétron e sua massa.

Por fim experimentalmente a relação se torna proporcional com a adição de um fator adimensional chamado fator-g, resultando para o elétron em**:**

$$\gamma = \mathrm{g}_e \cdot e/(2\mathrm{m}_e) \qquad \textit{Equação 3}$$

com o valor de g aproximado de g_e= –2.002290 (BROCKLEHURST, 2002).

Tentativas de teorizar os mecanismos envolvidos na germinação de radicais são discutidas. Brocklehurst (2002) afirma que a recombinação de pares de radicais é afetada pelo CME e o autor explica a susceptibilidade de radicais para o campo através da correlação entre spins de elétrons e fases relacionadas. Em um estado singlet os elétrons estão correlacionados com spin antiparalelo (relacionado em fase e com zero de momento de spin resultante). No estado triplet os elétrons também estão correlacionados, mas então eles são paralelos e com um spin resultante de um. O grau de interação, ou a energia total do sistema representado pelo Hamiltoniano (*H*), entre um elétron, um núcleo atômico próximo e do campo magnético externo (B) foi definida por:

$$H = g\mu_B BS + \sum_j a_j SI_j + \sum_j SA_j I_j \quad (Equação\ 4)$$

Onde: *a* é o termo de contato spin-spin; *A* é a clássica interação dipolar entre dois magnetos; μ_B (magnéton de Bohr) e *g* (fator g) expressa o momento magnético originado do momentum angular do spin; S e *I* são operadores dos spins do elétron e nuclear, respectivamente.

Os segundo e terceiro termos da equação 4 representa o somatório das duas partes de interações hiperfinas sobre todos os núcleos (o momento magnético dos núcleos mais o campo magnético produzido nos núcleos pelos elétrons) e o primeiro termo expressa a energia potencial de interação entre o campo externo e o spin do elétron (BROCKLEHURST, 2002).

Weller et al. (1984) definiram uma relação entre o CME e a recombinação de radical. Eles estudaram interações de radicais em solventes polares, sob um campo magnético externo, e determinaram a intensidade do campo externo $B_{1/2}$ (em que a extinção do estado triplet tem o seu valor de saturação pela metade) como um parâmetro para avaliar a influência do campo magnético sobre a produção de estados triplet (Equação 5).

$$B_{1/2} = \frac{B_1}{\bar{B}} B_1 + \frac{B_2}{\bar{B}} B_2 = 2\frac{{B_1}^2 + {B_2}^2}{B_1 + B_2} \quad (Equação\ 5)$$

Onde: $\bar{B}$= é a média aritmética dos dois valores experimentais B_1 e B_2.

B_1 e B_2, os campos magnéticos nucleares nos elétrons desemparelhados, são a medida para a mudança de spin-multiplicidade (modulado pelo CME) para ambos os radicais e calculado de acordo com:

$$B_i = \left(\sum_k a_{ik}^2 \, I_k (I_k + 1) \right)^{\frac{1}{2}} \quad (Equação\ 6)$$

Onde: a_{ik} = constante de acoplamento hiperfino isotrópica *e* I_k = spins nucleares

Os resultados experimentais confirmam que o magnetismo altera a formação de radicais livres. Chagovetz e Grissom (1993) investigaram a dependência de pares de radicais promovidos pela adenosylcob- (III) alamina (AdoCblIII) em meio à exposição magnética e o líquido móvel (glicerol aquoso, solução aquosa de Ficoll-400 e H_2O), quando uma fotólise com laser foi realizada. A taxa para (AdoCblIII) par de radicais geminados foi promovida de $1x10^{-9}$ para $4x10^{-9}$ sob 50 mT em glicerol e de $1x10^{-9}$ para $3x10^{-9}$ sob 80 mT em água, o que mostra o CME como influente. Eveson et al., (2000) estudaram a recombinação de radicais em micelas sob exposição magnética e descobriram que um campo fraco pode aumentar a concentração de radicais livres.

3.3.7 Influência de CMEs sobre a síntese de biomoléculas

Os CMEs podem modificar a maquinaria celular e estimular a síntese de biomoléculas. Mudanças nos processos enzimáticos e nas reações químicas são sugeridas como o caminho para alcançar o produto final. Gemishev et al. (2009) estudaram a síntese de endoglucanase sob campos estáticos de intensidades variáveis entre 5-70 mT e também tempos variáveis de exposição. Os autores, além disso, aplicaram um CME de 10 mT e observaram aumento na concentração de proteína total de 11,5%, para a exposição de 10 dias, de 15% durante 24 horas e 18% durante 1 hora. Da Motta et al. (2004) melhoraram 3,4 vezes a concentração de etanol através da aplicação de um campo magnético de 250 mT em fermentação em *Saccharomyces cerevisiae*. Os efeitos do álcool na organização da membrana e na inativação de proteínas, são prejudiciais para a célula e o magnetismo poderia proteger a célula dos efeitos nocivos destas permitindo que produza mais etanol. Esta constatação foi verificada por Galonja-Corghill et al. (2009), quando expuseram a mesma levedura a um campo de 150 mT e obtiveram um rendimento de etanol de 15% maior do que o controle.

Buchachenko e Kuznetsov (2008) estudou a síntese de ATP pela fosforilação enzimática (creatina quinase) e usando duas formas isótopas de magnésio: ^{25}Mg e isótopo não magnético $^{24,26}Mg$. O íon $^{25}Mg^{+2}$ é conhecido por integrar o sítio catalítico da enzima; na reação, um elétron é doado por oxiradicais ADP e assim forma par de radicais. Nesta situação, os pares podem assumir os estados singlet e triplet, com a fosforilação triplet sendo mais eficiente. A conversão singlet-triplet pode ser modulada por um CME externo e isso poderia explicar os efeitos experimentais obtidos. Experimentalmente, campos magnéticos alteraram a síntese de ATP, atingindo aumentos de 50% (55 mT) e 70% (80 mT) para a quinase com o isótopo ^{25}Mg.

4. MATERIAIS E MÉTODOS UTILIZADOS

4.1 Microorganismos

Estudamos a influência do CME sobre fermentações e extratos fermentativos por bactéria *Streptomyces* malayensis (em fermentações em meio líquido) e também pelo fungo *Mucor subtillissimus* (em fermentações em meio sólido).

A actinobactéria *Streptomyces* malayensis DPUA 1571 foi isolada de líquens da Amazônia. O gênero Streptomyces foi mencionado por Lopes et al. (1999) como fonte de várias proteases (principalmente de serina-proteases e metaloproteinases) e por Peng et al. (2005), especificamente como produtor de enzima fibrinolítica.

O fungo filamentoso *Mucor subtillissimus* UCP 1262 foi isolado do solo da caatinga, PE-Brasil. Diversas espécies do gênero Mucor são mencionadas como produtoras de proteases, inclusive a espécie *Mucor subtillissimus* é descrita por Alves et al. (2005) como fonte de enzimas proteolíticas.

4.2 Quantificação de Proteínas Totais

O consumo de proteína foi avaliado pelo método de ligação de Azul de Comassie G-250 à proteína tal como descrito por Bradford (1976). 1,5 ml de uma solução de reagente (100 mg de Azul de Comassie Brilliant G-250 foi dissolvido em 50 mL de etanol a 95% e 100 mL de ácido fosfórico a 85%) foram misturados com 0,05 mL do extrato fermentativo (amostra

líquida retirada da fermentação). A proteína total foi estimada através de correlação entre a absorbância (595 nm) e a concentração de proteína específica.

4.3 Proteases Gerais

A produção de proteases gerais foi medida tal como descrito por Ginther (1979). 0,25 ml de uma solução composta por 0,2 M de tampão Tris-HCl (pH 7,2); $CaCl_2$ 10^{-3} M e 1% de azocaseína foi misturada com 0,15 ml de extrato fermentativo centrifugado. A reação foi incubada durante 1 h (28 ° C) e foi pausada com 1 ml de uma solução de ácido tricloroacético a 10%. Depois o conteúdo foi centrifugado (14500 x *g*, 15 min) e o sobrenadante (0,8 ml) foi adicionado a 0,2 ml de solução de NaOH 1,8 M. 1 unidade de atividade da enzima correspondeu a cada 0,01 de alteração na absorbância (420 nm).

4.4 Protease Fibrinolítica

4.4.1 Método das Placas

Este método foi descrito por Astrup e Mullertz (1952). 4 mL de gel de fibrina foi produzido através de uma solução constituída por 2 mg/ml de fibrinogênio (obtido a partir de plasma bovino, e dissolvido em tampão Tris-HCl (150 mM) e tampão NaCl (150 mM), pH 7,75, adicionado de 200 µl de trombina de solução de plasma bovino (20 U/ml diluído em solução de cloreto de sódio 150 mM). A solução de fibrinogênio foi misturada, em placas de Petri de plástico, com 4 ml de agarose 2%, e 100 µl de solução 1 M de $CaCl_2$. Após a polimerização da fibrina, para cada placa, 6 orifícios com as mesmas dimensões (5 mm de diâmetro) foram feitos no gel de fibrina. 20 µl de extrato fermentativo foram adicionados a cada orifício e as placas foram incubadas a 37 ° C. O potencial de degradação foi determinado pelas superfícies de gel consumido em 18 e 24 horas.

4.4.2 Método de Agitação e Mistura Contínua

A quebra contínua de coágulos, com agitação permanente do extrato fermentativo e o gel de fibrina, também foi escolhido um método para estimar a atividade fibrinolítica. Este é um método espectrofotométrico descrito por Wang et al. (2011). Uma solução de 0,72% de

fibrinogênio (w/v), 0,4 mL de 0,15 M de Tris-HCl-NaCl e 0,1 ml de tampão fosfato 245 mM, pH 7, foi colocada em tubos de eppendorf e incubadas a 37 ° C durante 5 minutos. A formação de coágulo foi realizada por adição de 0,1 mL de solução de trombina (20 U / mL). A fibrinólise começa depois que 0,1 mL de extrato fermentativo é adicionado. Durante uma hora o gel de fibrina é quebrado em vórtex a cada 20 min até que 0,2 M de ácido tricloroacético é adicionado para finalizar o processo. A solução final é centrifugado a 14500 x *g* durante 15 min. 1 mL do sobrenadante é finalmente recolhido para ter a sua absorbância medida a 275 nm (1 unidade de degradação de fibrina é definida como um aumento na absorbância de 0,01 por minuto).

4.5 Análise de Imagem

O software ImageJ 1,48 foi usado para binarizar as imagens e delimitar a área de halo de degradação do gel de fibrina de cada orifício na placa (figura 4). A comparação dos grupos expostos e não-expostos foi feita através da uniformização dos diâmetros reais e diâmetros digitais das placas para ambos os grupos. A correlação pixel-cm foi feito pelo software a partir da associação entre o diâmetro medido diretamente com uma régua sobre a placa, bem como o número de pixel correspondente na imagem. Após a digitalização, cada um, halo, foi delimitado por uma circunferência e tinha o número de pixels estimada pelo software.

A imagem resultante do contraste ou binarização mostra o realce das regiões de consumo do gel de fibrina nas placas. Um modo simples de quantificar o número de pixels referentes a cada halo digital é utilizar a função *set scale* do programa ImageJ e relacionar o número de pixels (de uma medida real conhecida, como por exemplo uma fotografia de uma régua) a um valor em escala métrica previamente conhecido. As imagens a seguir mostram a imagem em contraste com um halo circulado a partir da ferramenta de seleção “Oval” do ImageJ (figura 4.a):

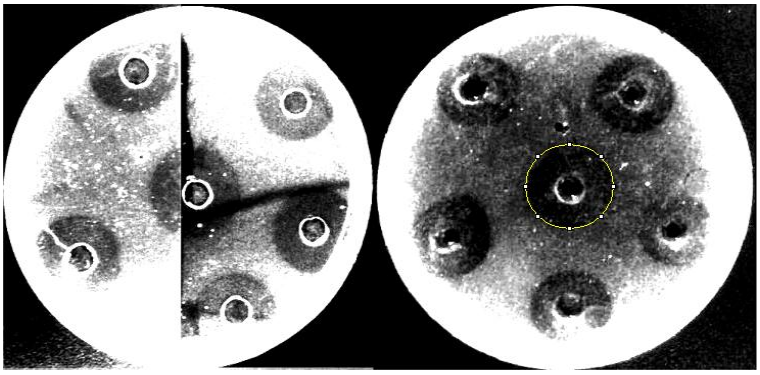

Figura 4.a Delimitação do halo de consumo de fibrina a partir da ferramenta de seleção oval do programa imageJ.

A utilização da função *set scale* para relacionar pixel e centrímetros (4.b). No exemplo 5 centímetros sobre a régua foram traçados com a ferramenta de seleção *straight* do imageJ e a correlação foi feita tal como:

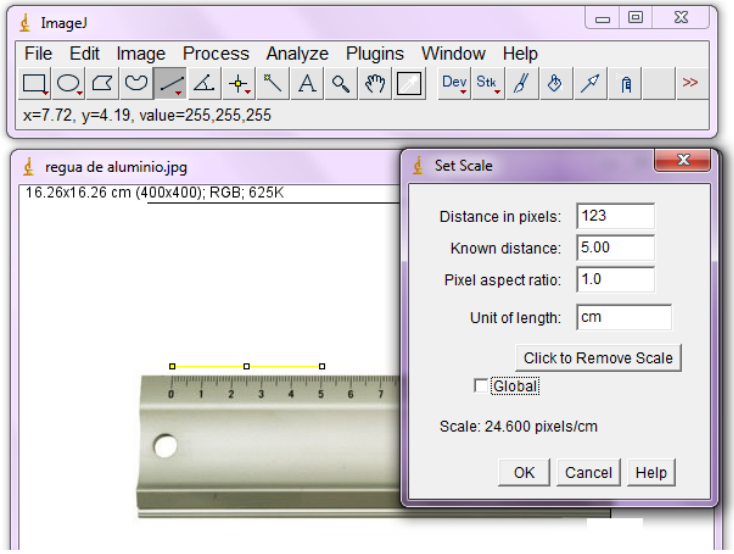

Figura 4.b Correlação do número de pixels com uma unidade métrica através da função *Set Scale* do software imageJ.

Por fim a utilização da função *Tools: ROI Manager* para fazer a medição do número de pixels estimados presentes na seleção oval (figura 4.c). É possível somar as áreas de todos os halos da figura e obter os resultados já em termos de média.

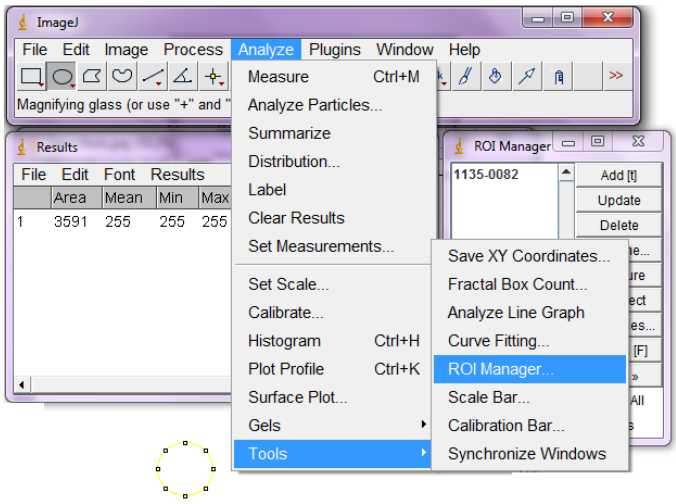

Figura 4.c Seleção de função de medição (ROI Manager) do software imageJ

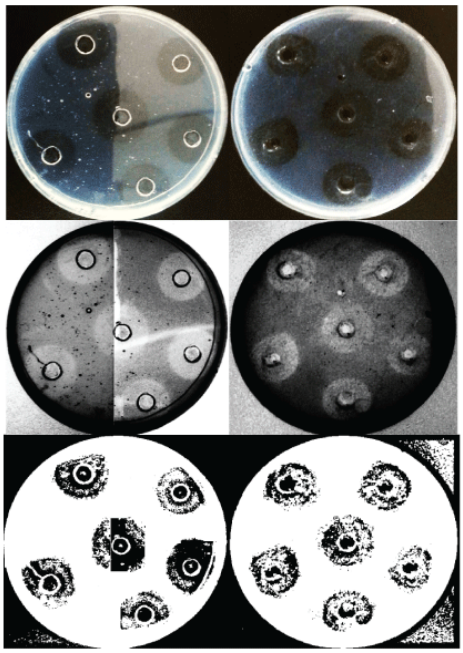

Figura 4.d Diferentes tratamentos (contraste, negativo e binário) de imagens para análise digital.

4.6 Cinética Enzimática

O gráfico de Michaelis-Menten foi usado como a correlação entre a concentração de substrato e a atividade enzimática para definir os parâmetros cinéticos. Para mais fácil estimar parâmetros como V_{max} e da constante de Michaelis (K_m) o gráfico de Lineweaver-Burk também foi traçado. Diferentes concentrações de substrato de azocaseína (dissolvido em tampão Tris-HCl) foram utilizadas para estes ensaios. O mesmo método para avaliar a atividade proteásica geral, baseado em Ginther (1979), foi usado para determinar a atividade e cinética nesses testes.

A determinação dos parâmetros cinéticos a partir do gráfico de Lineweaver-Burk foi feita através de manipulação da função de primeiro grau obtida com a equação de reta gerada a partir do gráfico. Após o plot correspondente aos dados 1/[S] versus 1/[V] obtemos um gráfico de uma linha reta a partir da linha de tendência referentes aos pontos plotados. Após obter a equação de primeiro grau: $ax + b$ onde a representa a relação K_m/V_{max} e b se refere a $1/V_{max}$. Obviamente obtém-se primeiramente o valor de V_{max}, isso se faz igualando o valor b da reta com $1/V_{max}$ ($b=1/V_{max}$). Em seguida com o valor de V_{max} conhecido, fazemos a operação $a = K_m/V_{max}$ e então obtemos o valor de K_m.

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CAPÍTULO 1

The Static Magnetic Field influence on the proteases production and activity

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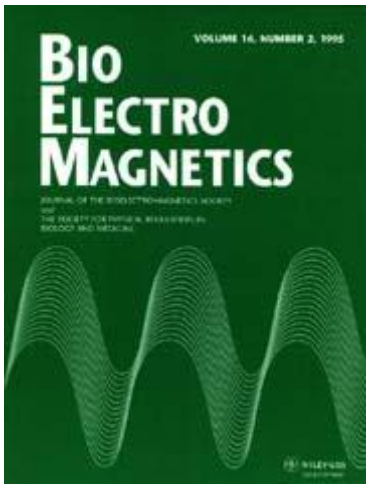

The Static Magnetic Field influence on the proteases production and activity

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Abstract

The chemical structure of living matter is known as susceptible to the static magnetic field (SMF) action. The enzymatic breakage of substrate generates free radicals with spins regulated by an external magnetic field. SMFs were studied as possible agents of changes in the activity of general proteases and specific fibrinolytic enzymes produced by *Streptomyces malayensis*. The fermentation processes occurred in bioreactor, lasting 120 hours and were exposed to a SMF gradient (peak of 100 mT). Magnets were composed by NdFeB alloy and dimensioned 5-5-2.5 cm. Many of the parameters in terms of production were not altered between exposed and non exposed treatments, although the fibrinolytic activity was enhanced in the last hours to the treated group. A 140 mT SMF was effective upon the proteolytic activity in vitro and altered its Michaelis-Menten enzyme kinetic.

Introduction

Static Magnetic fields (SMF) are often cited as having doubtful influential character upon biological systems. Much has been discussed regarding if the magnetic induction could alter intracellular components and at the same time some theories have proposed well established concepts, although without a consensus [Anton-leberre et al., 2010].

Enzymatic processes, production of biomolecules [Da Motta et al., 2004] and alteration of the cell growth and cycle [Vanags et al., 2010] are studied as susceptible to magnetism and with likely applicability in biotechnology. We studied the SMF influence in general proteases and specific fibrinolytic activity as a possible physical tool for biologic manipulation.

SMF promotes changes in the formation rate of enzymatic products through the radical-pairs recombination mechanism [Buchachenko and Kuznetsov, 2008]. Alteration of the electrons spin post enzymatic action defines the radical recombination rate from the

disproportion on raising triplet and singlet state molecules under an external magnetic field [Eichwald and Walleczek, 1996; Grissom, 1995; Taoka et al., 1997].

Proteases have been largely used in human and animal health as antiseptics [Fuglsang et al., 1995], anti-inflammatories [Valdivia et al., 2005], anticoagulants [Verheij et al., 1980] and also at cancer's therapy [Mohamed and Sloane, 2006; Talalay et al., 1995]. The relative importance of pharmacologic manipulation has highlighted the need of controlling the enzymatic biochemistry properties and their conditions of production.

Vajda (1980) studied the trypsin activity under a 1.4 T SMF in different pHs and substrates and could not verify difference between exposed and non-exposed essays. Nakamura et al., (1997) observed reduction of protease activity when exposed to an inhomogeneous magnetic field of 5.2-6.1 T. Iwasaka et al., (1994) applied a magnetic field of 8 T and observed that the level of fibrin degradation products increased relative to a control group and found that fibrin gels become more soluble under exposure. The authors explained the field influence through the diamagnetic anisotropy of intracellular components.

Secondary structures in proteins in helicoid format (alpha helix) are described as susceptible to the action of magnetic fields through the diamagnetic anisotropy phenomena [Pauling, 1979]. In this case, magnetic field lines induced a current along the helical peptide chain which functions as a solenoid creating a magnetic moment in the opposite direction and providing a torque throughout the chain. Such mechanism would result in changing its spatial orientation and promoting changes in structure of an enzyme or possibly altering the atomic arrangement in its catalytic center [Pauling, 1979; Worcester, 1978].

Material and Methods

Microorganism

Streptomyces malayensis DPUA 1571 was isolated from Amazonian lichens. The genus *Streptomyces* was mentioned by Lopes et al., (1999) as resource of several proteases (mainly serine-proteinases and metalloproteinases) and by Peng et al., (2005) specifically as producer of fibrinolytic enzyme.

Media and Operating conditions

Bacterial cultures were grown in 100 mL ISP-2 media (yeast extract 0.4%, malte extract 1% and glucose 0.4%), pH 7, in Erlenmeyer shaker flask during 5 days (120 rpm-

30°C) and further transferred (10% v/v) to 100 mL of the adaptation media MS-2 (Soy 2%, K_2HPO_4 0.4%, mineral solution 0.1%, NH_4Cl 0.1%, $MgSO_4x7H_2O$ 0.06% and Glucose 1%), pH 7.2, based in Porto et al., (1996), for 24 hours (120 rpm-30°). The inoculum amount was estimated by optic density aiming to reach the same initial biomass. The fermentation process (600 rpm-30°C) was performed by the bioreactor Tec-Bio TE-2003 (Tecnal-SP-Brazil) filled with 1 L of media MS-2 and inoculated with the 100 mL of the adaptation media. The bioreactor structure is composed by stainless steel (non ferromagnetic material).

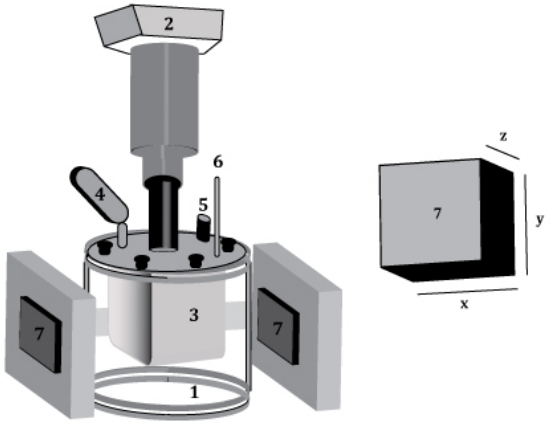

Figure 1. Bioreactor layout and the magnets arrangement. The bioreactor is composed by the steel support (1), Stirring motor (2), Vessel (3), Condenser (4), liquid input and output (5) and the temperature probe (6). Magnets dimensions are x=5, y=5, z=2.5 (7).

SMF arrangement

Two magnets composed by a NdFeB alloy (size 2.5x5.0x5.0 cm) were arranged as described in Figure 1. A Sypris T&M- FW Bell's model 7030 Gaussmeter was used to estimate the magnetic field gradient inside the Bioreactor along the 2 magnets. The field had maximum value of 100 mT at the 2 borders of the vessel and the exposition was continuous during the 120 hours of fermentation. The Intensities and vector field lines were displayed by the software Quickfield Student 6.0, as shown in Figure 2.

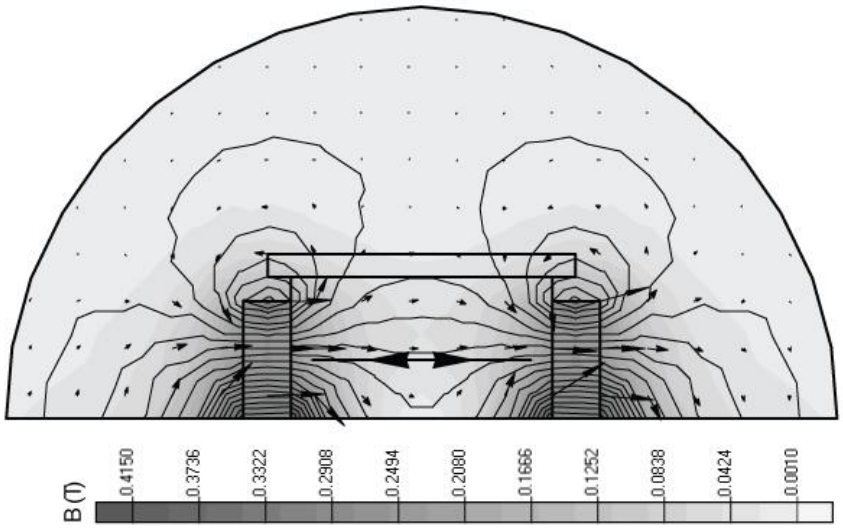

Figure 2. Static Magnetic Field Map. Magnetic field vectors are disposed to show opposite poles between 2 magnets separated by 11 cm through a Bioreactor. Field intensities are demonstrated by different gray tons.

Analytical Methods

Biomass, total protein, general proteases activity and specific fibrinolytic activity were daily measured for 120 hours.

The biomass was determined by the dry weight quantification described by Olsson and Nielsen (1997). Samples of 10 mL were filtered, dried and the difference on the membrane weight between post and pre filtration was correlated to the biomass.

The total protein was evaluated by the method of binding Comassie Brilliant Blue G-250 to protein as described by Bradford (1976). The total protein was estimated by correlation between absorbance (595 nm) and specific protein concentration.

The general proteases were measured as described by Ginther (1979)**.** 0.25 ml of a solution composed by 0.2 M of Tris-HCl buffer (pH 7.2); $CaCl_2 10^{-3}$ M and 1% azocasein was mixed with 0.15 ml of extract centrifuged medium. The reaction was incubated for 1 h (28°C) and paused with 1 ml of a trichloroacetic acid solution 10%. After centrifuged (14500 x *g* -15 min), supernatant (0.8 ml) was added for 0.2 ml of NaOH solution 1.8 M. 1 unit of enzyme activity corresponded to each 0.01 change in absorbance (at 420 nm).

The fibrinolytic activity was determined as described by Wang et al., (2011). 0.5 mL of a solution composed by fibrinogen 0.72% (Type 1, Bovine Plasm, Sigma-Aldrich, Brazil), 20% of phosphate buffer (pH 7) 245 mM and 80% of Tris-HCl buffer (pH 7.2) 0.7 M, was kept in bath(37° C) for 5 min and further added of 0.1 mL of a thrombin (Sigma-Aldrich, Brazil) solution 20 U/mL. After the clot formation, it was added 0.1 mL of the centrifuged metabolic extract and the reaction longed 1 hour (interrupted each 20 min to mix de solution in Vortex and stopped with 0.7 ml of 0.2 M trichloroacetic acid). The final solution was centrifuged (14500 x *g* -20 min) and the supernatant was collected to have the optic density measured at 275 nm. 1 unit of activity per minute was correlated to 0.01 of increase in the absorbance.

Enzymatic kinetics

To test whether the enzyme reaction follows the Michaelis-Menten law, the same method described to quantify protease activity was used but in eppendorfs tubes and at different substrate concentrations (from 0.2 to 2.5% of azocasein in interval of 0.2%). The magnets were disposed with opposite poles by the side of an eppendorf shelf ranging 140 mT (figure 3). Control groups were performed in the same conditions but without magnets.

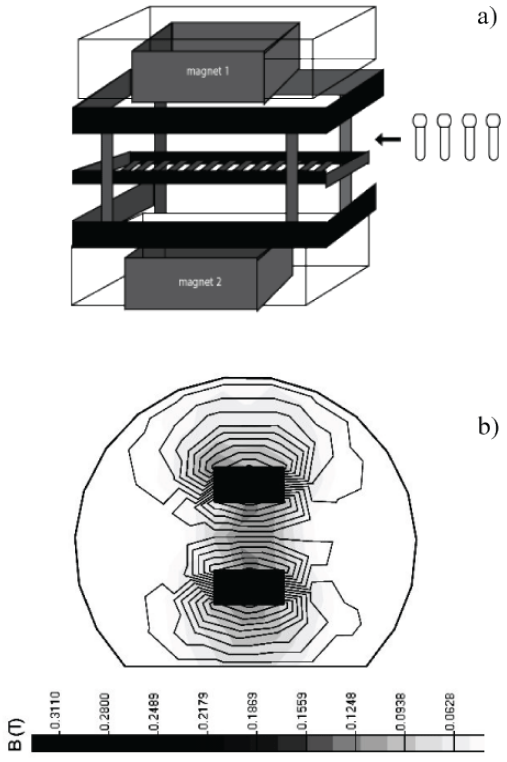

Figure 3. a) Shelf to the eppendorf tubes with 2 magnets at boards. b) SMF mapping

Statistics

Independent samples mean from magnetic and control groups were tested by Student's t-test (STATISTICA version 10.0) for each hour of collected sample. A p-value < 0.05 was considered statistically significant. Data are represented by mean ± SD.

RESULTS

Biomass

The effect of the SMF on the cell growth of *Streptomyces malayensis* cultures was studied in order to compare alteration in the normal bacterial phases curve. Biomass growth was quite similar regardless the growth phase observed and any of exponential or stationary phase were significant different. Figure 4 shows the comparison between groups in bioreactor and it was not found any significant statistical ($p<0.05$) difference.

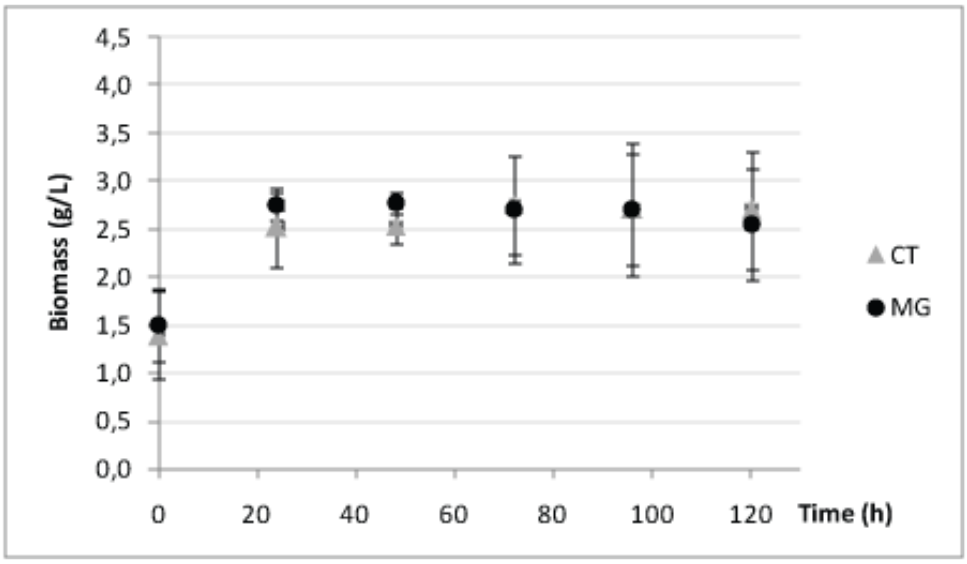

Figure 4. Biomass enhancement and the comparison between groups. Data are media of 4 independent essays.

Total Protein and Protease activity

To verify if the enzymatic concentration in the liquid media was altered we opted to verify its degree of azocasein hydrolysis and in order to verify whether the catalytic site was affected we determined the K_m of the exposed proteases.

The curves of figure 5 were averaged from the 4 independents essays of fermentations in bioreactor, each one comparing concomitantly exposed and non exposed groups. It is

possible to note that the increase in the general proteases activity was reflected in the consumption of total proteins. As seen, the behavior of both groups was similar during the 120 hours (only the protease activity at 72 hours presented significant differences between groups) of fermentation and the results do not indicate significant magnetic effects ($p<0.05$).

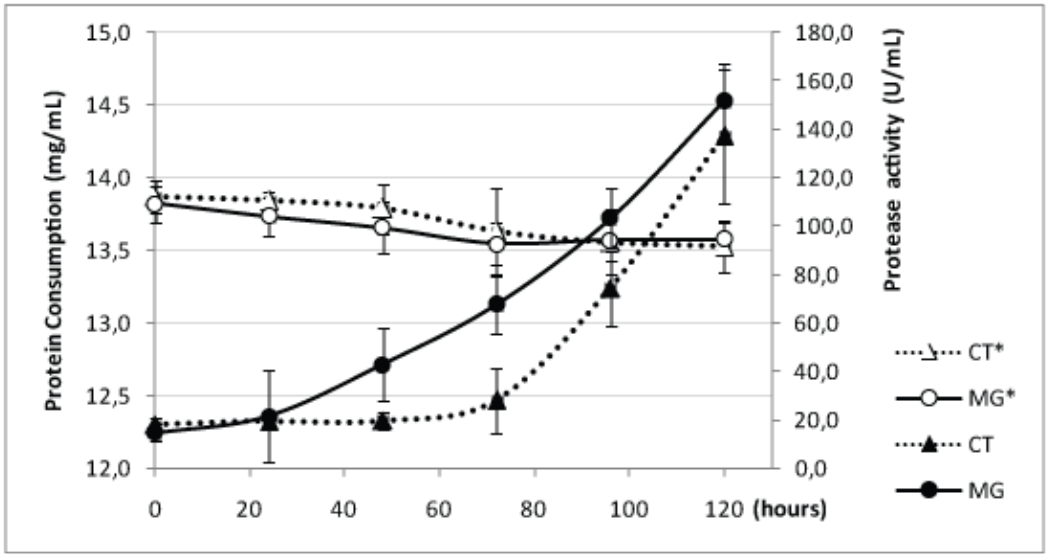

Figure 5. Protease enhancement (U/ml) (secondary axis) and protein consumption (mg/ml) (primary axis) for treated (MG) and non treated (CT) groups. In the legend, * differentiate to protein consumption curves. Data are media of 4 independent essays.

Fibrinolytic Activity

The degree of fibrin clot dissolution by magnetized fermentative extract was tested. As happened with general proteases, the fibrinolytic enzymes were exponentially produced after stationary phase of the bacteria metabolism. Figure 6 shows the curves for groups and differently from other protease essays, from 96 hours of fermentation, the extract reached values significantly higher to the fibrinolytic activity to the exposed group in comparison to the control group. Before 96 hours no one tests were significantly different ($p<0.05$).

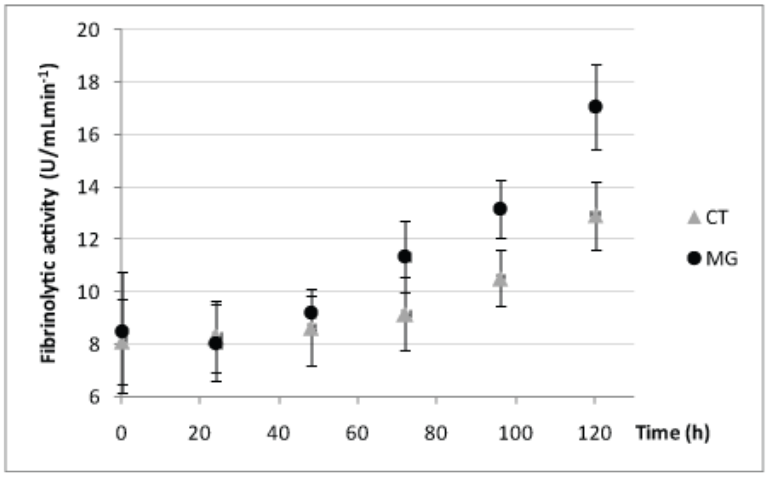

Figure 6. Fibrinolytic activity curves to control e magnetic treatments for fermentations in bioreactor. Data are media of 4 independent essays.

Kinetics of general proteases

Changes in the molecular structure of enzymes and physical properties at atomic level can alter their three-dimensional formation [Jia et al., 2009] and also affect their functional catalytic site [Buchachenko and Kuznetsov, 2008]. SMF could thus alter the protease activity by 2 paths: inducing changes in enzymes conformation or modifying the bacterial metabolism.

In both groups studied the enzymatic process obeyed Michaelis-Menten kinetics. Lineweaver-Burk plot [Lineweaver and Burk, 1934] was the method to linearize the relation between substrate concentration and enzymatic activity and it determined the apparent Michaelis constant (K_m) and maximum velocity (V_{max}).

The groups presented similar K_m (2.19 /L to control and 2.34 g/L to magnetic) but the V_{max} achieved was significantly different (242.7 U/mL to control and 290.5 U/mL to magnetic), as shown in figure 7.

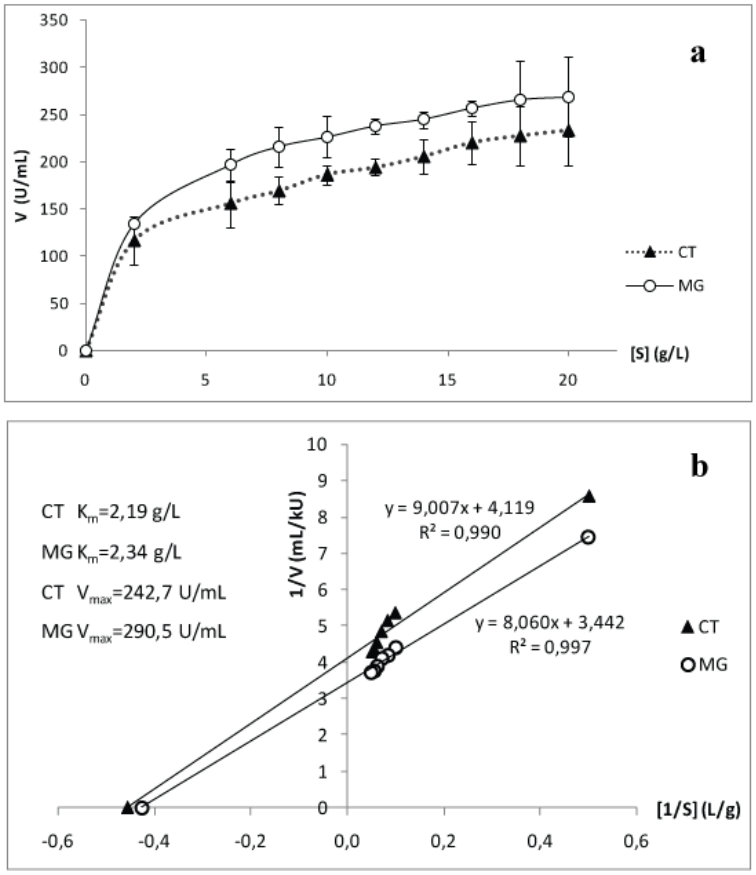

Figure 7. a) Michaelis Menten Plot. The curves represents V_0 (activity velocity) versus [S] to control and magnetic groups. b) Lineweaver-Burk plots for both groups.

Discussion

Once a SMF is applied through a fermentative medium and due the variety of susceptible elements to the field (medium components, exoenzymes, the bacteria intracellular medium and components), the magnetism could exert its influence as stochastic events involving many mechanisms as pair radical recombination [Eveson et al., 2000], gradual concentration of cells-nutrients following a magnetic gradient [Iwasaka et al., 2004], and changes in helix structures at secondary protein structure level [Worcester, 1978]. With many theories describing influence of the magnetism on the cell metabolism, we observed changes in the enzymatic activity although our experiments have failed to find changes in most of parameters of the enzymatic production.

With the advance of studies about the structure of matter in small scale, mainly due the research and applicability of nuclear magnetic resonance [Wiskirchen et al., 1999], SMFs

have got larger support as agent of changes in biologic systems. Thus, owing to the particular subatomic characteristics, it is necessary to add the SMF quantum effects in the biologic structures and components. The studies involving radical pair recombination is currently experimentally and theoretically well established [Grissom, 1995]. From the starting point that external SMF could alter the yield of products in chemistry reactions and promote difference in the products generation rate, we should admit that non-quantum scale effects could be also initiated in their electronic bonds, as for example changes in molecules conformation, break in DNA, bending in cytoskeleton structure and others [Rosenblatt et al., 1987].

The differences between the groups in the therms of enzymatic kinetics is characteristic of an alosteric regulation, where the regulated enzyme is each group reaches different levels of saturation cause molecular changes in a site other than the catalytic site. In this case, the alosteric agent would not be a chemical binder, but the magnetic field. At the same time it would not be necessarily an effect on enzyme structure, once the SMF could act post enzymatic action interfering in the products rate by the proportion between singlet and triplet radicals. This fact, therefore, shows us that SMF is able to modify the enzymatic structure and alter the way it binds with substrate as described in the lock key theory.

Liu et al., (2010) refers obvious changes in activity, K_m and V_{max} of α-amylase related to an external magnetic field acting as modulator of the enzyme conformation. Probably the peptide sequence of exposed enzymes should be studied more careful to identify those possible alterations. Jia et al., (2009) have proposed the same study of kinetic parameters under a SMF also, but with the α-amylase enzyme. The results showed significant differences between treated and non-treated enzymes affecting the activity, K_m and V_{max}. There was a good correlation between field intensity and enzymatic activity, with decrease of the parameters with increase on the SMF intensity. The authors justify the results by changes in the α-amylase secondary conformation.

Conclusion

The results presented here demonstrate that, at the conditions tested, the SMF had not capability to influence most of metabolic parameters of *Streptomyces malayensis*, although the fibrinolytic capability of the extract has enhanced significantly in the last hours of fermentation. SMF was able to improve the general protease activity in vitro and altered the enzymatic kinetics parameters, with possible changes in the enzyme catalytic sites.

Acknowledges

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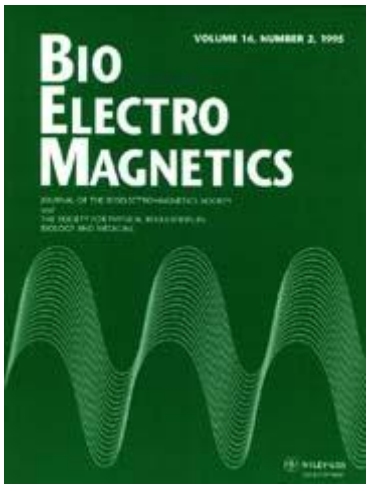
CAPÍTULO 2

Static Magnetic Field Effects on Proteases with Fibrinolytic Activity produced by *Mucor subtillissimus*

Artigo Submetido à Revista Bioelectromagnetics



Static Magnetic Field Effects on Proteases with Fibrinolytic Activity produced by *Mucor subtillissimus*

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Abstract

The influence of the SMF upon enzymes with proteolytic activity, obtained from fermentation extracts, is discussed. Several studies have highlighted the effects of the magnetism upon the spin properties of radicals and the consequent alteration in the rate in which products are released from the substrate-enzyme complex. Knowing the importance of proteases to the medicine and food industry, we attempted here to study if a SMF can truly interfere in tests in vitro and promote changes in the enzymatic performance (in general proteases and specific fibrinolytic activity). Metabolic extract, obtained from solid state fermentation by *Mucor subtillissimus* UCP 1262, was used to carry out essays under a gradient of exposition from 0 to 140 mT, promoted by 2 permanent magnets (NdFeB alloy). The results showed significant differences ($p<0.05$) in the fibrinolytic activity for the treated enzymes with enhanced halos of fibrin degradation. In the other hand, mixing the clots continuously to the fermentation extract and the kinetics parameters in general proteasic activity could not be related to any SMF action. We discuss the susceptibility of fibrin to the magnetism and the character non deterministic to arise effects from the SMF exposition.

Introduction

Studies at subatomic level have provided new tools to analyze the effects of SMF (Static Magnetic Field) upon organic systems, which depend on the spin properties of the particles [Köhler and Bässler, 2009]. For example, Nuclear Magnetic Resonance (NMR) and other advances in the spectrometry techniques have introduced advanced theories about the spin modification, electronic shielding and diamagnetic anisotropy of biological molecular structures and much has contributed to study how enzymes react to the SMF exposition.

The clear influence of magnetism upon spin properties of particles, as the so called Zeeman splitting effect, is important to discuss the real SMF influence upon orbital and spin

magnetic dipoles and correlate them with enzymatic catalysis, as showed by Buchachenko and Kuznetsov, [2008].

Alterations in the catalytic potential of fibrinolytic enzymes may be of use of many fields of interesting. Thrombosis and others disorders arising from the thrombus formation have become one of the most relevant health problem according to the World Health Organization (WHO) [Peng et al., 2005]. Fibrinolytic enzymes and enzyme activators have been focus of recent studies which aim to improve its efficiency and application. In this case, physical agents, as the SMF, could maybe help in the quality of the production and manipulation of drugs.

At the same time to evaluate the harmful character of SMF in macro-scale is also interesting. DNA damage, hormonal alterations and others potential effects is indeed worrying by the increasing electronic devices use and high dose of exposition for NMR equipments in laboratories or hospitals, what makes it a possible agent of health risk [Budinger, 1985]. The higher production of free radical by the SMF (through the pair recombination mechanism), for example, and its harmful aspect for humans has also been discussed in some studies [Formica and Silvestri, 2004].

Taking into account these factors, we proposed the study of the SMF as a suppositional physical promoter of alterations in the enzymatic performance. By treating the enzymatic extracts, we attempted to observe the degree of the SMF influence upon the general protease kinetics and also in the specific fibrinolytic activity.

Materials and Methods

Microorganism

The metabolic extract used in these experiments was obtained from fermentation in solid state by the filamentous fungi *Mucor subtillissimus* UCP 1262, isolated from soil Caatinga, PE-Brazil. The microorganism was maintained in stock in Czapek medium at 30 ºC for 7 days.

Inoculum

A nutrient solution composed by 0.5% (w/v) yeast extract; 1% (w/v) glucose and 0.01% tween 80 (w/v) was diluted in sodium phosphate buffer 245 mM (pH 7) and it was

used to collect the spores from the stock. Spores had the concentration estimated in 10^7 spores/mL.

Fibrinolytic protease by Solid-State Fermentation (SSF)

The spore solution previously described was added to 3 g of wheat bran as substrate moisture (50%) and it was incubated for 72 hours at 25°C.

Enzyme extraction

For the extraction, flasks were filled with 7.5 ml of sodium phosphate buffer 245 mM (pH 7) per 1 g of substrate and placed in an orbital shaker (150 rpm) for 90 min at environment temperature. Furthermore the content was centrifuged at 1250 x *g* for 10 min and the supernatant was collected for the experiments.

SMF Exposure

Two magnetic (NdFeB alloy, 5x5x2.5 cm) were disposed at the borders of a polyethylene shelf, displaced by 5.5 cm with opposite poles as shown in figure 1a. Tests were performed in the same support differing by plates or eppendorf tubes disposed in a gradient field from 0 to 0.14 T measured by a Sypris T&M- FW Bell's model 7030 Gaussmeter and with values computationally correlated (coercive field of 955 kA/m) to plot a map with magnetic vectors and field gradient by the software QuickField Student 6.0 (figure 1b).

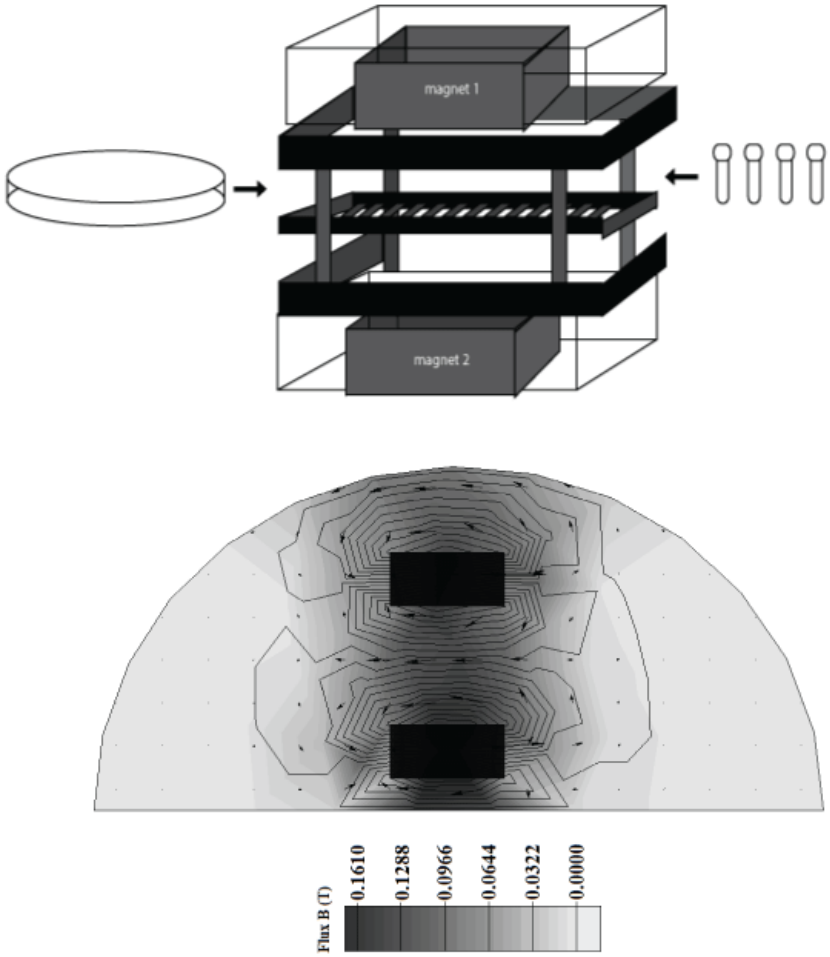

Figure 1. Magnet arrangement and Field Mapping. a) 2 magnets were disposed on the borders of a polyethylene shelf with the samples placed in an equidistant point. b) Vectors and field intensities map.

General Proteases Essays

To estimate the general proteolytic activity, bovine azocasein was used as substrate following the spectrophotometric method proposed by Ginther, [1979]. A mixture of substrate and the metabolic extract remained incubated in the dark for 1 hour, interrupted by addiction of 1 mL of Tricloroacetic acid (TCA). After centrifugation the supernatant was used to have its absorbance measured at 420 nm and 0.01 of absorbance was correlated to 1 U/mL.

Fibrinolytic Essays

Clot Breakage Method

The continuous breakage of clots, with permanent mixing of the enzymatic extract and the fibrin gel, was also a method chosen to estimate the fibrinolytic activity. This is a spectrophotometric method described by Wang et al., [2011]. A solution of 0.72% fibrinogen

(w/v), 0.4 mL of 0.15 M Tris-HCl-NaCl and 0.1 mL of 245 mM phosphate buffer, pH 7, was placed in eppendorf tubes and incubated at 37°C for 5 minutes. The clot formation was performed by addiction of 0.1 mL of thrombin solution (20 U/mL). The fibrinolysis begins after 0.1 mL of the enzymatic extract is added. During 1 hour the fibrin gel is broken in vortex each 20 min until 0.2 M trichloroacetic acid is added to end the process. The final solution is centrifuged at 14500 x *g* for 15 min. 1 mL of the supernatant is finally collected to have its absorbance measured at 275 nm (1 unit of fibrin degradation unit is defined as a 0.01 per minute increase in the absorbance).

Fibrin Plate Method

This method was described by Astrup and Mullertz, [1952]. Fibrin gel in plates was produced by 4 mL of solution composed by 2 mg/mL of fibrinogen (obtained from bovine plasma and dissolved in 150 mM Tris–HCl and 150 mM NaCl buffers, pH 7.75), added of 200 µl of thrombin from bovine plasma solution (20 U/ml diluted in 150 mM sodium chloride solution). The fibrinogen solution was mixed, in plastic Petri dishes, to 4 ml of 2 % agarose, and 100 µl of 1 M $CaCl_2$ solution. After the fibrin polymerization, for each plate, 6 holes with the same dimensions (5-mm-diameter) were made in the fibrin gel. 20 µl of enzymatic extract was added to each hole and the plates were incubated at 37 °C. The potential of degradation was determined by the superficies of consumed gel at 18 and 24 hours.

Image Analysis

The software ImageJ 1.48 was used to binarize the images and delimitate the halo area of the fibrin gel degradation of each hole in the plate. The comparison of exposed and non-exposed group was made by standardizing the real e digital diameters of the plates for both groups. The correlation pixel-cm was made by the software from the association between the diameter measured directly with a rule on the plate, and the pixel number correspondent in the image. After digitalization, each halo was delimited by a circumference and had the pixel number estimated by the software.

Enzymatic Kinetics

The Michaelis-Menten plot was used as correlation between the substrate concentration and enzymatic activity to define kinetic parameters. To easier estimate parameters as V_{max} and the Michaelis Constant (K_m) the Lineweaver-Burk graph was also

plotted. Six different concentrations (2, 4, 8, 10, 16 and 20 g/L) of azocasein substrate, dissolved in Tris-Hcl buffer, were used in these essays. The same method to evaluate general proteasic activity (based in Ginther, [1979]) was used to determine the activity and kinetics in these tests.

Statistics Method

The differences between groups were tested using the Student t test for independent groups performed by the software Statistica (version 10.0). The significance level was 5% and all the data are represented by the media and standard deviation.

Results

The influence of SMF upon Fibrinolytic Activity

Fibrin Plate Method

The halo of degradation in the fibrin gel is a good parameter to evaluate the degree of fibrinolysis and it was used to compare the enzymatic action between treated and non treated samples. The direct measure on the plate and the image analysis and the pixel quantization showed clearly that the SMF allowed significant ($p<0.05$) alterations in the diameters of the superficies of degradation (figure 2) in each hole ($n=12$) of the fibrin gel. Magnetic group presented media of 2.3 cm of diameter (measured directly to the plate) and pixel number media 20% higher than the control group, which presented media of 1.9 cm of halo (figure 2d and 2d).

The figure 2d shows the differences between halo diameters at 18 and 24 hours, and also that in these 6 hours the samples enhanced 18.3% in the exposed group and 13.1% in the control group, showing a high potential of gel dissolution in function of time.

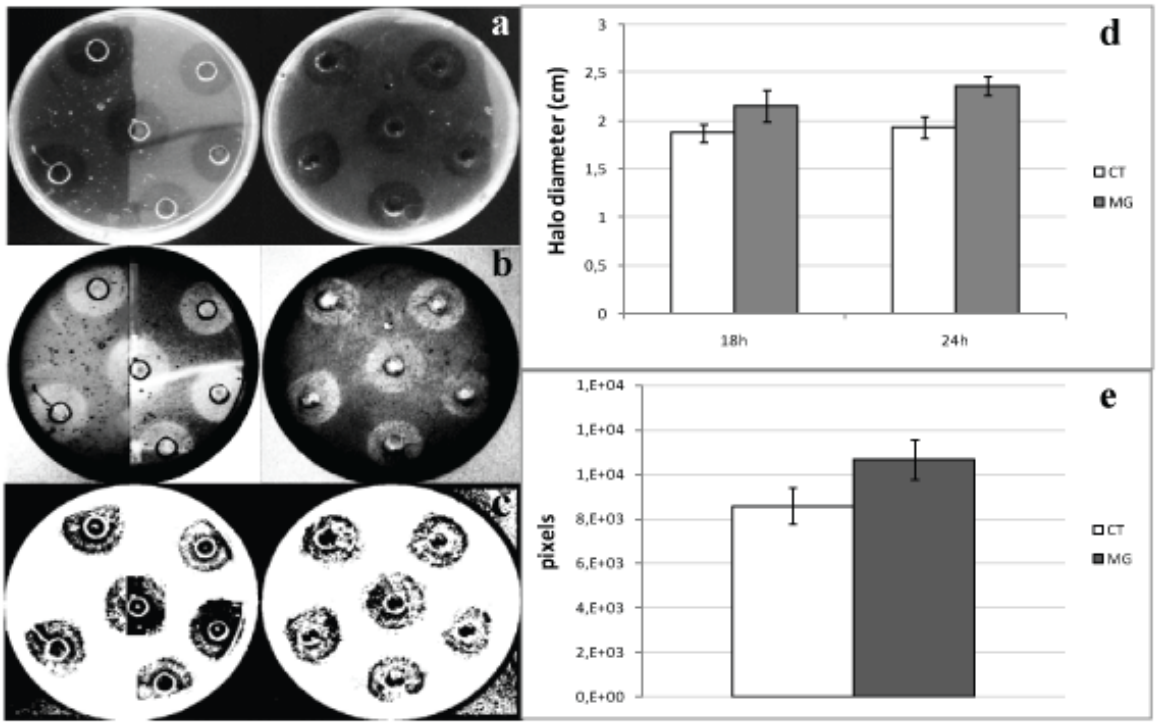

Figure 2. Fibrin Plate Method. a) Real picture of the Plates. b) Images in Negative. c) Binarization and halos delimitation. d) Halo diameter comparison between magnetized (MG) and control (CT) groups. e) Pixel number compared between groups. Results are represented by media ± standard deviation.

Fibrin Clot Mixture

The potential of fibrin clot degradation was evaluated by mixing the metabolic extract completely to the fibrin gel with continuous breakage of the clots by agitation in vortex. In these experiments, tests for both groups were performed in triplicate and repeated 3 times (n=9). As shown in figure 3 treated and non treated groups reached almost the same level of activity, around 23 U/mLs^{-1}. Test t student showed no significant interference in the clot degradation by the SMF.

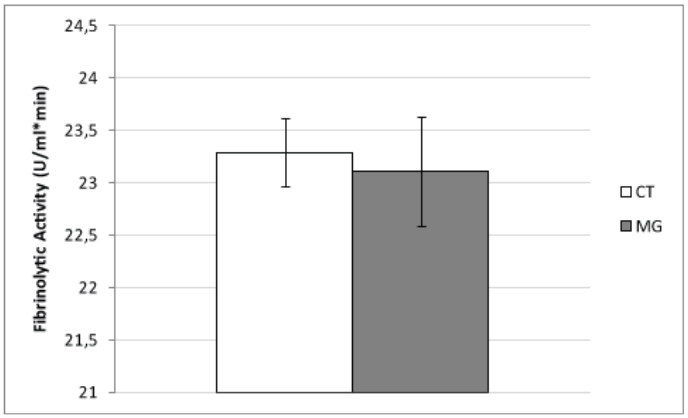

Figure 3. Fibrinolytic activity from continuous clot mixing method. Columns representing the exposed (MG) and non exposed (CT) groups. Results are represented by media ± SD.

General Proteases and Enzymatic kinetics

The relation between the substrate concentration and the enzymatic activity is a good parameter to identify the enzyme affinity and to track clues of possible effects of the SMF upon the enzymatic linkage with substrate or alterations in the enzymatic structure. The key-lock mechanism once altered by the SMF would present different patterns of curves in the Michaelis-Menten plot as well to the parameters K_m and V_{max} in the Lineweaver-Burk plot.

The figure 4 shows the Michaelis-Menten curves to both groups. The essays were performed in duplicate with 4 repetitions (n=8) for each concentration. The curves were practically similar, with saturation close to 600 U/mL.

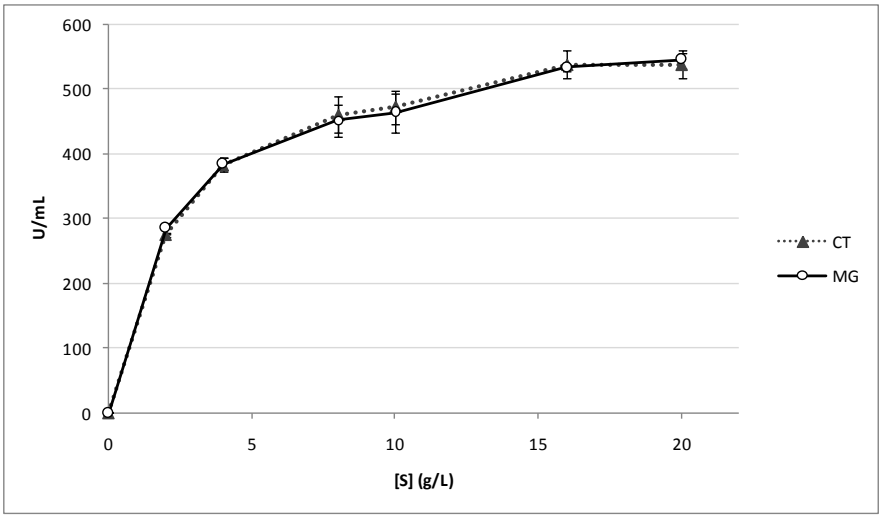

Figure 4. Michaelis-Menten Plot. Results are represented by media ± SD for Control (CT) e magnetized (MG) groups.

For consequence, Lineweaver-Burk plot presented similar kinetics parameters for both groups. For exposed samples it was obtained K_m=0.021 g/L and V_{max}= 580 U/mL for non exposed samples K_m was equal to 0.023 mg/ml and V_{max} reached 600 U/mL. SMF could not affect the kinetics parameters and it gives evidences of no alteration in the enzymes conformation during the exposition to the magnetic field, since in that case it could alter the interaction substrate-enzyme as an inductor or inhibitor of the activity. Figure shows the Lineweaver-Burk plot and presents the kinetic parameters.

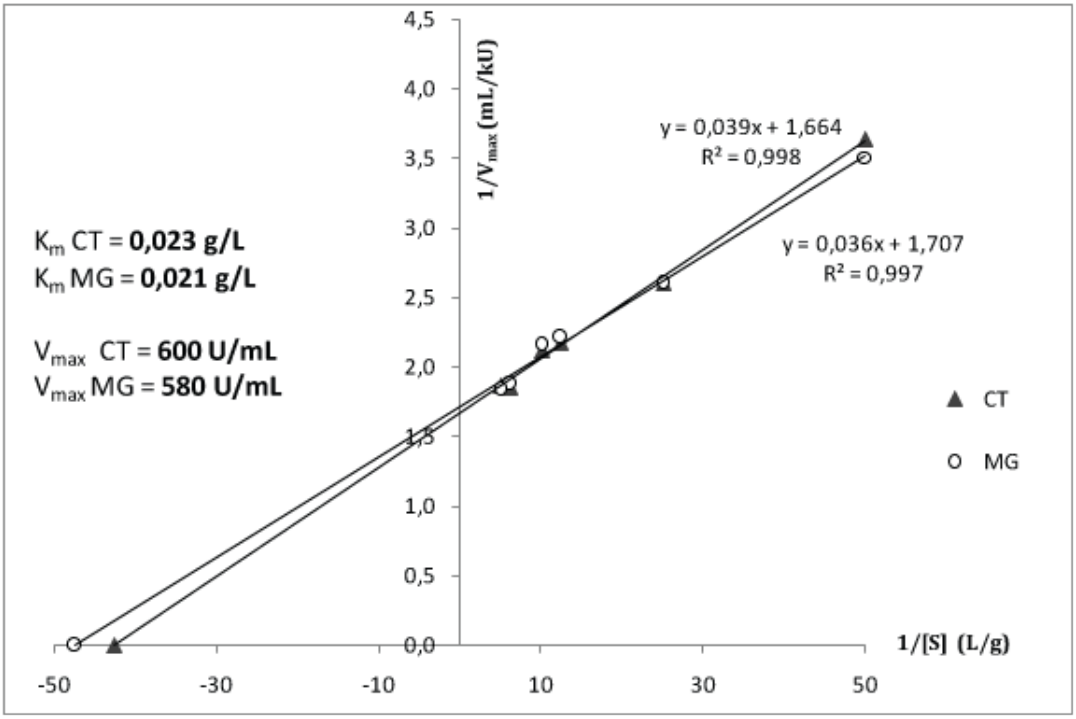

Figure 5. Lineweaver-Burk Plot. Kinetics parameters (K_m and V_{max}) are calculated and showed at left. Curves are represented by a trend line with respective equations and fit degree.

Discussion

The effects of the SMF upon biological systems arise from the interaction of the external field with the intrinsic magnetic dipole produced at subatomic level by the orbital moment of electrons and their spin moment.

The diamagnetic anisotropy of some helicoid structures can be used to discuss the susceptibility of fibrin monomers to the SMF, the alteration in the enzyme conformation and the modification of the interaction of substrates with their respective active sites. In proteins, the anisotropy is due to oriented aromatic groups and planar peptide bonds [Worcester, 1978].

The molecular arrangement in form of helix allows the summation of each magnetic field created by the electronic movement in the resonance structures (as a solenoid), and the alignment between the external field (SMF) and the intrinsic molecular magnetic dipole promotes a torque in the complete structure. Those mechanisms would explain the fact of changes in the fibrin conformation and the enhanced gel dissolution found under a SMF [Torbet et al., 1981].

Fibrin monomers arrangement provides a high anisotropic degree to the molecules [Hudry-Clergeon et al., 1983] . Actually the orientation of fibrin molecules were already studied by Ueno et al., [1993] when at 8 T, the intensity of refracted light (at 632.8 nm) was higher in fibrin gel than in non exposed samples. Iwasaka et al., [1994] evaluated the solubility and degree of dissolution of fibrin gel using the plate method. They submitted the samples to a gradient field with maximum around 8 T and found enhancement of 18% in the dissolution, with fibrin more soluble in the exposed samples. The authors justify the effects by 2 ways: The anisotropy diamagnetic of the fibrin monomers and the drifting of fibrin polymers under the SMF, allowing the increase of the substrate concentration in a local space.

As much as we know that proteolytic action infer in the hydrolyzes-breakage of a chemical bond, it results in the exposition of 2 unpaired electrons in each segregated radical. Once those electrons have a spin and consequently a magnetic dipole, it is possible to presume that an external SMF is able to interfere in the mechanism of recombination or non-recombination of radical pairs after breakage in the substrate molecule [Grissom, 1995]. Disposition of 2 different spins can exist as: 1 conformation (singlet) with no resultant angular moment (observable value of 0) and 3 conformations (triplet) with resultant angular moment (with observables values of 1/2ħ, 0, -1/2ħ) [Atkins and Friedman, 2011]. Chagovetz and Grissom, [1993] commented the idea that the conversion between the singlet and triplet spin states by a external SMF could alter the radical recombination rate and affect the proportion in which radicals may in fact re-link or not. They studied the recombination of geminate radical pairs by alkylcob(II1)alamin photolysis and verified increase of 18% in the germination rate under a SMF (range of 50-80 mT).

Eveson et al., [2000] have suggested parameters of how an enzymatic process could be influenced through the relation SMS-radicals spin orientation. They has tested the yield of radicals from micelles under magnetic fields, linked benzathe in proportion of 1:2 to each molecule of the anionic micelle and verified that a SMF can increase the radical liberation,

proving experimentally the alteration in radicals for the spin orientation. Pair Recombination theory was also proved by Buchachenko and Kuznetsov, [2008] when they observed higher rate of ATP synthesis proportional to the field intensity (from 0 to 80 mT).

Regardless of those some well established theories, we tested the possibility of mechanisms which interfere in the better accomplishment of the enzyme-substrate (key-lock) complex. We could not verify any influence of the SMF upon kinetics parameters with consequently no clue that the key-lock mechanism could be affected by changes in the enzyme structure. However, Liu et al., [2010] studied the kinetics of α-amylase by plotting the Lineweaver-Burk and found that a field of 0.15 T allowed different K_m and V_{max} to the groups exposed. Differences in these parameters suggest changes in the enzymatic structure, but the influence of the SMF could depend on the structural characteristics of each different enzyme, which in our experiment it was not verified.

Conclusion

The SMF was effective in enhance the dissolution of the fibrin gel. However the influence of magnetism is not very deterministic in enzymatic system as it was thought, once we have detected absence of effects in other proteolytic essays. The discussion about how the SMF affects the free radical production and the diamagnetic characteristic of molecular structures make us to believe that effects are result of special situation or arrangements which would allow the effects arise or not. Probably the fibrin itself and not the enzymes would be the one affected by the SMF to develop the effects observed.

Acknowledgments

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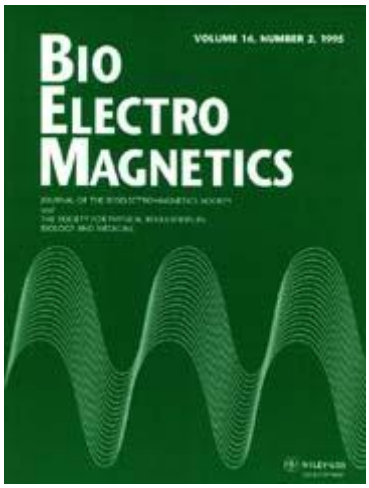
CAPÍTULO 3

Understanding the Static Magnetic Field Influence upon Biological Systems

Artigo Submetido à Revista Bioelectromagnetics



Bioelectromagnetics

Understanding the Static Magnetic Field influence upon biological systems

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Keywords:	static magnetic field, bioelectromagnetism, pair recombination, diamagnetic anisotropy, radiobiology

Title: Understanding the Static Magnetic Field Influence upon biological systems

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Abstract

Efforts to elucidate the doubtful character of the static magnetic field's (SMF) influence upon living cells have been made, although the topic still faces controversies owing to the confusing reports in the scientific literature. Once SMF is cited as modifier of gene expression, enzymatic activity and calcium metabolism, it can be consequently influential on cell growth, cycle and toxicity, as well trans-membrane transport and cytoskeleton frame, parameters in which we have tried to find clues to the phenomena explanation. The findings are in agreement that cell orientation through magnetite (Fe_3O_4) presence, pair recombination mechanism in chemical reactions and the diamagnetic anisotropy are the main mechanisms to produce biological alteration. To resolve the confusion of results it is proposed that scientists have been searching for linearity in what is actually a well characterized nonlinear molecular system and two outputs are considered: the variability of cell types used in experiments (cells differ in susceptible internal components and in enzymatic processes) and a kind of "window" of ideal intensities of field gradient and exposure time to trigger effects.

Keywords: static magnetic field, bioelectromagnetism, pair recombination, diamagnetic anisotropy

Introduction

Static Magnetic Fields (SMF) interact with matter and cause changes in its properties. The effects of magnetism upon biological systems have long been of interest to the scientific community and more recently to lay persons when magnetic fields have been shown to have practical applications in, for example, such diverse fields such as non-scientific community once the applicability could cover since the cancer treatment [Li et al., 2013; Raylman et al., 1996] and the alcohol production industry [Da Motta et al.,2004]. Reports, since the early twentieth century [Kimball, 1938], have tried to identify the action of SMFs on many kinds of cellular components. The literature relates experiments with widely diverse variables in intensities of fields (10^{-6} to 10^{1} T), time of exposure (real-time observation, or minutes, hours and days later), different organisms: microbial systems, plants, Drosophila, mammalian, human cells [McCann et al., 1993;] and also on intracellular constituents: microtubules [Chionna et al., 2005], proteins [Torbet and Ronzière, 1984] enzymatic activity [Maling et al., 1965] and nucleic acids [Potenza et al., 2004]. For this reason the results are confused and dispersed in an unclear and often unrelated constellation of effects.

Labes et al. [1966] cited there was a no satisfactory plausible mechanism for, or general proofs of, magnetic fields effects and recently Anton-leberre et al. [2010], studying yeasts, asserted that magnetism does not affect many cellular processes. By contrast, Short et al. [1992] found evidence of cellular degeneration, decrease viability, decrease in the rate of cell growth or even no effect and Zhang et al. [2003] commented that the reported effects have just not been evaluated sufficiently. This criticism is based on inefficient consensus on experimental design, absence of guideline to exposure range [Higashi et al., 1993b] and even cases of fraud [de Certaines, 1992]. However, in spite of the confusion, a large number of publications has suggested a positive action of magnetic fields.

Many researchers have concluded that magnetism is not cell growth modifier [Gamboa et al., 2007; Sato et al., 1992] but at the same time it is described as exerting an effect on DNA [Li and Chow, 2001; Potenza et al., 2004], and gene expression [Paul et al., 2006]. The orientation and alignment of biomolecules and unicellular microorganisms under exposure is considered the most traditional study and indicates real effectiveness [de Certaines, 1992; Miyakoshi, 2006].

Additional studies include toxicity [Christelle et al., 1998], changes in transmembrane channels [St Pierre and Dobson, 2000], alteration of Ca^{++} metabolism [Büyükuslu et al., 2006] and radical pair recombination on enzyme kinetics [Eichwald and Walleczek, 1996]. The cell could be considered as a system formed by a grouping of susceptible components to SMF through Faraday's law, such as electrical charges (ions, free electrons) in motion, molecules structured with magnetic moments [Teng, 2005], electrical potentials and transported proteins. All those elements could combine to produce cellular responses and, since the cellular environment comprises a non-linear system, a magnetic event could arise from combination of many ideal conditions.

SMF effects on DNA and gene expression

Differential gene expression plays an essential role in regulation of metabolism, biosynthesis and the response to cell stress. The SMF is cited as an agent of changes in those processes once it acts on DNA integrity [Amara et al., 2007]**,** mutation [Zhang et al., 2003]**,** and also in the processes of transcription [Paul et al., 2006] and translation [Goto et al., 2006]**.**

Studies were reviewed aiming to find some relationships between the arrangement of the magnetism and the expressed genes or the DNA damage. Experiments ranging from low

(15 mT) [Jouni et al., 2011] to high field (37 T) [Anton-leberre et al., 2010] intensities and over minutes, hours and days of exposure were evaluated and they do not present any linear correlation with the effects. *Escherichia coli* was often cited in these studies [Potenza et al., 2004; Zhang et al., 2003], for being a model and pioneer organism with a sequenced genome [Blattner, 1997], but observations have been made on many other organisms as diverse as *Salmonella enteric* [May and Snoussi, 2009]**,** *Drosophila melanogaster* [Kale and Baum, 1980]**,** plant cells [Paul et al., 2006] and human cells [Amara et al., 2007]**.**

The complex events and elements that form the machinery of DNA and RNA metabolism: multienzyme complex, transcription factors, transposition activity and mutagenicity are listed in the literature as subject to SMF action. Ikehata et al. [1999] studying bacteria**,** mentioned that the double strand DNA has probably an anisotropic character but, once the genomic DNA is generally located compacted with nucleoid proteins, it would lose its susceptibility to the field and thus the magneto-mechanic effect could not explain observed responses**.** The authors also suggest that electronic interactions, alteration in the mutation pathway of chemical mutagens, changes in membrane permeability and alkylation process are possible mechanism for action on the DNA.

The variability of reported effects is evident in these studies. Authors such as Anton-leberre et al. [2010] and Kale and Baum [1980] verified low or no sensitivity of DNA under a static field. Mahdi et al. [1994] found no evidence of DNA damage when exposing *Escherichia coli* to 0.5-3 T. Amara et al. [2007] failed to find significant DNA strand breaks in THP1 humans cells when exposed to SMF and Ikehata et al. [1999] could not demonstrate differences between exposed and unexposed *Salmonella typhimurium* and *Escherichia coli* in high magnetic fields. By contrast, Goto et al. [2006] reported a gene, Ntan1, responsive to an SMF of 100 mT in rat hippocampal neurons and Jouni et al. [2011] observed a significant enhancement of chromosomal aberrations (bridge, fragments and lagging chromosomes) and chromosome number when they exposed cells of *Vicia faba,* pre-cultivated in natural radiation background, to a field of 15 mT. Zhang et al. [2003] showed that high SMF (up to 9 T) can significantly increase the mutation frequency in soxR and sodAsodB mutants.

There is a consensual opinion that the influence of an SMF on DNA could be related to stress factors (intrinsic within the SMF field itself or oxidative stress induced within the cell). Potenza et al. [2004] found that an SMF of 300mT can work as a stress inducer of transposition activity and May and Snoussi [2009] reported that KatN gene expression could be associated with anti-oxidative response under an SMF of 200mT for 10 hours of exposure.

Examples of genes cited with enhanced expression are: rpoA, katN, dnaK, *Adh*/GUS transgene [Paul et al., 2006], soxR and sodAsodB. Several articles also mention alternating magnetic fields acting on the expression of heat shock protein genes [Goodman and Blank, 1998; Li and Chow, 2001; Lin et al., 1998], but alternating fields are outside the scope of this review.

Cell and intracellular component's orientation

The intercellular environment composition regulates the degree of cell orientation under the action of an SMF. The presence of biomolecules with paramagnetic or ferromagnetic character, such as magnetite crystals ($Fe_3O_{4)}$ [Erglis et al., 2007], and the scale of anisotropic diamagnetic of cell composition allows alignment following the magnetic field lines. Hemoglobin is an interesting example of this dependency, which can be affected differently when oxygenated (with diamagnetic character) and when deoxygenated (with paramagnetic characters) [Yamagishi et al., 1992].

Magnetosomes are vesicles synthesized in the periplasmic space and transferred to the cytoplasm [Erglis et al., 2007]. These compartments are membranes, enveloped organelles of prokaryotic organisms, that house nano-sized crystals of a magnetic iron mineral aligned in chains [Schüler, 2008] and the work created by the magnetic force in the magnetite crystal is capable of giving mobility to the cells.

The studies have mentioned the anisotropic diamagnetism of intracellular components as the most common factor of the cell orientation.

The reviewed articles agree that membranes, microtubules, proteins (mainly those with α-helix secondary structure) receive torques and consequently prompt the cell to movement. It is known that the electronic resonance in planar peptides bonds (figure 1-a) gives diamagnetic properties to the α helices of proteins [Pauling, 1979; Worcester, 1978], with the planar bonds oriented parallel to the helix axis. For example, microtubules (figure 1-b) assemble in the presence of an SMF so that their monomeric unit (tubulin), with α helices in its structure, aligns in a tubular arrangement along a common axis [Rosen and Rosen, 1990] and lipid tubules in the lipid bilayers self-organize as cylinders and are also often referred to as being oriented by magnetic fields [Rosenblatt et al., 1987]. Because the base pairs in part consist of aromatic rings [Maret, 1990], the nucleic acids are also mentioned as diamagnetically anisotropic.

High Magnetic Fields over 10 tesla are often cited as exerting positive effects although

weaker fields (below 1 Tesla) have also shown influences leading to the general view that stronger fields can more easily promote effects. Field intensities used experimentally range mainly between 1 and 10 Tesla.

We have defined 3 classes of the most often exposed objects: cells isolated from a organism (erythrocytes, muscle cells, Schwann cells and others), intracellular components (proteins, lipids, nucleic acids and others) and unicellular microorganisms.

The effects cited in the literature are mainly: orientation/alignment, cell mobility, torque and changes on particle trajectories. All the cells with no apparent ferromagnetic or paramagnetic properties (magnetosomes/magnetite, methemoglobin in erythrocytes [Higashi et al., 1993b] were considered to be altered by the interaction of the SMF and the diamagnetic anisotropy present in their structures. It is most rare to find reports of magnetic influence on the orientation of DNA [Emura et al., 2001], lipids [Higashi et al., 1993a], osteoblasts [Kotani and Kawaguchi, 2002] collagen fibrils [Torbet and Ronzière, 1984] and glial cells [Eguchi et al., 2003].

Cell growth and viability

The cell cycle pathway, growth factors signals and DNA transcription are reflected in the cell number and viability and provide useful parameters to detect changes in the cell machinery and estimate the influence of an extrinsic agent upon the intracellular system. Magnetism is supposed to promote changes in growth and this should prompt the cell into a proliferation state, from which [DeBerardinis et al., 2008] would follow elevated rates of glycolysis, biosynthesis of lipids, lactate production and other macromolecules. These will differ markedly from the metabolism observed in quiescent cells of the same types. Enzymes regulation also could promote changes in the cell cycle by the linkage of some specific ions as magnesium, manganese, calcium, iron and cofactors [Blanchard and Blackman, 1994] in the enzymatic structure under magnetic fields.

Recent works have speculated that changes in the cell cycle could be responsible for the connection between the SMF influence and the cell growth. Onodera et al. [2003] suggested that strong SMFs affect lymphocytes' division cycle by interaction of the field with DNA replication, mitosis and electrolyte balance. But, at the same time, Schiffer et al. [2003] exposed tumor cell lines to static fields of 1.5 T and 7.05 T and the results showed no alteration in the cell cycle transition between G_0/G_1, G_2/M nor in the S phase cell.

The absence of effects is actually not rare in these studies. Irrespective of the different

cell lines [Raylman et al., 1996], intensities [Ruiz-Gómez et al., 2004]or exposure duration [Wiskirchen et al., 1999] many studies have presented the SMF as having no influential. Gamboa et al. [2007], May and Snoussi 2009], Halpern and Greene [1964], Sato et al. [1992] and Malko and Constantinidis [1994] exemplify such studies with the most diverse exposure conditions yet without positive responses. In contrast, there are reports showing effectiveness of the magnetism in parameters such as: biomass enhancement [Muniz et al., 2007], higher optic density [Vanags et al., 2010], increased CO_2 production [Motta et al., 2001] and cell proliferation [Gruchlik et al., 2012].

The positives effects are contrasted by findings of growth inhibition or delay. Iwasaka et al. [2004] observed a decrease in the rate of yeast proliferation when applying strong fields of 14 Tesla for 16 hours. They proposed a mechanism whereby the magnetic flux density gradient works to disturb the O_2 transport and drift the yeast far through the buoyancy by diamagnetic forces, preventing faster cell multiplication by the oxygen action. Kimball [1938] reported that even a very weak SMF of 4×10^{-4} T can retard the yeast budding, which also would be the result of the magnetic gradient that can force the movement of the susceptible cellular components from the weaker to the stronger field.

An interesting suggestion was that in many studies it was proposed that the effects depend just on the cell type or organism. For example Aldinucci et al. [2003] could not find an influence of SMF on human peripheral blood mononuclear cells (PBMC) but found a decreased proliferation of Jurkat cells with 4.75 T. Nagy and Fischl [2004] used weak magnetic fields to obtain opposite conidia growth to *Alternaria alternata* and *Curvularia inaequalis* (increase of 68–133%) and *Fusarium oxysporum* (decreased of 79–83%). Sullivan et al. [2011] observed a 20% inhibition of growth of fibroblast, in human melanoma and yet no effects in stem cells when exposed to fields from 35 to 120 mT.

A search in MEDLINE database (accessed by PUBMED) using the search term "Static Magnetic Field Cell Growth" resulted in 31 references to direct studies relating SMF and cell growth between 1976 to 2012 and the effects on cell proliferation is not representative (figure 2).

The cell's viability and toxicity are reported as subjected to the SMF through changes in Ca^{+2} metabolism, phospholipid bilayer and enzymatic action (which covers the scavenger and anti-oxidant response) [Büyükuslu et al., 2006; Wang et al., 2006]. Buemi et al. [2001] applied a 0.5 mT field on renal cells and detected, after 2,4 and 6 days, an increase in cells with necrotic morphology and also a decrease in their apoptosis and proliferation. Valiron et

al. [2005] found that the focal adhesion and actin assemblies are inhibited by the field (10 T) and it results from the anisotropy of microtubules and actin filaments, with direct consequences in cell viability, organization and differentiation. Teodori et al. [2002] after applying a weak field of 6 mT to HL60 (human promyelocytic leukemia cell line) suggested that the SMF can not induce apoptosis or necrosis alone but acts synergistically with other agents (DNA topoisomerase I inhibitor CPT) through changes in the membrane phospholipids or in the Ca^{+2} metabolism. Gruchlik et al. [2012] and Khodarahmi et al. [2010] mentioned total absence of effects of SMF on cell viability. May and Snoussi [2009] conclude that bacteria growth and viability depend on the variability of conditions and also that cell type is a crucial factor determining whether the field exerts an influence.

Cell morphology

Intercellular components can be subjected to torques and change their alignment and conformation under a magnetic force. It is speculated that membranes, cytoskeleton and protein distribution (as actin filaments) could be directly affected by the SMF and that would consequently promote changes in the cell morphology [Dini and Abbro, 2005].

A relationship between calcium ion and the cell format is often mentioned. Changes in free Ca^{++} can affect cytoskeletal interaction or Ca^{++} binding proteins [Marhl et al., 2000]. The mobilization of Ca^{++} by the magnetic field promotes a cascade of microfilaments and microtubules reorganization and consequently the cell shape modification [Dini and Abbro, 2005]. Altered phosphorylation and dephosphorylation state of proteins can also interfere in the organization of the cytoskeleton elements when exposed to magnetic fields [Popov et al., 1991]

Pacini et al. [1999] reported that a 0.2 T SMF acting on human neuronal cells showed visible changes in morphology. They observed vortexes of cells, branched neurites and neurites featuring synaptic buttons after 15 minutes of exposure to the field. When Albertini et al. [2003] exposed the microorganism *Fusarium culmorum* to 0.3 T SMF they observed, through scanning electric microscopy, a considerable shriveling of the cells' walls. Additionally transmission electric microscopy showed that the magnetic field induced an increase of vacuoles and lipid bodies and organelle disorganization. However, no effects were reported when Khodarahmi et al. [2010] applied 2.1 T for 72 hours to in situ rat astrocytes and particularly no membrane protusion or changes in surfaces were found.

The diamagnetic anisotropy of the cytoskeleton structures varies from cell to cell and

those components are shown to be subjected to linear forces and torques which directly affect the cell morphology.

SMF and calcium metabolism

Ionic flux, apoptosis and release of proteins illustrate some of the calcium roles as secondary messenger and its important function as mediator of the intracellular signaling. It is known that an intrinsic relationship between Ca^{+2} and apoptosis is crucial for determining cell survival. Demaurex and Distelhorst [2003] have cited calcium content as responsible for the cell's ability to commit suicide. The relationship between increasing Ca^{+2} and the cell's decision to enter the apoptosis or anti-apoptosis pathway is actually ambiguous and dependent on the cell type observed [Chionna et al., 2005]

Magnetic field intensities in these studies ranged from $6x10^{-4}$ to 5 T. Unprecedentedly, it was mentioned by Fanelli et al. [1999] that the apoptotic rate of U937 monocytic cells (rat thymocytes) can be modulated by the field intensity. The anti-apoptotic reaction required 0.6 mT to be effective and it increased almost linearly with the SMF intensity. High intensities did not influence the apoptosis reduction. [Prina-Mello et al., 2005] reported that the extra cellular-regulated kinase (ERK) can be modulated by the field intensity and it reaches a maximum of activation at 0.75 T, reflecting directly at signaling cascade events associated with the cell differentiation.

Apoptosis, as mentioned, depends on the calcium concentration but it is not affected directly by the SMF. The field influences the Ca^{++} influx and consequently acts by inhibiting or inducing the programmed cell death depending on how the cell reacts to the calcium. Christelle et al. [1998] studied the effects of magnetism in different cells and observed a reduction in macrophage phagocytosis and enhancement of apoptotic death. Aldinucci et al. [2003], applying a 4,5 T field, could verify an increase of Ca^{++} in human peripheral blood mononuclear cells (PBMC) but conversely the ion concentration was reduced significantly to about one half in Jurkat cells.

The influx of Ca^{++} through the plasma membrane was the main responsible mechanism for the effects and can be associated with the activation of voltage-dependent calcium channels embedded in the plasma membrane by SMF [Prina-Mello et al., 2005]**.** The calcium concentration can be also regulated for ligands action on Ca^{++} channels and those ligands, as ATP, could be susceptible to a external magnetic field [Belton et al., 2008]. Fanelli et al. [1999] presented results showing that the SMF does not randomly mobilize Ca^{++}

(included intracellular extent) but increases its influx through the plasma membrane. This influx was also modulated by the field intensity, with a maximum reached (1.9-fold) at 6 mT.

SMF and enzymatic activity

Enzyme production and activity are reported as affected by static magnetic fields. However, as many contradictory results were found, it is not possible to be assured about the phenomena without considering what exposure conditions and cell types were employed in the experiments. The variety of cited enzymes includes: trypsin [Rabinovitch et al., 1967; Vajda, 1980], endo-1,4-β-glucanase, cellobiohydrolase, β-glucosidase [Manoliu et al., 2006], ribonuclease-rna and succinate-cytochrome c reductase [Maling et al., 1965]; plasmin [Iwasaka et al., 1994], malondialdehyde (MDA) [Amara et al., 2007], superoxide dismutase and catalase (SOD) [Büyükuslu et al., 2006; Çelik et al., 2009], glutamic pyruvic transaminase, glutamic oxalacetic transaminase and lactic dehydrogenase [Gorczynska and Wegrzynowicz, 1989], *α*-amylase [Yan et al., 1997], peroxidase [Atak et al., 2007], endoglucanase [Gemishev et al., 2009], ethanolamine ammonia lyase, methylmalonyl-CoA mutase [Taoka et al., 1997], carboxydismutase, glutamase dehydrogenase [Haberditzl, 1967].

Field intensities ranged from 10^{-4} to 10^{1} T with no dose-response relationship. Taoka et al. [1997] found no linear relationship between variable magnetic field intensity and enzymatic activity. Alternatively, some researchers reported strong magnetic fields as being influential [Ueno et al., 1993; Yan et al., 1997], but the same could not be reproduced by Maling et al. [1965] and Vajda [1980]. Contradictions are also reported with lower fields. Çelik et al. [2009] found that SMFs in the miliTesla range can increase activity of SOD and catalase and similarly Olgun and Rzakoulieva [2007] observed an increase on peroxidase's activity under a magnetic flux of 2.9-4.6 mT. Conversely Amara et al. [2007] showed that a 250 mT SMF did not cause oxidative stress and DNA damage in THP1 cells.

Overall, the influence of static magnetic fields upon 20 different enzymes were reviewed. The main focus of these studies was the antioxidant enzymes responsible for the cell scavengers: SOD, catalase and gluthatione peroxidase. Amara et al. [2007] found that the magnetism does not interfere in the oxidative stress and the enzymes SOD and catalase are not significantly affected. Results presented by Çelik et al. [2009], Büyükuslu et al. [2006] and Haberditzl [1967] reported an increase in the enzymatic activity. Trypsin was studied by Rabinovitch et al. [1967] and Vajda [1980] and both studies did not consider the enzyme to

be vulnerable to the SMF action. Table 2 shows a list of enzymes, intensities of exposure and their different effects on activity, production and kinetic parameters.

Discussions about the SMF action mechanisms upon enzymes are scarce. Iwasaka et al. [1994] defended the idea that diamagnetic components in the intracellular medium can be affected for a magnetic field gradient and drift in the buoyancy, thus, in weak magnetic fields region, they affect the local concentration of substrates and enzymes, promoting differences in their activity. Yan et al. [1997] proposed that the presence of the Ca^{++} ion in the enzyme α-amylase can alter its secondary conformation when exposed to a magnetic field and consequently change its activity and kinetic parameters. Studies by Çelik et al. [2009] and Büyükuslu et al. [2006] suggest that MF can orient unpaired electrons on metallic ions giving kinetic energy to the molecule and transferring energy to others, which in chain reactions forms more radicals.

The pair recombination mechanism

Spin orientation in the pair recombination mechanism is considered a very plausible example of how can magnetic fields alter the chemistry of reactions and cause biological effects. In the cell, enzymes alter chemical bonds allowing the formation of new molecules post-reaction with different characteristics than the initial substrate. The valence electrons are responsible for linkage in molecules and when a region of a orbital's overlap is broken, because of enzymatic action, the opposite spins of electrons (on the free radicals formed) pass from singlet state (antiparallel spin) to triplet state (parallel spin) because the influence of a nearby nucleus (interactions called hyperfine couplings) [Brocklehurst, 2002]. The balance of molecules in triplet and singlet conformation indicates the capability of re-reaction of the radicals and it is responsible for causing injuries in the cell. Magnetic fields are supposed to alter the spin of these valence electrons (post-reaction) and interfere with the reactivity of free radicals.

Attempts to theorize the mechanisms involved in the germinate radicals are discussed. Brocklehurst [2002] asserts that recombination of pair radicals is affected by MFs and the author explains the susceptibility of radicals to the field through the correlation between electron spins and related phases. In a single state the electrons are correlated with antiparallel spin (related in phase and with zero resultant spin moment). In the triplet state the electron are also correlated but then they are parallel and with a resultant spin of one. The powerful electron exchange interaction (J) determines the correlation. The break in the molecules to

form radicals can take the J value to zero and this determines whether the radical transferred will be a radical ion. The degree of interaction (H) among an electron, magnetic nuclei (as hydrogen) and the magnetic field (B) was defined by:

$$H = g\mu_B BS + \sum_j a_j SI_j + \sum_j SA_j I_j \quad (Equation\ 1)$$

Where: a = Spin-spin contact term; A = classical dipolar interaction between two magnets; μ_B = Bohr magneton (constant that express the electron magnetic dipole moment); g = g-factor (relation between the magnetic and angular moment) ; S and I = operators for electron and nuclear spin.

The second and third terms of the equation 1 represent the summation of the two parts of hyperfine interactions over all the nuclei and the first term express the potential energy of interaction between the external field and the electron spin [Brocklehurst, 2002].

Weller et al. [1984] defined a relationship between MFs and pair recombination. They studied radical interactions in polar solvents under an external MF and determined the external field strength $B_{1/2}$ (at which the triplet extinction has its half saturation value) as a parameter to estimate the influence of the magnetic field on triplet production (Equation 2).

$$B_{1/2} = \frac{B_1}{\bar{B}} B_1 + \frac{B_2}{\bar{B}} B_2 \ = 2\frac{{B_1}^2 + {B_2}^2}{B_1 + B_2} = \ (Equation\ 2)$$

Where: $\bar{B}$ *= is the arithmetic mean of 2 experimental values* B_1 *e* B_2.

B_1 e B_2 , the nuclear magnetic fields at the unpaired electrons, are the measure for the spin-multiplicity change (modulated by the MF) for both radicals and calculated according to:

$$B_i = \left(\sum_k a_{ik}^2 \, I_k (I_k + 1) \right)^{\frac{1}{2}}$$

Where: a_{ik} = isotropic hyperfine coupling constant and I_k = nuclear spins

The experimental results confirm that magnetism alters the formation of free radicals. Many of them agreed with the method to solve the stochastic Liowville equation (SLE), developed by Shushin [1990]. [Chagovetz and Grissom, 1993] investigated the dependence of pair radicals arising from adenosylcob- (III) alamin ($AdoCbl^{III}$) in relation to the magnetic exposure and the mobile liquid (aqueous glycerol, aqueous Ficoll-400 and H_20) when photolysis by laser was performed. The rate for ($AdoCbl^{III}$) geminate radical pair

recombination was improved from $1x10^{-9}$ to $4x10^{-9}$ under 50mT in glycerol and from $1x10^{-9}$ to $3x10^{-9}$ under 80 mT in H_20, which shows the MF as being able to exert an influence. Eveson and Timmel [2000] studied the radical recombination in micelles under magnetic exposure and found that a weak field could increase the concentration of free radicals.

SMF influence on biomolecules synthesis

SMFs can modify cell machinery and stimulate synthesis of biomolecules. Changes in enzymatic processes and chemical reaction are suggested as the way to reach final product. Gemishev et al. [2009] studied the synthesis of endoglucanase under static fields with variable intensity between 5-70mT and also variable times of exposure. Applying a 10 mT field produced increases in the total protein of 11,5% for 10 days exposure, 15% for 24 hours and 18% for 1 hour. Da Motta et al. [2004] improved by 3.4-fold the ethanol concentration by applying a 250mT magnetic field in fermentation by *Sacharomyces Cerevisiae*. The effects of the alcohol on membrane organization and in the inactivation of proteins are injurious to the cell and the magnetism could shield the cell from these deleterious effects allowing it to produce more ethanol. This finding was verified by Galonja-Corghill et al. [2009] when they exposed the same yeast to a field of 150 mT and obtained an ethanol yield of 15% higher than the control.

Magnetism has been also mentioned to act at ATP enzymatic synthesis. Buchachenko and Kuznetsov [2008] studied ATP synthesis by phosphorylating enzyme (creatine kinase) and using 2 isotopic magnesium forms: ^{25}Mg and nonmagnetic isotope $^{24,26}Mg$. The $^{25}Mg^{++}$ ion is known to integrate the catalytic site of the enzyme; in the reaction one electron is donated by ADP oxyradical and so forming radical pair. In this situation the pairs can assume the singlet or triplet states, with the triplet phosphorylation being more efficient. The conversion singlet-triplet can be modulated by an external magnetic field and that would explain the experimental effects obtained. Experimentally, MF altered the synthesis of ATP, reaching increases of 50% (55 mT) and 70% (80 mT) to the kinase with ^{25}Mg isotope.

Absence of effects was verified ,for example, during analysis of SMF influence upon prostaglandin synthesis in mouse cells by [Breunig et al., 1993].

SMFs, membranes and channel properties

Membranes and embedded channels are reported to be susceptible to a SMF. The consistency of these studies is based on focusing on only ion channels and lipid molecules and avoiding contamination from cell signaling processes, enzymes and other cell components [Hughes et al., 2005]. Rosen has made considerable contributions in this area by studying channels of sodium [Rosen, 2003], calcium [Rosen, 1996] miniature endplate potentials (MEPPs), synaptic events, discharge frequency and discharge pattern [Rosen and Lubowsky, 1990]**.**

A consensus exists regarding the mechanisms of interaction of the SMF. The diamagnetic anisotropic lipid molecules are described as changing their orientation under the field action and, as they comprise the membrane, altering the regular channel function [Hughes et al., 2005]. Rosen [1993] confirmed that the packing of the membrane lipid molecules in the gel phase causes an increase in the diamagnetic anisotropy of the phospholipid bilayer and the membrane rigidity prevents deformation by the field action. Schwartz [1979] hypothesize that the effects do not result from alteration in the number or size of channels (in this case an higher response in the transmembrane current would be detected), but otherwise a temporary modification in the conformation of the pores would alter the detected electrical signal. However, Maret and Dransfeld [1977] failed to prove that hypothesis when they applied magnetic fields to artificial phospholipid membrane (lamellar multilayer's of hydrated egg lecithin) by the Cotton-Mouton-method.

The magnetic fields applied in these studies have low intensity due the sensitivity of the exposed object. Field intensities were from 10 mT [McLean et al., 1995] and 1.2 T [Schwartz, 1979] and they are applied during seconds or under real time observation. A theoretical study [St Pierre and Dobson, 2000] showed that none of the magnetic field intensities are plausible to justify the Lorentz Force on the mechanisms described here.

The effects detected are, in general, related to a deficiency in the normal pore path deriving from the mechanisms already mentioned. Rosen [2003] found an higher activation time constant (the response time to changes in the injected current) during 100 s of exposure and changes in the current-voltage relationship. McLean et al. [1995] verified that exposure to SMF reduced the ability to fire action potentials in sensory neurons. Rosen and Lubowsky [1990] could evidence changes in the discharge frequency and pattern in a large number of cells in the cat's lateral geniculate body, in addition to changes in their interspike interval histogram. In spite of the consistency of the studies, they still raise controversy. For example,

Schwartz [1979] verified that both membrane potential and the transmembrane currents were not affected by the field and Hughes et al. [2005] reported that a 80 mT SMF can not induce activation in bacterial mechanosensitive ion channels of large conductance (MscL) and changes in recorded baseline current when the applied pressure is absent.

Conclusions

Unseen magnetic forces and their visible considerable effects still cause scientific disquietude and led us to inquire how can life be affected by fields of action-at-a-distance . The confusion of experimental findings does not permit a convincing pattern of effects and it makes necessary a discussion for understanding the phenomena. Living matter is subjected to the electromagnetic fields action by the presence of electric and magnetic properties resultant from mobile ions, unpaired spins, paramagnetic elements and the diamagnetic anisotropy.

The published articles have confirmed the effectiveness of static magnetic fields at the cellular level. By data analysis of field intensities, cell type exposed, durations of exposure and main mechanisms suggested, it is possible to obtain a picture of how the scientific community has tried to solve the problem. One first question , as mentioned by Hore [2012], is that, too many studies, seem to have no hypothesis for a mechanism to guide the experimental plan and clarify the experimental aims. The biggest consensus found in literature is the controversy of results and the majority of papers shows concomitantly both presence and absence of the field influence.

The connection between SMFs and the observed cellular responses are suggested to be explained by: magnetosome presence, pair recombinant mechanism in chemical reactions, drifting of molecules in buoyancy following a field gradient, torques in charged molecules, linkage of ion to the enzyme active site, ion-protein attachment [Binhi et al., 2001] , calcium mobilization and diamagnetic anisotropy of: lipid bilayer, mitochondria, DNA helix and cytoskeleton.

A second question is to understand why experimental results present so many controversial reports. It is not possible to verify any correlation of effects with an exact field intensity. Regardless of few tesla or high intensity field with variable exposure times (days or even minutes) the data do not seem to be in agreement. The findings suggest that the variability of cell types used in experiments could be a possible explanation. Different organisms have been compared in the same conditions of exposure [Aldinucci et al., 2003; Nasher and Hussein, 2008; Sullivan et al., 2011] and it is commonly observed that different

results are obtained by just altering the object of exposure. The differences in experimental objects' presence or absence of susceptible magnetic elements, such as ferro-paramagnetic components and anisotropic diamagnetic structures, for example, could explain the presence or absence of demonstrable effects. Nagy and Fischl [2004] emphasize this idea when they observed inhibition, stimulation or zero effects in different cell classes under the same field parameters.

But what to say about divergent results obtained from the same cell type? For example, *Saccharomyces cerevisiae* was studied by Anton-leberre et al. [2010]; Egami et al. [2010]; Iwasaka et al. [2004]; Malko and Constantinidis [1994]; Motta et al. [2001]; Muniz et al. [2007] and Ruiz-Gómez et al. [2004], with no agreed results. This leads one to suppose that many conditions act together to interfere in the cell metabolism and the modification of one simple parameter can bring about a completely different outcome. This would give the SMF unpredictability and a nonlinear character to be influential upon the cellular system. Phair [1997] comments that molecular cell processes are well described by nonlinear features and nonlinearity is better seen the as rule than the exception. Some publications mention a kind of "windows" effect [Grissom, 1995; Morrow et al., 2007] of ideal conditions that includes ideal field gradient [Egami et al., 2010; Kimball, 1938], ideal field intensity[Fanelli et al., 1999; Nasher and Hussein, 2008], ideal exposure duration [Hirai et al., 2005; Wiskirchen et al., 1999], and ideal organism exposed.

We hope that this review has highlighted important factors that will enable a better understanding of the discord that permeates published studies and point to important features that should be considered in future experimental designs.

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Table 1. Survey Data of the Association Between SMF and Cells Orientation

Reference	Intensity (T)	Cell	Mechanism
Guevorkian and Valles, 2006	**3 to 25**	Organism (Paramecium)	- SMF cause torque in anisotropic diamagnetic constituents
Eguchi et al., 2003	**8**	Cell (Schwann)	- Anisotropic diamagnetism of membrane phospholipids, actins and microtubules
Iwasaka and Ueno, 2003	**10**	Cell (muscle cell)	- Diamagnetic force on the cell components
Yamagishi et al.,1992	**0 to 8**	Cell (blood cells)	- Microtubules interfere in the anisotropic diamagnetic susceptibility
Egami et al., 2010	**2.93**	Organism (yeast)	- The magnetic properties of the yeast microtubule
Higashi et al.,1993a	**0 to 8**	Cell (platelets)	- Hemoglobin does not participle in the orientation, but the diamagnetic anisotropy from membrane lipid bilayers and membrane proteins.
Torbet and Ronzière, 1984	**1.9 and 5.6**	Protein (collagen)	- The summation of the molecular diamagnetic anisotropies
Hirose et al., 2003	**10**	Cell (human glioblastoma)	- The arrangement of microtubules under the influence of magnetically oriented collagen fibers.
Yamagishi et al.,1990	**0 to 8**	Protein (fibrin fibers)	- A full orientation is achieved when the diamagnetic molecules aggregate in the magnetic field, keeping their diamagnetic principal axes along the field direction.
Rosen and Rosen,1990	**0.126**	Organism (Paramecium)	- Anisotropies of constituents could interfere with ion channels which would lower the membrane resting potential. A lowered resting potential reduces ciliary beat frequency/angle and alters velocity and mobility.
Higashi et al.,1993b	**0 to 8**	Cell (erythrocytes)	- The orientation of erythrocytes is determined by the paramagnetic properties of the methemoglobin.
Iwasaka and Ueno, 2005	**8**	Cell (fibroblast)	- The diamagnetic anisotropy of the lipid bilayer in cell membrane
Blakemore, 1975	**~ $5x10^{-5}$**	Organism (*Spirochaeta plicatilis*)	- Novel structured particles, rich in iron, impart to cells a magnetic moment
Higashi et al., 1997	**0 to 8**	Cell (Erythrocytes and platelets)	- The diamagnetism of lipid bilayer and transmembrane proteins and additionally of microtubule
Emura et al., 2001	**0-1.7**	Cell (bull sperms)	- If DNA lines up tidily in the nucleus it could orientate the cell. Diamagnetic components as cell membrane, DNA in the head and microtubule in the tail contribute to orientation.

Some reviewed papers are listed showing the correlation between intensity of the field, the cell type exposed and the main suggested mechanisms of action.

Table 2. Survey Data of the Association of SMF with Effects on Enzymatic Activity

Reference	Intensity (T)	Enzyme	Effects
Manoliu, 2007	**0.08**	Celulases, Peroxidase and Catalase	- Activity alternated between inhibition and stimulation depending on the time of exposure of 7 or 14 days.
Maling et al., 1965	**0 to 4.8**	Ribonuclease-RNA and Succinate-cytochrome c reductase	- The reactions remained constant after the exposure.
Amara et al., 2007	**0.25**	Malondialdehyde, Glutathione peroxidase, Peroxidase, Catalase and SOD	- SMF failed to cause oxidative stress and DNA damage.
Rabinovitch et al.,1967	**22**	Trypsin	- No effect on reactivity was observed.
Iwasaka et al., 1994	**8**	Plasmin	- Fibrin degradation products had higher levels in exposed cultures.
Vajda, 1980	**1.4**	Trypsin	- Detectable changes were not observed when cultures were exposed to the field.
Çelik et al., 2009	**2.9-4.6×10^{-3}**	Catalase SOD	- SOD and catalase activities were increased 21.15% and 15.20%.
Yan et al., 1997	**0 to 0.20**	α-amylase	- The activity was increased by 27%, 34.1%, 37.8% compared with the control.
Olgun and Rzakoulieva, 2007	**2.9-4.6×10^{-3}**	Peroxidase	- Peroxidase activity significantly increased in all magnetic field treatments.
Gemishev et al., 2009	**5-70×10^{-3}**	Endoglucanase	- *Under the applied pretreatment of the spore inoculum, a higher activity of endoglucanase was observed.*
Taoka et al., 1997	**0 to 0.25**	Ethanolamine ammonia lyase and Methylmalonyl-CoA mutase	- The enzymes did not exhibit a magnetic field effect that could be greater than about 15%.
Büyükuslu et al., 2006	**2.9-4.6×10^{-3}**	SOD	- SOD activity increased significantly.
Haberditzl, 1967	**5 – 7.8**	L-glutamic dehydrogenase and Catalase	- The experiments presented increases in the activity of the enzymes in non-uniform fields.

The SMF effects on enzymatic activity are related to the field intensity for each enzyme observed in some reviewed experimental studies.

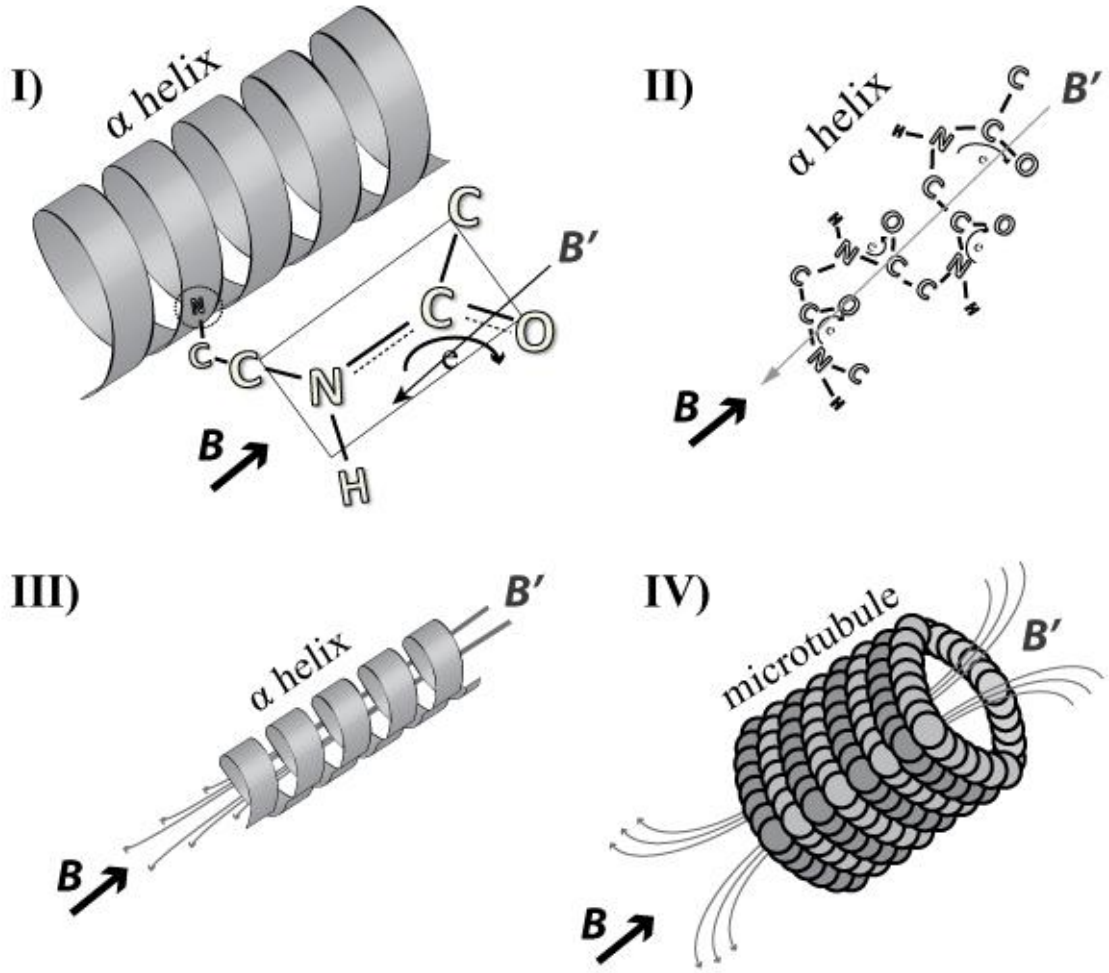

Figure 1. Anisotropy diamagnetism in biological structures. Planar Peptide bonds present in α-helix give it large diamagnetic anisotropy (a). In microtubules (b), for example, the parallel alignment of the peptide bonds with the α-helix axis and their assembly internally to the circular structure increase the magnitude of the magnetic anisotropy as a summation of each secondary magnetic fields B'.

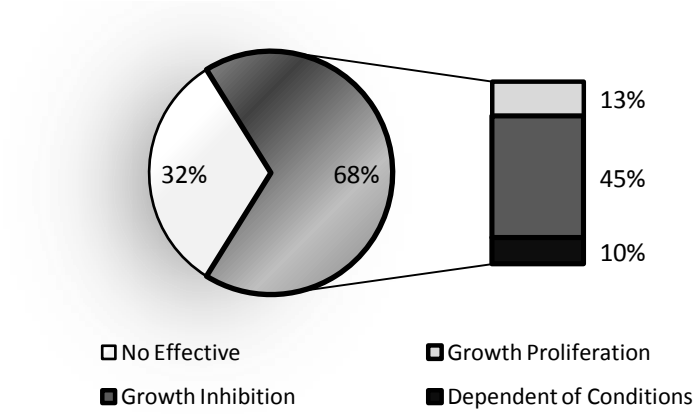

Figure 2. Percentage distribution of SMF effects on cell growth obtained from a search on the MEDLINE database. Perturbations are reported as mainly inhibitory (45%) but 13% were stimulatory and a further 10% reporting changes intermittently depending on exposure conditions and/or cell type specific. About one third (32%) of studied reported no effect.

6. Conclusões

A energia proveniente do campo magnético e seus efeitos consideráveis ainda causam inquietação científica e levou-nos a procurar saber como sistemas biológicos podem ser afetados por campos de ação à distância. A confusão de resultados experimentais na literatura não permite um padrão convincente dos efeitos e ainda se faz necessário uma discussão maior para a compreensão dos fenômenos. Uma revisão de literatura permitiu-nos destacar que a matéria viva é dita como sujeita à ação de campos magnéticos por apresentar propriedades elétricas e magnéticas oriundas de íons móveis, modulação de spins, elementos paramagnéticos e a anisotropia diamagnética.

Os resultados aqui apresentados demonstram que, nas condições testadas, o CME não teve capacidade de influenciar a maioria dos parâmetros metabólicos da bactéria *Streptomyces malayensis*, embora a capacidade fibrinolítica do extrato fermentativo melhorou significativamente nas últimas horas de fermentação. O CME foi capaz de melhorar a atividade proteásica geral in vitro e alterou os parâmetros de sua cinética enzimática.

Por outro lado, utilizando extratos obtidos por fermentações em meio sólido pelo fungo *Mucor subtillissimus*, o CME foi eficaz em aumentar a dissolução do gel de fibrina. No entanto, a influência do magnetismo não é muito determinística no sistema enzimático como se pensava, uma vez que detectamos ausência de efeitos na atividade proteolítica geral. A discussão sobre a forma como o CME afeta a produção de radicais livres e a característica diamagnética de estruturas moleculares nos faz acreditar que os efeitos apresentados são bastante dependentes do tipo de extrato fermentativo ou do arranjo do campo magnético para que assim se permita surgir os efeitos observados.

7. Apêndice

Uma revisão básica sobre cinética enzimática

O perfil de catálise enzimática é delineado a partir da análise quantitativa de fatores que nos permitem relacionar a velocidade da reação com a concentração de substrato, grau de interação enzima-substrato, regulação alostérica e outros.

Um modelo bastante utilizado para a cinética de reações enzimáticas foi sugerido por Leonor Michaelis e Maud Menten. Eles mediram a velocidade inicial das reações como uma função da variação da concentração de substrato, no caso a sacarose, e propuseram que essa relação hiperbólica entre atividade catalítica e concentração de sacarose era real, eles comprovaram a dependência da velocidade da reação pelo complexo enzima-substrato através de uma equação. Isso foi decisivo para provar a magnitude da afinidade de uma enzima por um substrato (MICHAELIS et al., 2011).

A relação entre reagentes, o complexo enzima-substrato e a formação de produtos é explicitada pela interpretação da equação 7 (ROGERS; GIBON, 2009).

$$\boldsymbol{E + S \underset{k_1}{\overset{k_{-1}}{\leftrightarrows}} ES \xrightarrow{k_2} E + P} \quad (7.1)$$

A segunda etapa dessa reação é irreversível e considerada a etapa limitante do processo, havendo a dissociação do complexo enzima-substrato (ES) e formação do produto (P), liberando a enzima (E). As velocidades de cada etapa do processo são representadas por:

$$\boldsymbol{v_1 = k_1[E][S]} (7.2)$$
$$\boldsymbol{v_{-1} = k_{-1}[ES]} (4.3)$$
$$\boldsymbol{v_2 = k_2[ES]} (4.4)$$

Num estado estacionário a taxa de formação do complexo [ES] se iguala a taxa de dissociação em sentidos dos produtos ou sentido reverso:

$$\boldsymbol{k_1[E][S] = k_{-1}[ES] + k_2[ES]} (7.5)$$

E tendo em vista que a concentração de enzima livre [E] é a diferença entre a concentração de enzimas totais [E_T] menos a de complexo enzima substrato [ES], podemos escrever:

$$\boldsymbol{k_1[E_T - ES][S] = k_{-1}[ES] + k_2[ES]}(7.6)$$

Agrupando-se as contantes achamos a constante de Michaelis $K_{m:}$

$$\frac{\boldsymbol{[E_T - ES][S]}}{\boldsymbol{[ES]}} = \frac{\boldsymbol{k_{-1} + k_2}}{\boldsymbol{k_1}} = \boldsymbol{k_m}(7.7)$$

Assim:

$$\boldsymbol{[E_T][S] - [ES][S] = [ES]k_m}\ (7.8)$$

$$\boldsymbol{[E_T][S] = [ES](k_m + [S])}\ (7.9)$$

$$\frac{\boldsymbol{[E_T][S]}}{\boldsymbol{k_m + [S]}} = \boldsymbol{[ES]}\ (7.10)$$

Como foi visto que $\boldsymbol{v_2 = k_2[ES]}$ logo, a velocidade de formação de produtos no modelo de Michaelis-Menten é:

$$\boldsymbol{V = \frac{k_2[E_T][S]}{k_m + [S]}}(7.11)$$

E uma vez que V_{max} é a velocidade máxima observada quando todas as enzimas têm seu sítio ativo ocupado tem-se que$\boldsymbol{V_m = k_2[E_T]}$ e então:

$$\boldsymbol{V = \frac{V_{max}[S]}{k_m + [S]}}(7.12)$$

E esta é a equação de Michaelis-Menten onde se demonstra intuitivamente que se o valor do k_m for igualado ao do substrato, a velocidade instantânea encontra-se à metade da velocidade máxima. Portanto o k_m pode ser usado como parâmetro para estimar a concentração de substrato necessária para que a enzima atinja metade de sua velocidade máxima de ação.

Com k_m= [S] temos:

$$\boldsymbol{V = \frac{V_{max}[S]}{[S] + [S]}} \text{ (7.13)}$$

$$\boldsymbol{V = \frac{V_{max}[S]}{2[S]}} \text{ (7.14)}$$

$$\boldsymbol{V = \frac{V_m}{2}} \text{ (7.15)}$$

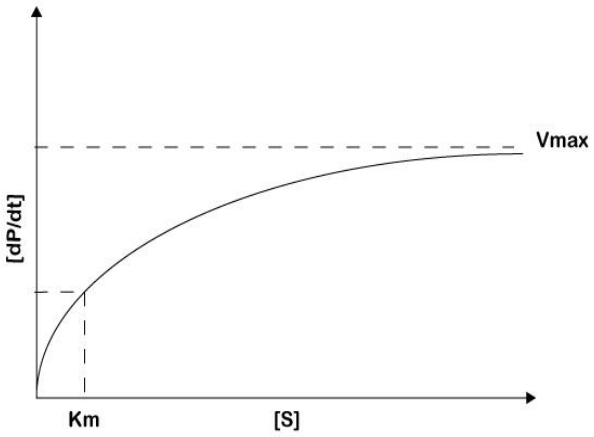

Figura 1. Cinética de Michaelis-Menten. O gráfico define os parâmetros de V_{max} e K_m a partir do ponto de saturação da reação.

Pela dificuldade experimental e de análise gráfica para se determinar o Vmax, opta-se por arranjar linearmente a equação de Michaelis Menten através de inversão de índices para as razões descritas, o que resulta na equação conhecida por Lineweaver-Burk (LINEWEAVER; BURK, 1934):

$$\boldsymbol{\frac{1}{V} = \frac{k_m}{V_{max}[S]} + \frac{1}{V_{max}}} \text{ (7.16)}$$

Através da análise da reta gerada podemos definir os parâmetros cinéticos de Km e Vmax a partir dos pontos em que interceptam os eixos ou pela angulação da reta que é relacionada a $K_m/V_{máx}$.

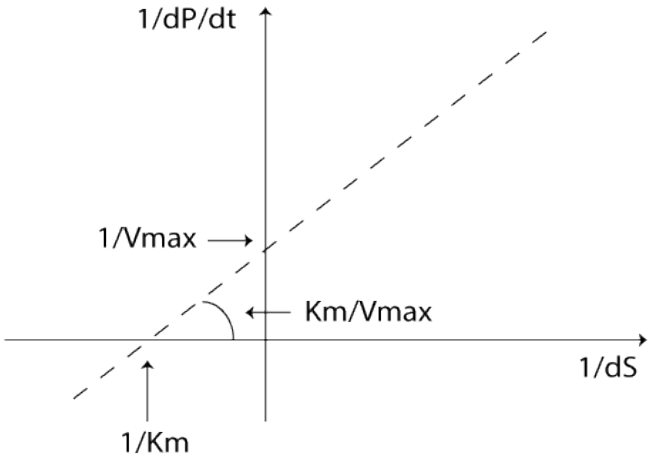

Figura 6. Lineweaver-Burk Plot. O Gráfico mostra a linearização da equação de Michaelis-Menten e os parâmetros definidos a partir das interseções dos eixos e da angulação da reta.

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